Effects of dietary polyphenols on vascular endothelium and cardiovascular health

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Abstract

Introduction: Polyphenols have been suggested as being able to modulate the risk of cardiovascular disease. However, the molecular mechanisms underlying these effects are unclear and the literature lacks consistency and reproducibility, with *in vitro* studies being unable to account for factors such as bioavailability and metabolism seen *in vivo*.

Methods: This doctoral thesis was a clinic-laboratory body of work investigating the effects of polyphenols (Oligopin®) on endothelial function using a dose of Oligopin® in the physiological range. Human umbilical vein endothelial cells (HUVEC) were used for the *in vitro* work. To examine the possible translation of the *in vitro* work, endothelial function assessed by flow mediated dilatation (FMD) and reactive hyperaemia peripheral arterial tonometry (RH-PAT) was compared in individuals with and without polycystic ovary syndrome. A double blind placebo controlled phase 1 clinical trial (ClinicalTrails.gov Identifier: NCT02116816) was undertaken in healthy volunteers to investigate the effects of the Oligopin® *in vivo*. Endothelial function was determined by the reactive hyperaemic index (RHI) and augmentation index (AI).

Results: Oligopin® improved endothelial function *in vitro* by increasing endothelial nitric oxide synthase activity (p-value=0.0066) and decreasing superoxide production (p-value=0.0361) resulting in increased nitric oxide concentration (p-value = 0.0286). There were no differences in endothelial function measurements between PCOS and controls for either FMD (6.9±3.1 vs 5.7±3.1% (p-value=0.14) and RHI (2.0±0.7 vs 2.2±0.7 (p-value=0.51) respectively. There were no differences observed after Oligopin® treatment in the clinical trial in healthy volunteers for either marker of endothelial function.

Conclusions: Treatment of HUVEC in an *in vitro* model showed that Oligopin® may have a beneficial effect on endothelial function by increasing endothelial nitric oxide production. Both FMD and RH-PAT were shown to be equivalent in measuring *in vivo* endothelial function. However, in a phase 1 clinical trial, Oligopin® appeared not to have any *in vivo* biological activity on the cardiovascular risk factors measured.

Publications arising from this research

Abstract

1) **Ehtesham E**, Javed Z, Kilpatrick ES, Atkin SL and Sathyapalan T 2015 Intra-Individual correlation between flow mediated dilation and reactive hyperaemia peripheral arterial tonometry in PCOS. Presented at Society for Endocrinology BES 2015, Edinburgh, UK. Endocrine Abstracts (2015) 38 P270. DOI:10.1530/endoabs.38.P27010.1530/endoabs.38.P270.

Poster Presentation

1) **Ehtesham E**, Javed Z, Kilpatrick ES, Atkin SL and Sathyapalan T. Intra-Individual Correlation Between Reactive Hyperaemia Peripheral Arterial Tonometry and Flow Mediated Dilation in Women with Polycystic Ovary Syndrome. Poster presentation ENDO 2016 Boston, Massachusetts. Available:https://endo.confex.com/endo/2016endo/webprogram/Paper25334.html.

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List of Abbreviations

AI	Augmentation Index
Ang II	Angiotensin II
AT_1	Angiotensin receptor type 1
AT_2	Angiotensin receptor type 2
BH_4	Tetrahydrobiopterin
BMI	Body mass index
BP	Blood pressure
CaM	Calmodolin
CO_2	Carbon dioxide
DAF-2DA	4, 5 diaminofluorescein diacetate
DHE	Dihydroethidium
DMF	Dimethylformamide
DRT	Dérivés Résiniques et Terpéniques
DTPA	Diethylenetriamine-pentacetic acid
ECG	Electro cardiogram
ECGM	Endothelial cell growth media
ED	Endothelial dysfunction
EDRF	Endothelium derived relaxing factor
eNOS	Endothelial nitric oxide synthase
ET-1	Endothelin 1

FBS	Foetal bovine serum
FCS	Foetal calf serum
FDA	Food and drug agency
FMD	Flow mediated dilatation
GCP	Good clinical practice
HBSS	Hank's balanced salt solution
HUVEC	Human umbilical vein endothelial cells
iNOS	inducible nitric oxide synthase
LC-MS	Liquid chromatography -Mass spectrometry
NADPH	Nicotinamide adenine dinucleotide phosphate
NIH	National Institute of Health
nNOS	neuronal nitric oxide synthase
NO	Nitric Oxide
PAT	Peripheral arterial tone
PBS	Phosphate buffered saline
PCOS	Polycystic ovary syndrome
PGI_2	Prostacyclin
PVDF	Polyvinylidene difluoride
R & D	Research and development
RHI	Reactive hyperaemic index
RH-PAT	Reactive hyperaemia peripheral arterial tonometry

RIPA	Radio immune precipitation assay
ROS	Reactive oxygen species
RT	Retention time

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Author’s Declaration

I confirm that this work is original and that if any passage(s) or diagram(s) have been copied from academic papers, books, the internet or any other sources these are clearly identified by the use of quotation marks and the reference(s) is fully cited. I certify that, other than where indicated, this is my own work and does not breach the regulations of HYMS, the University of Hull or the University of York regarding plagiarism or academic conduct in examinations. I have read the HYMS Code of Practice on Academic Misconduct, and state that this piece of work is my own and does not contain any unacknowledged work from any other sources’. ‘I confirm that any patient information obtained to produce this piece of work has been appropriately anonymised.

CHAPTER # 1 INTRODUCTION

1 Introduction

Polyphenols are structurally diverse secondary metabolites produced in plants. They play a range of important roles in processes such as protection against herbivores, ultraviolet radiation and infections, act as attractants for pollinators and as antioxidants against oxidative stress (1). It has been proposed that dietary polyphenols modulate the risk of cardiovascular diseases in humans (2, 3), and have been suggested to exert beneficial effects on the vascular system by improving endothelial function (4). The molecular mechanisms underlying the beneficial effects of polyphenols are the subject of debate as the literature lacks consistency and reproducibility.

Although the available *in vitro* research suggests that polyphenols improve endothelial function, most of these studies have failed to take into account factors such as metabolism and bioavailability seen *in vivo.* A better understanding of the underlying mechanisms is therefore required in which these metabolic processes are taken into account and where a dose in the physiological range is used. Such an approach may lead to preventive strategies applicable to cardiovascular diseases. In this thesis work human umbilical vein endothelial cells (HUVECs) were used as an *in vitro* model to investigate the effects of polyphenols on endothelial function while a double-blind placebo controlled phase 1 clinical trial (ClinicalTrails.gov Identifier: NCT02116816) was conducted to investigate the effects of polyphenols on the endothelium *in vivo*.

1.1 Dietary Polyphenols

Polyphenols are naturally occurring organic compounds found mostly in fruits, vegetables, cereals and plant-derived beverages such as tea, wine and coffee. They are abundant in plants such that in excess of 8000 polyphenolic compounds have been identified in various species. They all have a common intermediate either phenylalanine or shikimic acid and occur in a conjugated form having one or more

sugars linked to a hydroxyl group (5, 6). Polyphenols can be classified into four broad groups depending on the number of phenol rings and the chemical structures that attach them to each other. These groups are phenolic acids, stilbenes, lignans and flavonoids (5) and their representative structures are illustrated in Figure 1.1.

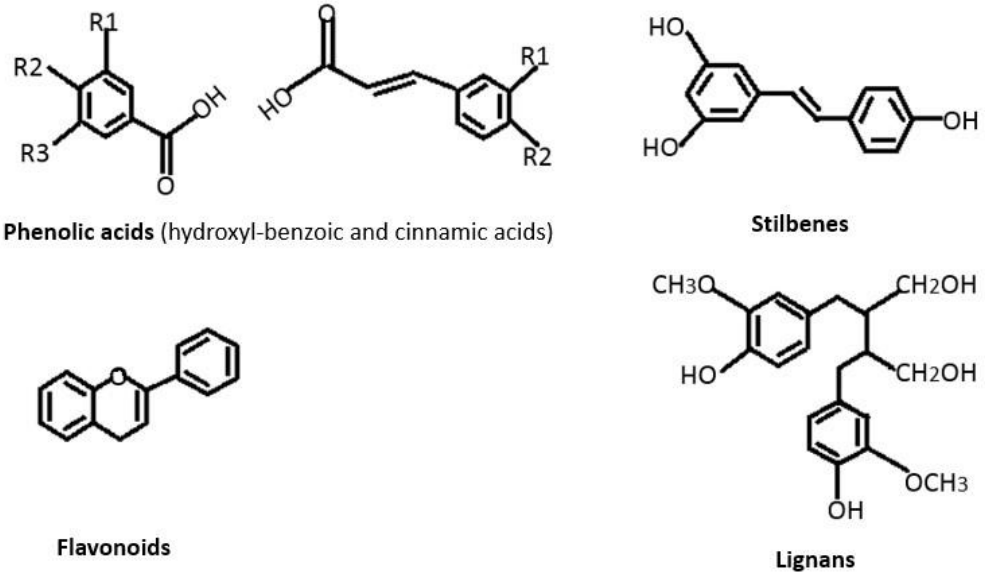


Figure 1-1 Four major classes of polyphenols; namely phenolic acids, stilbenes, Flavonoids and Lignans with their representative chemical structures (7)

Over the past two decades, the food production industry and researchers have become more and more interested in polyphenols, the reasons being their abundance in the diet, their antioxidant properties (8, 9) and their suggested role in prevention of diseases such as cardiovascular, cancer and other neurodegenerative diseases (10-14).

1.1.1 French maritime pine (*Pinus pinaster*) bark extract

The antioxidant properties of pine bark extracts have been known for some time and their health benefits can be traced back to Hippocrates (400 BC) who found they

had inflammation reducing properties (15). *Pinus pinaster* is a medium sized pine which reaches up to 30 meters in height and has paired needles in the leaf spur, brown oval cones and a reddish brown bark as shown in Figure 1.2 (16). The standardized French maritime pine bark extract (*Pinus pinaster*) is a mixture of flavonoids mainly procyanidins (oligomeric catechins) and phenolic acids which are available commercially under different trade names such as Oligopin® and Pycnogenol®. These polyphenol mixtures are claimed to limit/reduce oxidative stress in cardiovascular, cancer and other degenerative diseases such as Alzheimer's disease (15, 17).

Figure 1-2 French maritime pine leaf spurs with paired needles, brown oval cones and a reddish brown bark and powder.

1.1.1.1 Pharmacokinetics of the French maritime pine bark extract

A brown powder obtained on drying the bark extract from this plant is stable in the dark and under dry storage conditions. As discussed earlier the extract is rich in procyanidins and is therefore highly soluble in water. Great care during the extraction process is needed to avoid exposure to moisture because a high water content would encourage enzymes that can degrade the native polyphenols. The same is true for light, heat and oxygen exposure (18). Compounds derived from the extract are readily absorbed from the gut after oral ingestion and their distribution to the body tissues is rapid. Thus, in 2006 Grimm et al (19) reported that compounds such as catechin, caffeic acid, taxifolin, ferulic acid and 10 other previously unknown compounds reached maximum concentration in blood within 5 hours after ingestion of a single dose of 300 mg or multiple doses of 200 mg of maritime pine bark extracts. Moreover, most of these derived compounds remained detectable for up to 14 hours after intake. Compounds in their conjugated states (sulfates and/or glucuronic acids) were also detected suggesting that a significant phase II metabolism takes place in the liver.

1.1.2 Dietary polyphenols and Cardiovascular Diseases

Epidemiological evidence suggests that diets rich in polyphenols such as those containing a large proportion of fruits and vegetables improve health and prevents or delay the onset of cardiovascular diseases (20-22). Intervention studies in animals and humans reinforce this conclusion by showing that ingestion of polyphenol rich diets improved cardiovascular health. Various authors (23-26) have reported these effects in detail but the underlying mechanisms are still not clear. However, the general public health advice nowadays is to eat “five portions a day” of fruits and vegetables for better health (27). A better understanding at the molecular level could result in identifying the mechanisms involved in giving rise to these health benefits and thus provide insight into the formulation of diets that promote cardiovascular health.

1.1.3 Suggested mechanisms underlying the beneficial effects of polyphenols

There is still ambiguity regarding the molecular mechanisms through which polyphenols exert health benefits. However, as a mixture of numerous biologically active compounds, it is of no surprise that the pine bark extracts could have various modes of action.

1.1.3.1 Dietary polyphenols as antioxidants

The most widely suggested mechanism behind the health benefits of polyphenols is their capability to scavenge reactive oxygen species (ROS) and prevent and/or reduces the risk of various oxidative stress related diseases including cardiovascular disorders such as atherosclerosis (28-30). Polyphenols are the major contributors to the total antioxidant activity of fruits (31) and mostly act by neutralizing free radicles by donating an electron or hydrogen atom. They frequently act as chain breakers whereby they directly scavenge radicals of the lipid peroxidation chain reactions by donating an electron to the free radical, thus neutralizing and stabilising them (32, 33).

In addition to direct free radical scavenging, polyphenols also act as metal chelators. They chelate transition elements such as iron (Fe^{+2}) and/or copper (Cu^{+1}) which act as catalysts in reactions that produce free radicals and thus reduces oxidative stress (34).

1.1.3.2 Other mechanisms that may be involved in the health benefits of dietary polyphenols

Most information on the antioxidant activity of the polyphenols is derived from *in vitro* studies but there is an increasing body of evidence that other mechanisms may be involved *in vivo*. Polyphenols such as flavonoids can be absorbed through the gastrointestinal tract but their plasma concentrations are in such a low amount (usually less than 1 µ mole/L) that is very low for most polyphenols to exert any significant and direct antioxidant effects. The low plasma concentrations are partly a result of rapid metabolism of the polyphenols by human tissues (35, 36).

Polyphenols block enzymes that are responsible for generation of reactive oxygen species such as superoxide. These include enzymes such as xanthine oxidase and protein kinase C (37). Polyphenols also increase the production of endogenous antioxidants such as glutathione peroxide, catalase and superoxide dismutase which are responsible for the degradation of hydroperoxides, hydrogen peroxide and superoxide respectively (38).

1.2 Endothelium

1.2.1 Overview

Endothelium comprises a single layer of endothelial cells lining blood and lymphatic vessels. The endothelium is strategically located between blood/lymph and tissues and is directly exposed to the cells in circulation. There are approximately ten trillion (10^{13}) endothelial cells in an adult human and collectively they weigh around a kilogram (39). Wilhelm His first proposed the name endothelium as early as 1865 to describe the barrier between the blood and tissues, which prevented the circulating cells from leaking out into the vascular matrix. Surprisingly, it was not until over a hundred years later that the endothelium was considered more than a mere barrier through the discovery in 1980 that an intact endothelium was required for acetylcholine to elicit vasodilation (40). Since then the role of endothelium in vascular haemostasis has been recognised and it is now consider as an organ that has multiple functions (41).

1.2.2 Structure

Endothelial cells show heterogeneity in their structure and shape which vary between different vascular beds depending on the tissue requirement, with most having a continuous non-fenestrated cell layer with others having a continuous fenestrated or discontinuous cell layer (41, 42). This variability in shape is shown in Figure 1-3 where different type of endothelial cells are shown lining different types of capillaries.

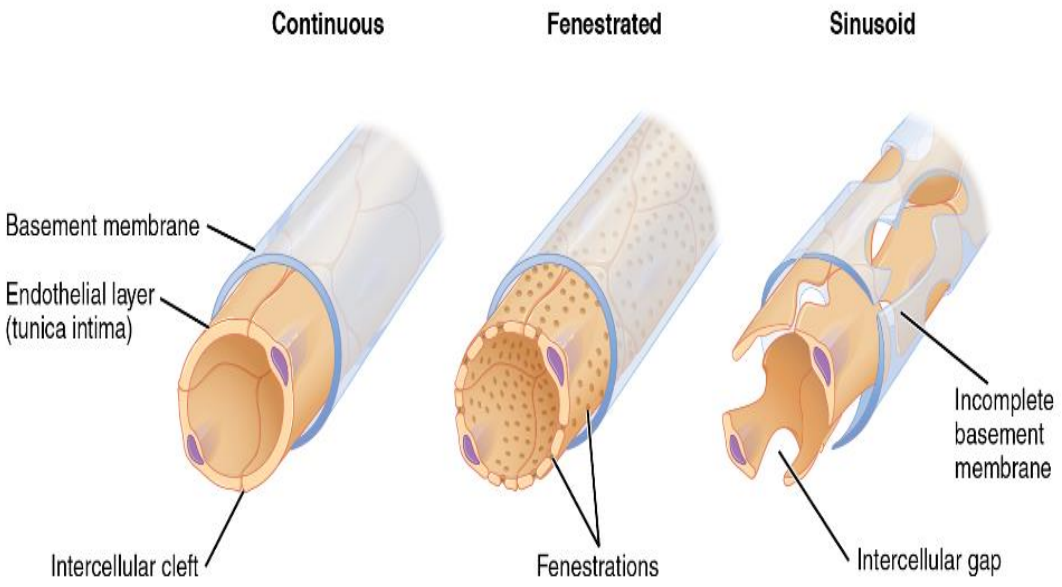


Figure 1-3 Different types of endothelial cells lining various types of capillaries (43).

1.2.3 Function

While the endothelium was initially considered as only a selectively permeable membrane responsible for transport of molecules between blood and tissues, research in the last few decades has led to a better understanding of the endothelium and several additional functions have been attributed to the endothelial cells. These include important roles in vasomotor tone and blood flow regulation, serves as an anti-coagulant surface preventing blood coagulation, plays a central role in vascular growth and inflammation (44, 45).

1.2.4 Endothelium derived vascular mediators

The Endothelium secretes a variety of mediators including vasoactive substances such as nitric oxide (NO), prostacyclin (PGI_2), epoxyeicosatrienoic acids,

endothelin-1 (ET-1), prostaglandin H_2, endothelium-derived hyperpolarizing factor (EDHF), platelet-activating factor and reactive oxygen species (ROS) (46, 47). The endothelium helps in maintaining an anti-atherogenic environment under resting conditions (48).

1.2.5 Nitric oxide

1.2.5.1 Overview

NO is one of the most important mediators produced by the endothelium that are said to be protective in that it causes vasodilatation, growth inhibition, is antithrombotic, anti-inflammatory and an antioxidant . Although NO production by the endothelium can be triggered by a variety of pharmacological agents however physiologically the most important stimuli for its release from the endothelial cells is shear stress (or viscous drag) and increased blood pressure.

1.2.5.2 Brief history

Although Joseph Priestly discovered Nitric oxide in 1772 as a colourless gas having 6-10 seconds lifetime *in vivo*, its vascular smooth muscle relaxant properties were not discovered until late in 1979 in pre-contracted bovine coronary artery in an organ bath (49). Moreover in 1980, Furchgott and Zawadzki's observation that acetylcholine results in relaxation of rabbit aorta only in the presence of intact endothelium led to the recognition of its importance in regulating vascular tone (40). They suggested that this relaxation is caused by a diffusible factor, which was called endothelium derived relaxing factor (EDRF), but a few years later, it was identified as nitric oxide (50-52). In 1998 Robert Furchgott, Luis Ignaro and Ferid Murad received the Noble prize in physiology and medicine for their contribution to the discovery of the important role of nitric oxide in vascular regulation (53).

1.2.5.3 Nitric oxide synthesis

Endothelial nitric oxide synthase (eNOS) is one of the three different subtypes of NO synthases the others being inducible nitric oxide synthase (iNOS) and neuronal

nitric oxide synthase (nNOS). eNOS predominantly produces NO in endothelial cells from L-arginine in the presence of molecular oxygen and other co-factors such as tetrahydrobiopterin (BH_4) and calmodulin (CaM) (54).

1.2.5.4 Role of Nitric oxide in endothelial function

NO has multiple cellular functions apart from a direct action on vascular smooth muscles leading to their relaxation resulting in vasodilatation. Thus, availability of NO in the vasculature also inhibits nuclear factor- kB dependent expression of chemoattractant and adhesion molecules (55), inhibits platelet activation (56) and tissue factor expression and is responsible for suppression of abnormal smooth muscles proliferation (57, 58). All these functions of NO make it a critical factor in the endogenous defence system against inflammation, vascular injury and thrombosis, i.e., the key steps involved in atherosclerosis development and progression.

1.2.6 Endothelial Dysfunction

1.2.6.1 Overview

Endothelial dysfunction (ED), a failure of the endothelium to suppress oxidative stress, inflammatory and thrombotic processes, plays a key role in the development and progression of atherosclerosis. ED has been detected in coronary and resistance vessels as well as in peripheral arteries and is thus regarded as a systemic condition (59-61). Endothelial function is usually assessed as a reduced endothelium dependent (NO mediated) vasodilatory response of the peripheral vasculature *in vivo* (57) and serves as a surrogate for bioavailability of NO.

1.2.6.2 Cardiovascular risks and endothelial dysfunction

Endothelium plays a vital role in vascular homeostasis by maintaining a balance between endothelium-derived relaxing and contracting factors (62-64). In the

context of cardiovascular disease, this balance is shifted in favour of the contracting factors. Cardiovascular risk factors, such as smoking, high Low-density Lipoprotein cholesterol and triglycerides, hypertension and diabetes mellitus may disrupt this balance and predispose the endothelial cells to adopt a pro-atherogenic phenotype and thus resulting in endothelial dysfunction that may lead to vasoconstriction, platelet activation, inflammation, coagulopathy and atherosclerosis (65-67).

1.2.6.3 Predictive value for future cardiovascular events

Endothelial damage reflects the damaging effects that cardiovascular risk factors have on the endothelium and endothelial dysfunction is an early sign of atherosclerotic disease progression (68). Endothelial dysfunction has been shown to be an independent predictor of future cardiovascular events and assessment of endothelial function can identify individuals at risk of developing cardiovascular disease (69, 70).

1.2.6.4 Nitric oxide bioavailability and endothelial dysfunction

Although other vasodilators secreted by the endothelium such as endothelium derived hyperpolarizing factors and prostaglandins may have a role, NO is considered mainly responsible for the endothelial dependent vasodilatation (71, 72). There is a large body of evidence supporting the proposition that accelerated degradation of NO by reactive oxygen species is underlies the reduced NO bioavailability seen in various disease states (73, 74).

1.2.6.5 Angiotensin II and endothelial dysfunction

Angiotensin II is an octapeptide hormone that is the major effector of the renin-angiotensin blood pressure control system, plays a role in the development and progression of cardiovascular diseases such as hypertension, atherosclerosis, myocardial infarction and vascular hypertrophy (75-77). Angiotensin II mediates its

actions through its plasma membrane receptors, namely Angiotensin receptor type 1 (AT_1) and Angiotensin receptor type 2 (AT_2) (78).

Most of the known functions of angiotensin II are mediated by AT_1, and past research has shown that AT_1 activation results in the production of reactive oxygen species (ROS) in vascular smooth muscle (79). Associated eNOS dysfunction also results in decreased NO levels and increased superoxide production (73, 80). Increased superoxide production by any mechanism may ultimately result in eNOS uncoupling (when there is altered eNOS function, which starts producing superoxide instead of NO) and thus increased superoxide production (81). Figure 1.4 outlines this important role of endothelial NO synthase (eNOS) uncoupling in the pathogenesis of endothelial dysfunction. The presence of superoxide in the vasculature greatly alters the bioavailability of NO, and increased reactive oxygen species (ROS) is a hallmark of cardiovascular diseases (47, 57).

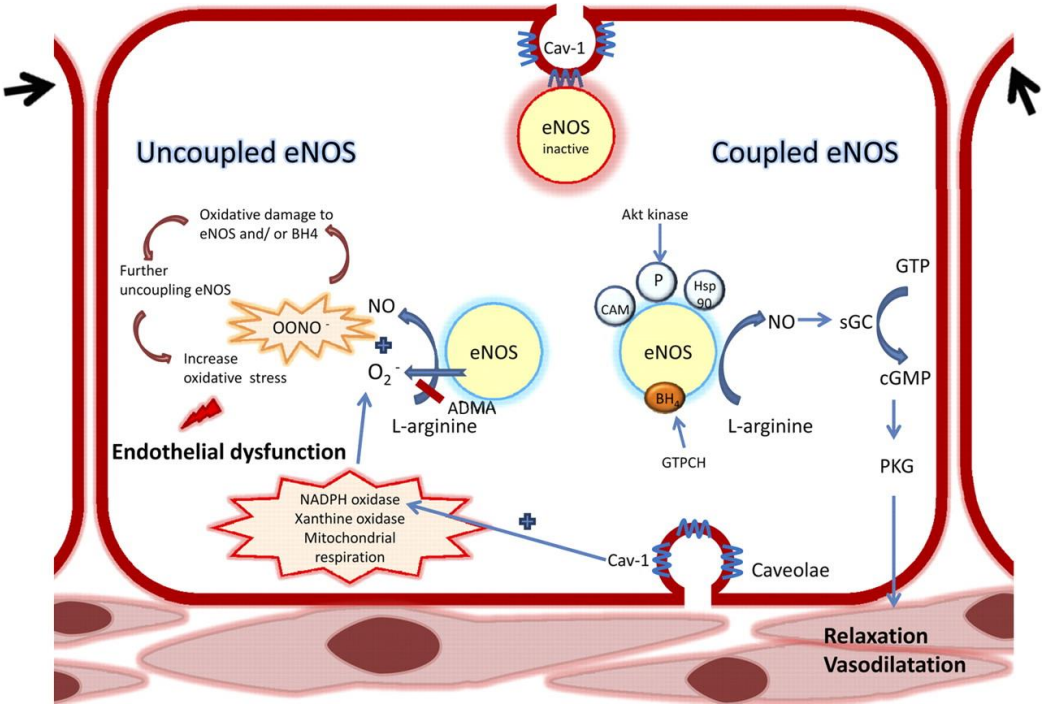


Figure 1-4 Central role of endothelial NO synthase (eNOS) uncoupling in the pathogenesis of endothelial dysfunction.
Adopted from (82)

1.2.6.6 NADPH oxidase and endothelial dysfunction

There are many enzyme systems (including xanthine oxidase, uncoupled NO synthase and mitochondrial sources such as mitochondrial electron transport and mitochondrial DNA damage) which can lead to increase ROS in the vasculature. One such system is NADPH oxidase, a major contributor to ROS production (83, 84). The molecular mechanisms are still poorly understood but previous studies have shown that NADPH oxidase is activated in hypertension induced by angiotensin II (85-87), and that AT_1 is linked to the activation of NADPH oxidase in vascular cells (88).

NADPH oxidase is found in numerous cell types as well as endothelium and has several enzymatic subunits. It has membrane-located components including $p22^{phox}$ and $gp91^{phox}$ and cytosolic components, $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and rac as shown in Figure 1.5. In the resting state, $gp91^{phox}$ and $p22^{phox}$ form an inactive membrane complex called cytochrome b_{558}. $P47^{phox}$ that forms a trimer in the cytosol with $p40^{phox}$ and $p67^{phox}$, facilitates NADPH oxidase assembly by binding both $p67^{phox}$ and $p22^{phox}$. The binding with $p22^{phox}$ results in the activation of $gp91^{phox}$, aided by the interaction of GTP-bound rac with $p67^{phox}$. Molecular oxygen is then reduced by the activated $gp91^{phox}$ in a NADPH-requiring reaction (89, 90).

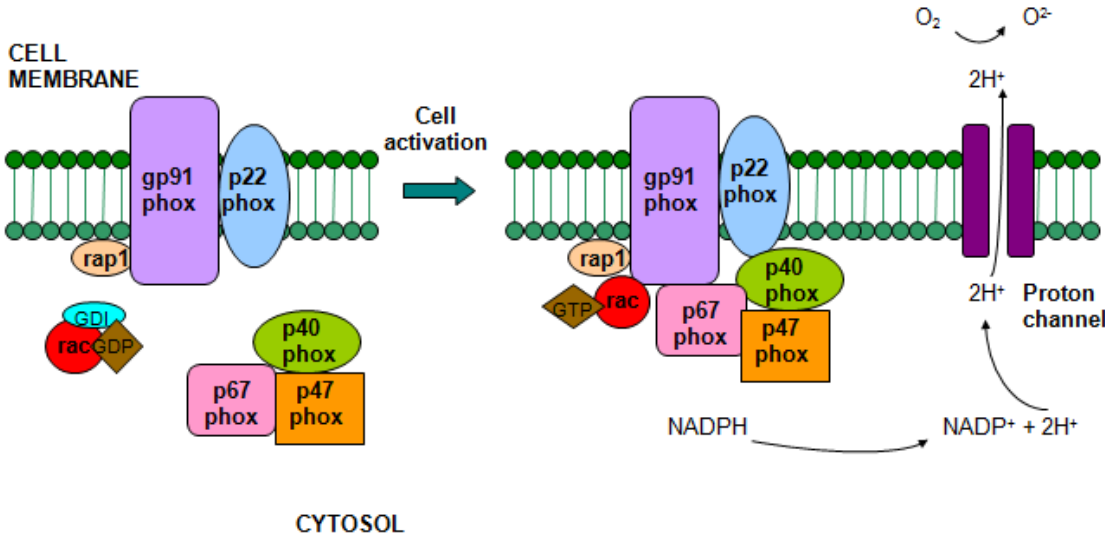


Figure 1-5 NADPH oxidase activation.
Adopted from (91)

1.2.6.7 Effects of dietary polyphenols on NADPH

Dietary Polyphenols such as quercetins have been suggested to modulate NADPH activity. A finding well supported by studies showing that a decrease in NADPH oxide activity and selective reduction in the protein expression of its subunits $P47^{phox}$, $P22^{phox}$ and $gp91^{phox}$ occurs *in vitro* in human endothelial cells in response to red grape juice polyphenols (92). The same is also true in aortic rings (93) where quercetin and isorhamnetin prevented Ang II-induced increase in superoxide production and over expression of $P47^{phox}$.

1.2.7 Endothelial function assessment

1.2.7.1 Overview

With the recognition of endothelial dysfunction as an early event in atherosclerosis, its role in the progression of the atherosclerotic process and capacity to increase the risk of cardiovascular diseases (94-96), it was a logical choice for researchers to target endothelial function assessment as a tool that could possibly reveal the earliest signs of atherosclerosis.

The first endothelial function assessment in humans was performed almost 3 decades ago by Ludmer and colleagues in 1986 (97) where an intracoronary infusion of acetylcholine resulted in paradoxical vasoconstriction of the coronary arteries in patients with atherosclerosis. Since then research has led to the development of a number of reliable methods for the measurement of endothelial function. These include the induction of endothelial dependent vasodilation by infusing vasoactive agents using coronary angiography and a Doppler flow guide wire (97, 98), venous occlusion plethysmography (99, 100), flow mediated dilatation (FMD) (101, 102), pulse wave analysis (103), peripheral arterial tonometry (104) and laser Doppler flowmetry (105).

1.2.7.2 Non-invasive techniques used for assessing endothelial function

As explained earlier vascular function includes the regulation of blood flow and pressure, capillary recruitment and filtration. The mechanisms are complicated and includes interactions between intrinsic (such as NO and prostaglandins) and extrinsic factors (including sympathetic and parasympathetic system, adrenaline and angiotensin). Arterial stiffness (106), hypertension (107) and endothelium dependent vasodilation (108) are important indicators of vascular function and health, are associated with increased cardiovascular risk and mortality. They can therefore be targeted with interventions such as diet and lifestyle modification.

The coronary arteries are the most important site for endothelial dysfunction due to their proximity to the heart and thus as such makes them the prime target for assessment. However, their assessment requires an invasive as well as expensive technique that makes its function assessment complicated. Thus, the availability of techniques that are inexpensive and minimally or non-invasive is important for endothelial function assessment in people at risk that are more vulnerable to invasive procedures. There is a positive correlation between non-invasive conduit vessel function and invasively measured coronary arteries endothelial function (109-111). The fact that endothelial dysfunction precedes gross morphological and clinical symptoms of atherosclerosis makes non-invasive endothelial function assessment more important (66, 112-114).

Endothelial function is widely studied in peripheral arteries mainly using non-invasive techniques and provides a surrogate measure for coronary artery function (111). Although carotid, femoral and radial arteries have also been used, flow mediated dilatation (FMD) assessed by brachial artery ultrasound scanning is one of such techniques that is used most frequently. FMD is an endothelium dependent process where the vessel dilates in response to shear stress stimulus produced by an increased blood flow. The brachial artery FMD technique is reliable and reproducible (109, 115). See Figure 1-6 below for a typical FMD setup.

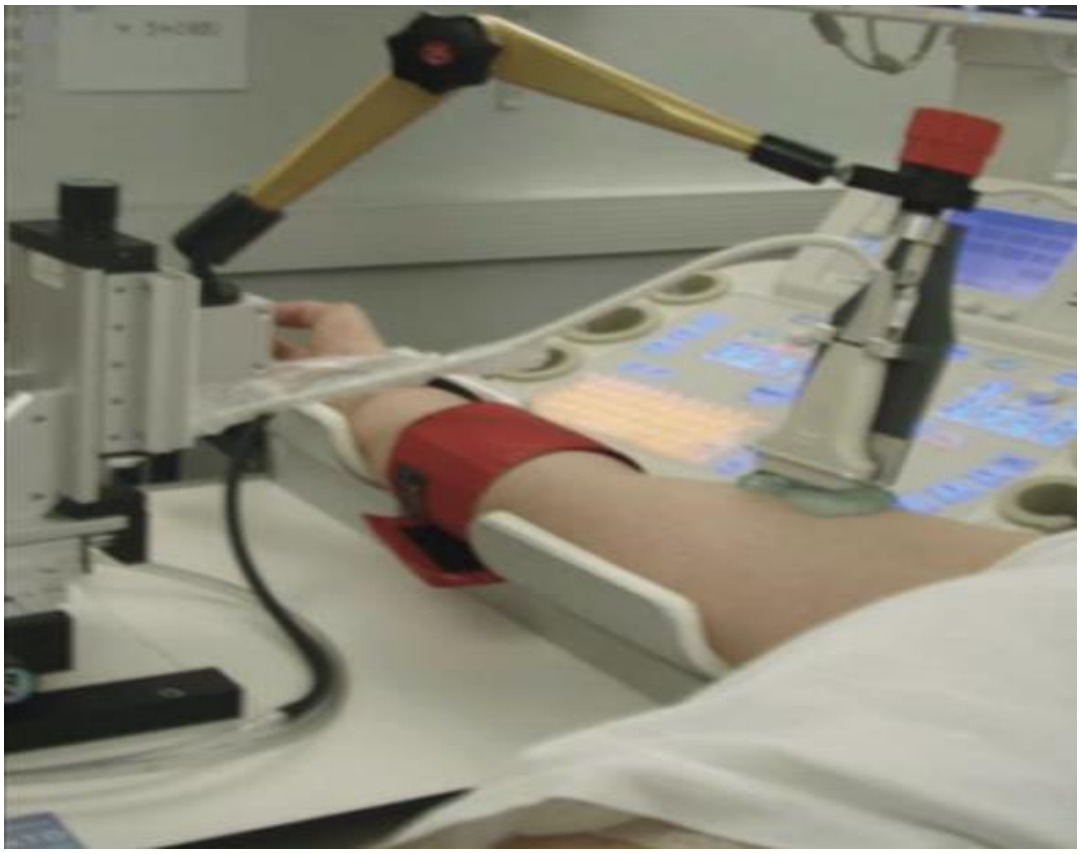


Figure 1-6 Brachial artery diameter measurements using ultrasound scanning for Flow Mediated Dilation (114)

Although several physiological, equipment and operator-related factors can affect the reliability of this technique (71) comparison with intravascular ultrasound ("the gold standard" technique) baseline measurements shows good reproducibility (116). The intra and inter-session variation in baseline diameter for the FMD technique as used in this thesis was acceptable having a coefficient of variation 1.1% and 4.1% but there was considerable variability between sessions in the same individuals, with coefficient of variation of 13.9% for FMD (116). The recommendation is therefore to use FMD for group measurements only as the poor intra individual reproducibility may limit its use as a follow up parameter/marker in individual subjects.

Reactive hyperaemia peripheral arterial tonometry (RH-PAT) is an emerging non-invasive technique that has recently been reported to be an alternative, rapid, operator independent method of assessing endothelial function by calculating the reactive hyperaemic index (RHI) from pulse volume changes at the fingertips using EndoPAT 2000 (113).

EndoPAT 2000 (Itamar Medical Ltd, Caesarea, Israel Figure 1-7) is a non-invasive medical device approved by the FDA for research purposes. It uses Peripheral Arterial Tone (PAT) signal technology for the assessment of endothelium mediated changes in vascular tone using specially designed plethysmographic sensors placed on the fingertips. The PAT signals are a measure of the changes in the digital pulsatile volume.

Figure 1-7 EndoPAT 2000 device

A standard upper arm blood flow occlusion of the feeding artery (applying a standard blood pressure cuff) for 5 minutes creates ischemia down-stream. Releasing the cuff pressure results in a shear stress exerted by the increased blood flow on the endothelium, causing the release of mediators resulting in endothelium-dependent dilatation. These reactive hyperaemic changes are recorded by EndoPAT as an increase in the amplitude of the PAT signal.

1.2.8 Polycystic Ovary Syndrome

1.2.8.1 Overview

PCOS is the most common endocrine disorder in women in their reproductive years that leads to irregular periods, infertility and increased androgen levels causing hirsutism and acne (117, 118). Obesity affects the majority of women with PCOS, and they have a higher prevalence of both impaired glucose tolerance and type 2 diabetes (119, 120). Women with PCOS show increased cardiovascular risk through a higher incidence of hypertension, an adverse lipid profile, and insulin resistance (IR) (121, 122). Insulin resistance is thought to be central to the underlying cause of PCOS, but the underlying pathophysiological mechanisms at the cellular level remain poorly understood. Insulin signalling is normal in PCOS and there are no abnormalities in insulin binding, insulin receptor expression or in second messenger intracellular signalling. However, tissue changes in the adipocyte function, such as glucose transport enhancement (123)and GLUT4 production (124), have been described (125-127). It is also recognized that there is an increased risk of endometrial cancer due to amenorrhoea and oligo menorrhoea leading to relatively unopposed estrogen (128). More recently, it has been recognized that there is an increased risk of non-alcoholic fatty liver disease that may lead to hepatic inflammation and cirrhosis (129).

It is recognized that PCOS is common in women of reproductive age (130); however, prevalence estimates range from 2.2% to as high as 26% (131, 132) due to the variability of symptoms and the different diagnostic criteria used. Differing diagnostic criteria have contributed to the confusion regarding prevalence; for example, use of the Rotterdam criteria led to over twice the prevalence compared to that of the National Institute of Health (NIH) criteria for the diagnosis of PCOS (133). The prevalence of PCOS also differs according to ethnic background; for example, women from South East Asia often present at a younger age and with a more severe phenotype resulting in more severe symptoms (134, 135). The Rotterdam criteria suggest a wider definition for PCOS that requires two of three criteria be present to make the diagnosis of PCOS and therefore leads to four

potential phenotypes (136). These four different phenotypes may have different metabolic profiles. For instance, it has been shown that those women with the non–hyper androgenic phenotype show a milder phenotype with a milder metabolic profile compared to the other phenotypes (137).

Two genome wide association studies in Chinese populations have identified 11 risk loci for PCOS that count for 17 single nucleotide polymorphisms (SNPs) that are related to genetic variants of the disease (138, 139). These SNPs have been related to glucose and lipid metabolism as well as ovarian hormonal regulation and cell cycle regulation. These studies are supported by studies relating the genes to PCOS and in non-Chinese populations (140-143). Its diagnosis and management have been estimated to cost around $ 4 billion per annum to the USA healthcare system (144) while such data for Europe is unavailable.

1.2.8.2 Pathophysiology of PCOS

The primary pathophysiology of PCOS is unknown but there is strong evidence that complex interactions between genetic, environmental and behavioural factors are involved. There is generally an increased amount of Luteinizing hormone (LH) production relative to follicle stimulating hormone (FSH) so that the ratio between these two (LH : FSH) is increased (145) due to changes in the GnRH pulse generator. As a result, there is also an impairment in the production of the sex hormones testosterone and estrogen. Under normal conditions, LH activates ovarian theca cells resulting in testosterone production while FSH stimulates ovarian granulosa cells to convert testosterone into estrogen (146).

The increased LH production relative to FSH in PCOS results is an increased amount of testosterone. Obese patients however have a large amount of adipose tissue and adipocytes contain the aromatase enzyme that converts testosterone into estrone and this increased estrogen can lead to endometrial hyperplasia (147).

In addition, there is insulin resistance in PCOS, which is suggested to be due to a post receptor transduction defect, and occurs in both lean and obese PCOS patients (148). This contributes to the development of the metabolic syndrome that is associated with a cluster of conditions including hypertension, diabetes mellitus,

obesity and dyslipidemia, where there is decreased high-density lipoprotein (HDL) and increased low-density lipoprotein (LDL) cholesterol. All these factors confer an increased risk of atherosclerosis, plaque formation and future cardiovascular events (122, 149).

There is also a decrease in sex hormone binding globulin (SHBG). SHBG binds testosterone and its reduction will ultimately result in increased free testosterone. This may explain why some PCOS patients with normal total testosterone levels have androgenic symptoms such as hirsutism and acne (150).

1.2.8.3 Diagnosis of PCOS

The 1990 NIH preliminary consensus definition has now been replaced by a more recent definition by the Rotterdam European Society for Human Reproduction and Embryology (ESHRE) and the American Society of Reproductive Medicine (ASRM) PCOS Consensus Workshop Group (151, 152).

The Rotterdam criteria (153) have suggested a broader definition for PCOS with two of the three following criteria being diagnostic of the condition (154).

- Polycystic ovaries (either twelve or more follicles, or increase ovarian volume; > 10 cm3)
- Oligo ovulation or anovulation
- Clinical and/or biochemical signs of hyperandrogenism

The use of Rotterdam criteria has led to four PCOS phenotypes (155):

- 'Classical' oligo menorrhea, hyperandrogenism and polycystic ovaries.
- Oligo menorrhea and hyperandrogenism.
- Oligo menorrhea and polycystic ovaries.
- Hyperandrogensism and polycystic ovaries without menstrual irregularity.

These different phenotypes may have different metabolic profiles and the non–hyper androgenic phenotype may represent a form of PCOS associated with a milder metabolic profile compared to the other phenotypes (137, 156).

It should be noted that the diagnosis of PCOS can only be made when other aetiologies for irregular cycles such as thyroid dysfunction or hyperprolactinaemia have been excluded (157, 158).

1.2.8.4 PCOS and Cardiovascular risks

Apart from the hormonal abnormalities of hyperandrogenism and chronic anovulation, most women with PCOS also have insulin resistance that alone confers increased risk of cardiovascular disease irrespective of the presence of other risk factors (159, 160). Other independent risk factors such as dyslipidaemia, hypertension and central obesity in women with PCOS further increase the risk of developing cardiovascular disease (161-163).

1.2.8.5 Endothelial dysfunction in PCOS

Endothelial damage is one of the early signs of cardiovascular disorders and studies in women with PCOS have shown evidence of cardiovascular disorder in the absence of clinically evident disease, by assessing endothelial function (164-167). There is also evidence of elevation in the levels of circulatory markers associated with endothelial dysfunction in PCOS (168, 169). The available literature has contrasting results for brachial artery flow mediated dilatation (FMD) with some reporting lowered FMD (167, 170) while others found no changes (171). This may be due to the high variability between studies as has been pointed out in a recent meta-analysis (172). The results from this meta-analysis of twenty-one published studies showed that participants with PCOS had reduced FMD.

1.3 Aims of the thesis

1) The primary aim of the thesis was to investigate whether Oligopin® when used in a physiologically relevant dose influenced nitric oxide bioavailability, and thus, endothelial function.

2) To determine the effect of Oligopin® on plasma nitrite/nitrate levels, blood pressure and endothelial function compared to a methylcellulose placebo in healthy volunteers.

3) To investigate the association between two non-invasive techniques, namely brachial artery FMD and reactive hyperaemia peripheral arterial tonometry RH-PAT measurements in the same individual with the secondary aim of discovering whether endothelial function assessed by these techniques differs in individuals with PCOS.

4) To develop and validate a standardized, easily reproducible in vitro, model for hypertension to study the effects of dietary polyphenols on endothelial function.

2 General Methods

2.1 *In Vitro* study procedures and protocols

2.1.1 Cell Culture and treatments

Human umbilical vein endothelial cells (HUVECs, Promo cell C-12203) were used for cell cultures in all the *in Vitro* experiments. HUVECs have played a major role as a model system for studying the regulation of endothelial cell function. HUVECs were grown in endothelial cell growth media (ECGM) containing a supplement cocktail (Sigma Aldrich) and 20% FBS (Life Technologies). All cultures were incubated at 37 °C in a humidified atmosphere containing 5 % CO_2 in air. The assays were performed precisely 3 days after seeding to ensure similar cell growth conditions for all experiments.

2.1.1.1 Cell Culture conditions for the Nitric oxide assay

For the nitric oxide assay experiments the HUVEC cells were seeded in 96 well plates at a density of 0.5×10^4 cells per well and were grown in endothelial cell growth media containing supplement cocktail and 20% FBS (200 µl/well) for 2 days. 24 h before assay, the cells were serum deprived in Medium-199 (Invitrogen 11043-023) containing 0.5% foetal bovine serum (FBS). Cells were then treated with either DMF (0.1%), or Oligopin® for 8 hours before assay in Medium-199 containing 0.5% FBS.

2.1.1.2 Cell Culture conditions for Superoxide assay

For the Superoxide assay experiments the HUVEC cells were seeded in 6 well plates at a density of 0.141×10^6 cells per well and were grown in endothelial cell growth media containing supplement cocktail and 20% FBS (2 ml/ well) for 2 days. 24 h before assay, the cells were serum deprived in Medium-199 containing 0.5% foetal bovine serum (FBS). Cells were then treated with either DMF (0.1%), or Oligopin® for 8 hours before assay in Medium-199 containing 0.5% FBS.

2.1.1.3 Cell Culture for endothelial nitric oxide synthase (eNOS) activity assay

For the eNOS activity assay experiments, HUVEC cells were seeded in 6 well plates at a density of 0.141×10^6 cells per well and were grown in endothelial cell growth media containing supplement cocktail and 20% FBS (2 ml/ well) for 2 days. 24 h before assay, the cells were serum deprived in Medium-199 containing 0.5% FBS. Cells were then treated with either DMF (0.1%), or Oligopin® (500 nM) for 8 hours before assay in Medium-199 containing 0.5% FBS.

2.1.1.4 Cell Culture for angiotensin II-induced endothelial hypertension model

HUVEC cells were cultured in T-75 flasks at a density of 1-1.1 x 10^6 cells per flask and were grown in endothelial cell growth media (20% FCS). The cells were serum starved using phenol red free medium 199 + 0.5 % FCS (Invitrogen 11043-023) for 24 hours and were treated with angiotensin II (100 nM) or left untreated for 8 hours before protein extraction.

2.1.2 Nitric oxide assay

2.1.2.1 Overview

The effect of Oligopin® on nitric oxide availability was assessed *in vitro* using the fluorogenic dye 4, 5-diaminofluorescein diacetate (DAF-2DA). DAF-2DA is a non-fluorescent cell permeable reagent that can measure free NO in living cells.

2.1.2.2 The technique's working principles

DAF-2DA, once inside the cell is hydrolysed by cytosolic esterases releasing DAF-2. NO produced in the cells then converts this non-fluorescent dye into its fluorescent triazole derivative that can then be observed fluorometrically.

2.1.2.3 Protocol

Human Umbilical Vein Endothelial Cells (HUVEC, Promo cell) were cultured in 96 well plates at a density of 0.5×10^4 cells per well for 3 days in endothelial cell growth media containing supplement cocktail and 20% FBS. 24 hours before assays, the cells were serum deprived in Medium-199 containing 0.5% foetal bovine serum (FBS). Cells were then treated with either DMF (0.1%, Sigma Aldrich), or Oligopin® for 8 hours before assay in Medium-199 containing 0.5% FBS. These cells were washed in Hank's balanced salt solution (containing magnesium and calcium, HBSS, Sigma Aldrich) and then incubated with 2 µM DAF (Enzo life sciences) in HBSS and the rate of fluorescence accumulation was measured over 30 minutes using a Tecan infinite 200 plate reader ($\lambda_{excitation}$ = 485 nm, $\lambda_{emission}$ = 520 nm). The rate of fluorescence accumulation was calculated for each sample and expressed as a percentage of the control samples.

2.1.3 Superoxide assay

2.1.3.1 Overview

Superoxide ($O_2^{\bullet -}$) is one of the major reactive oxygen species (ROS) generated by endothelial cells (173). Potential endothelial sources of $O_2^{\bullet -}$ include mitochondria, NADPH, xanthine oxidase and cytochrome P-450. Endothelial NOS dysfunction also results in increased superoxide production at the expense of Nitric oxide (173). Increased superoxide production by any mechanism may ultimately result in eNOS uncoupling (i.e. altered eNOS activity, which result in superoxide production instead of NO) and thus more superoxide production leading to oxidative stress. The presence of superoxide in the vasculature greatly alters the bioavailability of NO, and increased reactive oxygen species (ROS) is a hallmark of cardiovascular diseases (174).

2.1.3.2 Protocol

The cells were washed with phosphate buffered saline (PBS, containing 100 µM Diethylenetriamine-pentacetic acid, DTPA) and incubated with 20 µM

Dihydroethidium (DHE) in HBSS and the plate kept at 37 ° C in an atmosphere containing 5 % CO_2 and well protected from light. The supernatant was removed and the cells lysed in 100 % Methanol. The resulting precipitated protein was removed by centrifugation (16 100 × g, 10 minutes at - 4 °C) and the recovered supernatant was used for LC-MS analysis. Chromatography was done using a Shimadzu LC20-AD quaternary pump, SIL-20A HT auto sampler and CTO-10A column oven connected via a FCV-20AH_2 switching valve to a LC2020 single quadrupole mass spectrometer. An Agilent Eclipse-XDB-C_{18} column (5 µm pore size, 4.6 x 150 mm) was used for separating the analytes, with solvent C comprising of 18.2 Ω water containing 0.5 % formic acid, and solvent D comprising methanol containing 0.5 % formic acid. The column was maintained at 40 °C and the flow rate was 0.5 ml/min. An injection volume of 15 µl was used for these experiments.

$O_2^{\bullet-}$ production was measured by a highly specific novel method, which quantifies the reaction product of hydroethidine and NADPH oxidase-derived $O_2^{\bullet-}$ (2-OH-E+) by LC-MS. For HE and 2HE+ separation a gradient of 43 % of solvent D was maintained for 3.35 minutes, followed by an increase in solvent D from 43 % to 85 % over 3.65 minutes. Solvent D was then maintained at 85 % for 4.5 minutes before returning to 43 % over 0.25 minute. The initial starting conditions were re-equilibrated over 7 minutes. Under these conditions ethidium eluted at a retention time (RT) = 3.6 min, dihydroethidium at RT = 6.8, fluorescein (internal standard) at RT = 11.2 and detected at *m/z* = 316, 330 and 333 respectively.

2.1.4 L-Citrulline detection method using LC-MS

2.1.4.1 Overview

Endothelial nitric oxide synthase (eNOS) produces L-citrulline and NO in endothelial cells from L-arginine in the presence of oxygen and co-factors including tetrahydrobiopterin (BH4), NADPH, Calcium and calmodulin (CaM).

2.1.4.2 Working principles of the technique

The assay makes use of a heavy isotope of L-arginine (^{13}C L-arginine). The conversion of L-arginine by eNOS in the presence of the co-factors produces equimolar amounts of NO and L-citrulline thus the amount of ^{13}C L-citrulline produced can be quantified as an indirect measure of NO production.

2.1.4.3 Method development

The various developmental stages of the ^{13}C L-citrulline detection method are graphically represented in Figure 2-1 to Figure 2-7 below.

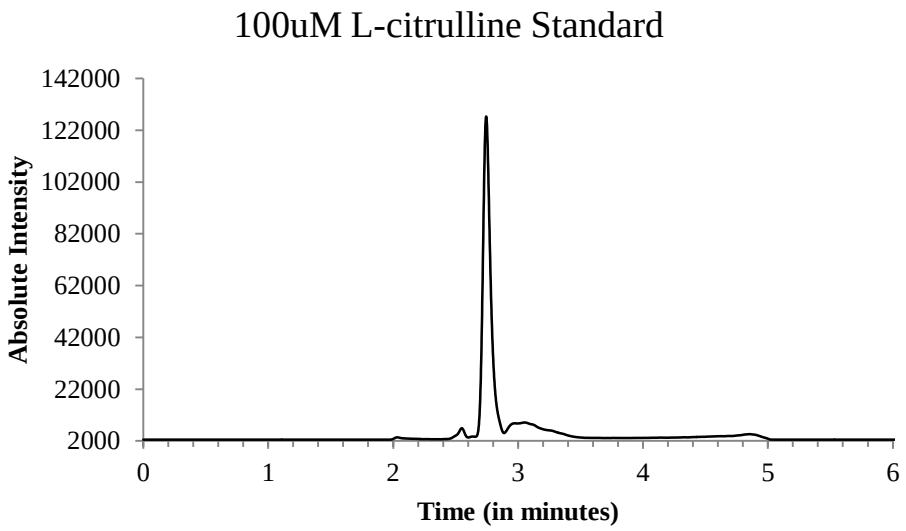


Figure 2-1 Chromatograph showing L-Citrulline.
Note the peak at 2.8 minutes.

C + S - L + I -

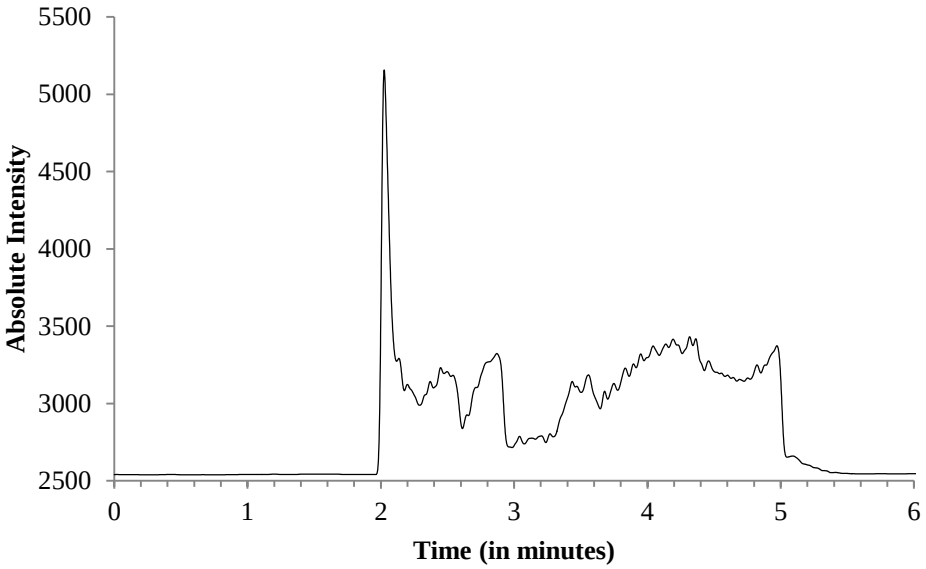


Figure 2-2 Chromatograph without the substrate (^{13}C L-arginine)
Note the lack of a peak at 2.8 minutes. Where + means with and – without C Co-factors. S, Substrate. L, Lysate, I, Inhibitor

C + S + L + I -

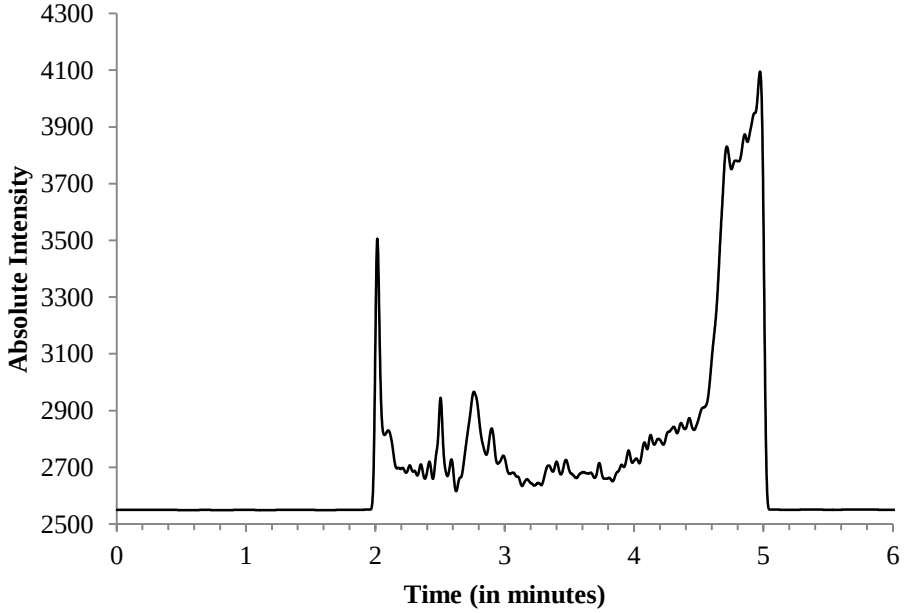


Figure 2-3 Chromatograph of the substrate (^{13}C L-arginine).
Note the appearance of a peak at 2.8 minutes. Where + means with and – without C, Co-factors. S, Substrate. L, Lysate, I, Inhibitor

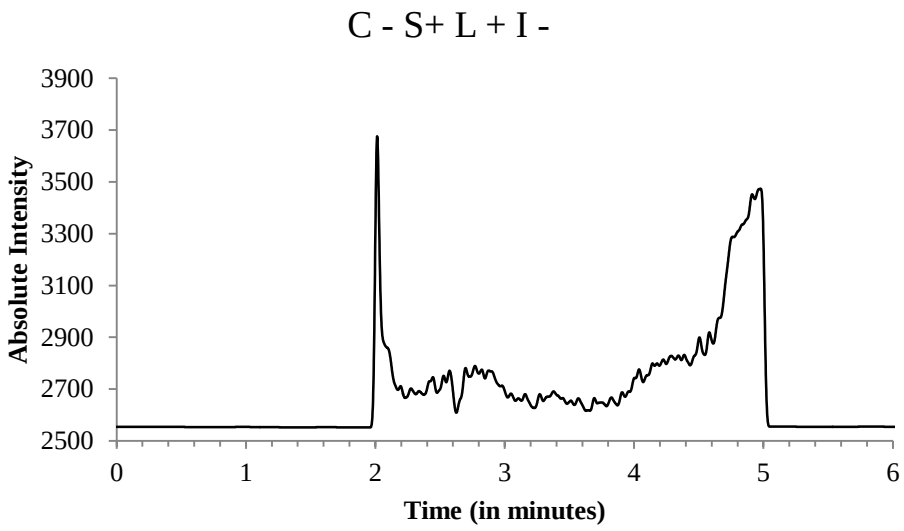


Figure 2-4 Chromatograph showing the effect of omitting the Co-factors for the substrate (^{13}C L-arginine).
Note lack of a peak at 2.8 minutes. Where + means with and – without C, Co-factors. S, Substrate. L, Lysate, I, Inhibitor

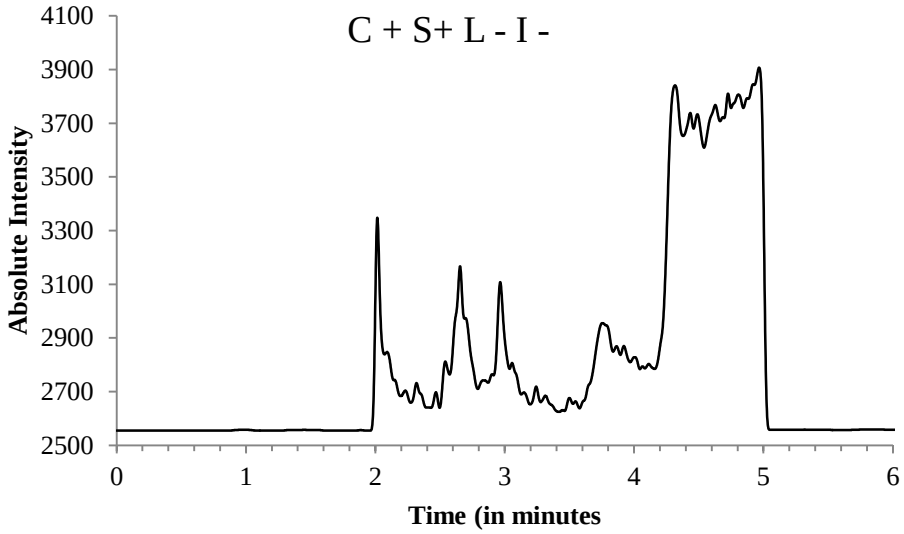


Figure 2-5 Chromatograph showing the effect of omitting the cell lysate substrate sample.
Note the lack of a peak at 2.8 minutes. Where + means with and – without C, Co-factors. S, Substrate. L, Lysate, I, Inhibitor

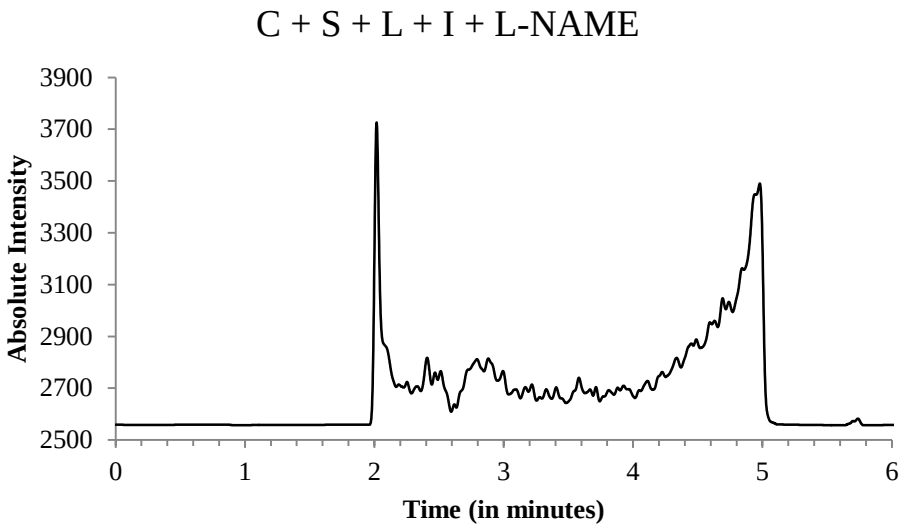


Figure 2-6 Chromatograph showing the effect of adding an inhibitor (L-NAME) of the substrate (^{13}C L-arginine).
Note a diminished peak at 2.8 minutes. Where + means with and – without C, Co-factors. S, Substrate. L, Lysate, I, Inhibitor

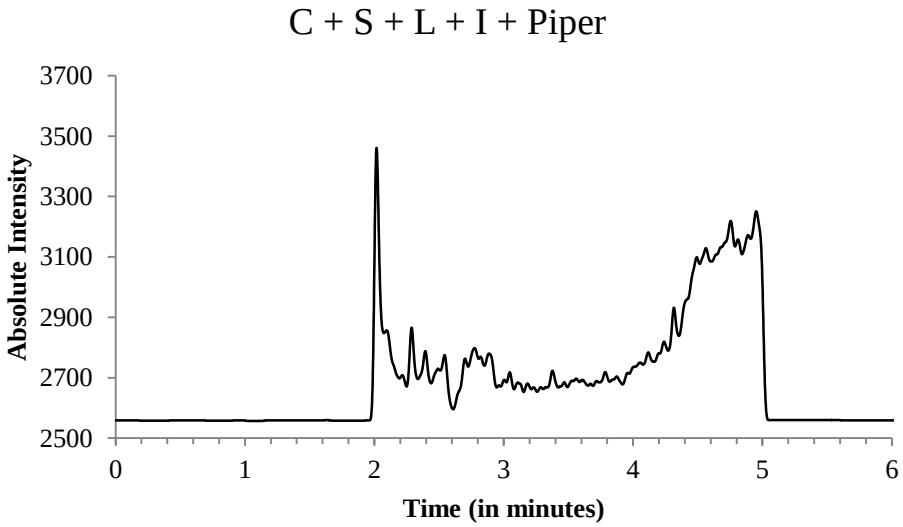


Figure 2-7 Chromatograph showing the effect of adding an inhibitor (Methyl Piperidine) of the substrate (^{13}C L-arginine).
Note a diminished peak at 2.8 minutes. Where + means with and – without C, Co-factors. S, Substrate. L, Lysate, I, Inhibitor

2.1.4.4 Protocol

Overnight serum starved HUVEC cells were treated with or without Oligopin® for 8 hours in 6 well plates. The cell lysate obtained from these experiments was spiked

with a heavy isotope (^{13}C labelled) of the L-arginine amino acid and the product L-citrulline (^{13}C labelled) quantified on the LC-MS.

Briefly, the reaction mixtures (50 µl lysate + 50 µl master mix, 100 µl in total) were incubated for 2 h at 37 °C. The reaction was stopped by adding an equal amount of 100 % methanol and keeping the samples at - 20 °C for at least 1 hour. The resulting precipitated protein was removed by centrifugation (16 100 × g, 10 minutes at - 4 °C) and the recovered supernatant used for LC-MS analysis. Chromatography was performed using a Shimadzu LC20-AD quaternary pump, SIL-20A HT auto sampler and CTO-10A column oven connected via a FCV-20AH$_2$ switching valve to a LC2020 single quadrupole mass spectrometer. An Agilent Eclipse-XDB-C_{18} column (5 µm pore size, 4.6 x 150 mm) was used for separating the analytes as detailed below, with solvent A comprising 18.2 Ω water, and solvent B comprising 100 % methanol. The column was maintained at 40 °C and the flow rate was 0.5 ml/min. An injection volume of 20 µl was used for these experiments.

For ^{13}C labelled L-citrulline separation a gradient of 2 % of solvent B was maintained for 8 minutes, followed by an increase in solvent B from 2 % to 85% over 1 minute. Solvent B was maintained at 85% for 7 minutes before returning to 2% over 1 minute. The initial starting conditions were re-equilibrated over 8 minutes. Under these conditions L-citrulline eluted at RT = 2.8 min and could be detected at *m/z* = 180.

2.1.5 Extraction of protein from HUVEC cells cultured in T-75 flasks

The monolayer of HUVEC cells in each flask was washed with 20 ml ice cold PBS. The PBS from the wash was discarded by pouring it into 2 % Virkon solution. Radio Immune Precipitation Assay (RIPA) Lysis buffer was prepared by adding 1 ml non-Idet P-40 (1% v/v final), 50 ml of PBS, 500 mg sodium deoxycholate (0.5 % w/v), 1 ml 10 % SDS (0.1 % w/v) and top up with PBS to make 100 ml total volume) and then 20 µl of protease inhibitor cocktail/ ml of RIPA added. 200 µl of the RIPA buffer cocktail added to each flask, which were kept on ice until scraping. Each flask scraped on ice with a scraper (applying windscreen wiper technique) to cover all corners of the growth surface. The suspension was transferred to an

Eppendorf tube and allowed to lyse on ice for 5 minutes. Each sample was sonicated (at 25% Ampl) on ice for 3 times (each of 10 seconds) using a SONICS machine (Vibra Cell™). The samples were centrifuged at a speed of 10,000 g for 10 minutes at 4°C and the supernatant transferred to 1.5 ml micro centrifuge tubes, and were stored at -20 °C.

2.1.6 Measurement of protein concentration

The samples were subjected to DC protein assay (Bio Rad®). All samples and standards (Bovine serum albumin) within a concentration range of 0.2-1.5µg were prepared in RIPA buffer. The protein concentration of each sample was determined by comparing their absorbance at 750 nm to that of the known standards using an absorbance plate reader FLUO star Omega (BMG LAB Tech).

2.1.7 Western Blotting

2.1.7.1 Historical Background

Western blotting evolved from Southern blotting named after Southern who invented the technique in 1975 (175) and from Stark's invention of the Northern blotting technique in 1977 (176). Although Stark and his group succeeded in developing an early technique of protein blotting in 1979 (177) Towbin's simpler and faster method for electro blotting proteins which was developed in the same year, is preferred today (178). Burnette in 1981 (179) was the first to give the name "Western blotting" to the technique in recognition of Southern's work and due to the United States west coastal location of the place of its invention.

2.1.7.2 Brief description of the Western Blot Technique's working principles

Western Blotting is very widely used in biochemistry, molecular biology and immunogenetics for the detection of a specific protein from a mixture of different proteins in a given sample. The technique uses gel electrophoresis to separate

denatured proteins based on their size. The proteins are transferred from the gel onto a membrane that is either a nitrocellulose or a PVDF membrane. The membrane is treated with an antibody specific to the target protein (called primary antibody) and a secondary antibody that is usually pre-stained, is introduced and it combines with the primary antibody. This can be detected on a film and used to quantify the protein level in the sample.

2.1.7.3 Protocol

Samples were mixed 1:1 with lamelli buffer (Bio Rad®) and boiled for 10 minutes at 95 ºC. Equal amounts of protein (10 µg) were run on 12% SDS polyacrylamide gels (Bio Rad®) and electro blotted onto 0.2 µm PVDF membranes using a Trans blot turbo transfer system (Bio Rad®). Equal rates of transfer were confirmed by staining with Ponceau S (Sigma). Membranes were blocked with 5% milk (Marvel® (Original) dried skimmed milk) solution prepared in 1xTBS-t (containing Trizma HCl, NaCl and Tween 20) for 30 minutes at room temperature. Membranes were then washed with 1xTBS-t for 10 minutes.

Primary antibodies were prepared ($P47^{phox}$ 1:2000, $gp91^{phox}$ 1:5000 and AT_1 1:2000) in 1% milk solution and the membranes incubated for 1 hour at room temperature. Membranes were washed with 1xTBS-t for 3 x 10 minutes. Secondary antibody (Goat anti Rabbit in 1:30000) was prepared in 1% milk solution and the membranes incubated for 1 hour. Membranes were washed for 3 x 10 minutes with 1xTBS-t. A Clarity Western ECL substrate kit (Bio Rad®) was prepared in a 1:1 ratio of both reagents and applied to the membranes for 5 minutes at room temperature. Membranes were placed in a plastic film in a cassette and exposed to an x-ray film in the dark (2.5 minutes for P47, 1 minute for gp91, 10-20 minutes for AT_1 and 10 seconds for b-actin). The x-ray film was developed and fixed before washing in water. The scanned film was quantified using ImageJ software.

2.2 *In Vivo* study design and protocols

2.2.1 Approvals

All studies were approved by the Research and Development (R & D), Hull and East Yorkshire (HEY) Hospitals NHS trust. The FMD and EndoPAT in PCOS comparison was part of the PCOS genetic bio-bank study approved by NRES Committee North East – Newcastle and North Tyneside 1 (REC reference: 10/H0906/17). NRES Committee Yorkshire and the Humber – South Yorkshire, approved the polyphenol absorption profile study (REC reference: 14/YH/0084).

2.2.2 Recruitment methods

Healthy volunteers were recruited through posters and advertisements on the University of Hull and HEY hospitals intranet while the PCOS patients were recruited from the endocrine clinics. Informed consent was obtained from all study participants and their general practitioners (GPs) were informed about the study.

2.2.2.1 Recruitment Criteria

For the FMD and EndoPAT comparison study in PCOS bio bank, pre-menopausal women aged 18 to 40 years with or without PCOS were approached. The diagnosis of PCOS was based on the Rotterdam criteria (fulfilling two of the following three criteria: 1) Polycystic ovaries 2) Oligo/anovulation and 3) Clinical or biochemical hyperandrogenism). Other causes of hyperandrogenism such as non-classical 21-hydroxylase deficiency, hyperprolactinaemia, Cushing's disease and androgen-secreting tumours were excluded by appropriate tests (136).

For the polyphenol absorption profile study a total of thirteen healthy volunteers (7 males and 6 females) were screened after identification from the diabetes research database of healthy volunteers at the Michael White Diabetes Centre, Hull Royal Infirmary. One subject (P004) was excluded during screening because of difficult venous access so only 12 subjects entered the clinical phase.

For the polyphenol Oligopin® study 24 participants were recruited having the following criteria

2.2.2.2 Inclusion criteria

- Male or Female subjects between the age of 18-65
- No concomitant medication including herbal medicines and food supplements
- No concomitant disease processes
- Body Mass Index 21- 29kg/m^2
- Systolic blood pressure ≤150 mm Hg and diastolic pressure <90 mm Hg
- Subjects who have given informed consent

2.2.2.3 Exclusion criteria

- Patients not wishing to allow disclosure to their GPs.
- Concomitant medication including herbal medicines and food supplements
- Concomitant disease processes
- History of drug/alcohol abuse or Alcohol intake within 24 hours of dosing visit (visits 2-4)
- Body Mass Index <21 and > 29kg/m^2
- Systolic blood pressure >150 mm Hg and or a diastolic pressure>90 mm Hg
- Unable to tolerate polyphenol products or adhere to low polyphenol diet
- Vegetarian
- Subjects not willing or able to fast until 12 noon (a total of 14 hours).
- Pregnant females or planning to conceive in the next 3 months.
- Participation in any other study currently or in the last three months.

2.2.3 Protocols used

2.2.3.1 Polyphenols (Oligopin®) absorption profile study

All the participants attended four visits and the study design is shown below as a flowchart in figure 2.8.

Flowchart of subject enrolment and preparations received through the study:

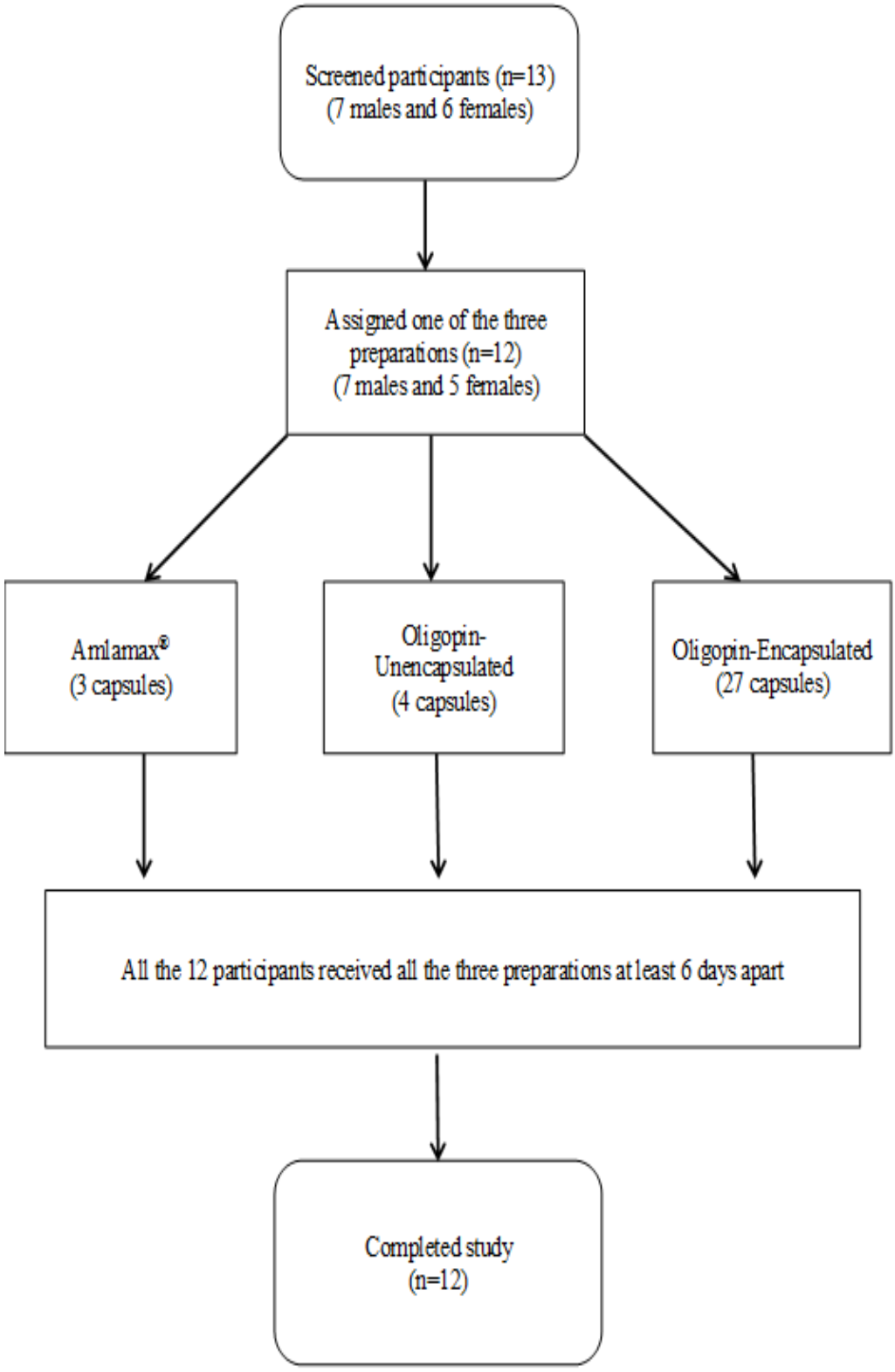


Figure 2-8 Flowchart showing subject enrolment and the polyphenol preparations received

2.2.3.2 Assessment of endothelial function in PCOS and control patients using EndoPAT and FMD.

All the participants in the assessment of endothelial function in PCOS and controls using EndoPAT and FMD study followed the timetable shown in Table 2-1

Table 2-1 Study time table for the EndoPAT and FMD in PCOS

Visit No.	Where	Duration	Requirements	Purpose
Visit 1	Diabetes Centre Hull Royal Infirmary (HRI)	4 hour	Fasting 12 hours except water	To give consent Responding to questionnaire Baseline physical measurement Blood Pressure Blood tests EndoPAT 2000 Collection of saliva Collection of urine Tape to assess greasiness of skin Collection of hair Flow Mediated Dilatation (FMD)
Visit 2	Diabetes Centre, HRI	1 hour	Will arrange at convenience of participant	Vaginal ultrasound of ovaries

2.2.3.3 Effects of Polyphenols (Oligopin®) ingestion on endothelial function

All the participants attended five visits and the protocol followed is shown below in Table 2-2.

Table 2-2 Effects of Polyphenols (Oligopin®) ingestion on endothelial function.

	Visit 1	Visit 2	Visit 3	Visit 4	Visit 5
Informed Consent	x				
Medical History	x				
Inclusion/Exclusion	x				
Full physical examination	x				
BP, Pulse	x	x		x	
Height and weight	x				
Screening blood tests	x				
Fasting before visit		x	x	x	x
EndoPAT, Blood pressure & blood samples before preparation or placebo		x		x	
Polyphenol preparation or placebo given		x		x	
Blood samples at 2 hours		x		x	
EndoPAT, Blood pressure & blood samples at 4 hours		x		x	
Meal (Standardized low polyphenol lunch)		x		x	
EndoPAT, Blood pressure & blood samples at 8 hours		x		x	
Blood samples at 9 hours		x		x	
EndoPAT, Blood pressure & blood samples at 24 hours			x		x
Adverse event recording & Concomitant medication review		x	x	x	x

2.2.4 Dosage and administration of Polyphenols Preparations used

2.2.4.1 Polyphenol Preparations and their respective doses used in the absorption profile study

The doses detailed below have been calculated using data from published peer-reviewed studies and the phenol explorer database, to result in a circulating plasma concentration within the range used for the in vitro screening. Details of the three polyphenol preparations selected for this study are in Table 2.3.

Table 2-3 Polyphenol preparations and their doses used

Internal Code	Product	Manufacturer	Estimated Single dose in the form of capsules	Major constituents that will be quantified by LC-MS*
NP1 (Un-encapsulated)	Amlamax 15% hydrolysable gallo ellagic tannins	Frutarom	**1.47 g** (**3 capsules**** with 470mg per capsule)	Gallic acid Ellagic acid
NP7 (Un-encapsulated)	French Maritime pine bark extract	Oligopin	**1.11 g** (**4 capsules**** with 278mg per capsule)	Catechin Epicatechin
NP7 (Encapsulated)	French Maritime pine bark extract	Oligopin	**7.33 g** (**27 capsules**** with 272mg per capsule)	Catechin Epicatechin

*****Liquid Chromatography-Mass Spectrometry (LC-MS)
****** Each capsule has the same size as a standard Paracetamol 500mg capsule product.

2.2.4.2 Effects of Polyphenols (Oligopin®) on endothelial function

Oligopin® was used at a dose of 1.14g and administered orally. Oligopin® has a high content of catechins and epicathechins and lack tanins. The antioxidant capacity (ORAC value) of Oligopin® is 15000 µmol TE/g.

2.3 Non-invasive Endothelial Function assessment

2.3.1 EndoPAT 2000

2.3.1.1 EndoPAT Protocol followed

Reactive hyperaemic index (RHI) was assessed using a plethysmographic device Endo-PAT 2000 (Itamar Medical Ltd, Caesarea, Israel) (180). The study participants attended the centre at 08:00h after an overnight fasting of at least 10 hours. The participants were asked to relax for at least 15 minutes in a quiet, temperature (22 – 24 ^{o}C) controlled room. Blood pressure readings were recorded (mean of 3 measurements) using Mindray Data scope Duo™ (Mindray DS USA, Inc., Mahwah, NJ 07430) machine. A blood pressure cuff was then secured on the non-dominant arm (study arm), while the other arm served as a control (control arm).

The fingers were inspected for any injuries or deformities that can affect the test results. Endo-PAT bio-sensors were then placed on the index fingers of both hands. The participants were asked to relax and refrain from talking or making any sudden movements during the test. The probes were inflated and the signals recorded on the computer following the manufacturer's (Itamar Medical Ltd, Caesarea, Israel) instructions. Briefly, 5 minutes of baseline recording, followed by blood pressure cuff inflation to a supra-systolic level (at least 60 mmHg above systolic pressure and no less than 200 mmHg) sustained for 5 minutes and then deflated and PAT readings recorded for 5 more minutes.

The data was then analysed by using an automated computer software (Endo-PAT2000 version 3.3.2 Itamar Medical Ltd) the software also gives a value for

Augmentation Index (AI), a measure of the stiffness of the vessel, along with the RHI.

2.3.2 Flow Mediated Dilatation

2.3.2.1 FMD protocol followed

Flow Mediated Dilation (FMD) was assessed using techniques consistent with guidelines discussed elsewhere (181). All tests were performed with a Toshiba Aplio™500 ultrasound system (Toshiba Medical Systems Corporation) using a 14–7.2 MHz linear transducer (Toshiba Aplio 500 PLT-1204BX transducer). The participants were asked to relax for at least 15 minutes in a quiet, temperature (22 – 24 ^{o}C) controlled room. Blood pressure readings were recorded (mean of 3 measurements) using Mindray Data scope Duo™ (Mindray DS USA, Inc., Mahwah, NJ 07430) machine.

The tests were performed with the participants in the supine position, with the right arm placed in a supporting cradle, a blood pressure cuff placed around the forearm and the ultrasound probe placed on the arm proximal to the elbow supported in a stereotactic stand. 3 lead ECG was connected to the ultrasound machine to enable display of the cardiac cycle. Baseline scanning of the brachial artery for a period of 5 minutes was recorded prior to cuff inflation. The cuff was then inflated to a supra-systolic level (at least 60 mmHg above systolic pressure and no less than 200 mmHg) sustained for 5 minutes to achieve total brachial artery occlusion. Recording recommenced at 15 seconds post occlusion, continued for 5 minutes, and stored on a computer connected to the ultrasound machine.

The pre and post occlusion clips were then analysed for diameter measurement by automated edge detection software (Brachial Artery Analyser, MIA-LLC, Coralville, USA). All measurements were obtained during diastole. The point of maximum dilatation was identified and the maximum diameter was obtained by averaging images obtained from 60 to 120 seconds after the release of cuff pressure. The FMD was calculated using the equation:

$$\text{FMD (\%)} = [(\text{peak diameter} - \text{baseline diameter})/\text{baseline diameter}] \times 100$$

2.3.3 Measurement of plasma nitrate and nitrite levels

Nitrate and nitrite concentrations were determined using the Cayman Chemicals Fluorometric Assay kit (catalogue number 780051). Plasma samples (100 µl) were filtered by centrifugation (14,000 x g, 30 minutes) through 10 kDa molecular weight cut-off filters (Millipore, catalogue number MRCPRT010) and the filtrate was used for further analysis. The kit reagents were prepared as directed by the manufacturer protocol with the following modifications.

For nitrate measurements the sample filtrate was diluted 1 to 1 using assay buffer prior to addition to the appropriate wells (10 µl of diluted sample per well). Enzyme cofactors (10 µl) and nitrate reductase (10 µl) were added to each well, and the content of each well was mixed by pipetting (avoiding the formation of bubbles). Following the 2 hour incubation at room temperature the protocol was followed as directed by the manufacturer.

For nitrite measurements the sample filtrate was added without dilution to the appropriate wells (10 µl of filtrate per well). The kit instructions provided by the manufacturer were then followed for the remainder of the assay.

All samples and standards were measured in duplicate wells, with the appropriate standard curve included for every plate. Fluorescence was measured using a Tecan Infinite MX200 plate reader with the following settings: excitation wavelength = 360 nm, emission wavelength = 430 nm, gain setting = 50, multiple reads per well (3x3 filled circle grid), 25 flashes. Standard curves were plotted and used for the quantification of nitrite or nitrate in samples via rearrangement of the equation of a straight line ($y=mx+c$) solved for x.

2.3.4 Statistical Analysis

Normality of distribution of the data was tested using the Shapiro-Wilk test. For parametric data, a t-test or ANOVA of repeated measurements with appropriate post-hoc test was used. If the data was not normally distributed, ANOVA of repeated measurements on ranks was done with comparisons versus control or

baseline samples were done using an appropriate post-hoc test. The Sigma plot v.13 software was used to do these statistical tests.

3 Open label, dose response study of absorption profiles of three polyphenol preparations with and without sporopollenin exine encapsulation

3.1 Introduction

Many polyphenol extracts are sold as ingredients in a large number of dietary supplements and cosmetics but there is still little data on their comparative absorption to whether there is a linear absorption profile or does it plateau? These are lipid soluble compounds and therefore a secondary question is whether pollen shells that have been shown to enhance the absorption of fish oils can enhance their absorption. These studies may then inform on how polyphenols can be used to maximise their health benefits.

A range of commercially available plant extracts were screened, both under basal as well as stress (angiotensin II (a physiological regulator of blood pressure)) conditions for any effects on nitric oxide availability. Only Amlamax® and Oligopin® were observed to maintain or increase nitric oxide availability in the *in vitro* model (HUVECs) and thus were selected for this clinical study. The details of these preparations and their doses used are detailed in **Chapter 2**.

The main objectives of this open label, dose response study of absorption profiles were

1) To determine the basic pharmacokinetic profile for Oligopin® and Amlamax® (amla fruit extract rich in polyphenols) in healthy volunteers to inform the selection of appropriate time-points for the clinical and biochemical assessment of the effects on vascular tone in healthy volunteers

2) To assess the effect of encapsulation of the preparation in sporopollenin exines as a potential method for overcoming the limited bioavailability of polyphenolic chemicals

3) To collect pilot biochemical data that allows the ranking or shortlisting of a single candidate preparation for a full clinical assessment of the effects on vascular tone.

3.2 Methods

12 healthy volunteers were enrolled in the study. Each participant received all the three polyphenol preparations (1 encapsulated (Oligopin®) and 2 unencapsulated (Amlamax® and Oligopin®) on three separate visits, after an overnight fast. Each visit was a week apart. The details of all the methods and protocol used in this study are in **Chapter 2**.

3.3 Results

3.3.1 Demographics and baseline clinical data

There were seven males and five females with an average age of 28.67±7.14 (Mean ± SD), BMI 25.73±2.67, SBP 117.92±13.79, DBP 74.83±8.97 and HR 75.33±14.97. The baseline characteristics are given in Table 3.1.

Table 3-1 Subject characteristics on screening

S #	Age	Sex	SBP	DBP	Weight	Height	BMI	Hip	Waist	HR	Temp
P001	40	M	107	59	78.65	1.8	24.3	100	89	59	35.9
P002	18	F	127	87	73	1.62	27.8	105	81	92	36.9
P003	19	M	118	69	82	1.72	27.7	108	91	62	36.2
P004	43	F	107	67	72	1.65	26.4	109	90	73	37
P005	30	M	124	88	99	1.87	28.3	112	98	84	35.8
P006	36	F	113	69	58	1.58	23.2	100	71	82	36.4
P007	25	F	140	86	90.2	1.78	28.5	118	86	95	37.2
P008	21	M	130	76	75.3	1.8	23.2	101	82	55	35.5
P009	31	M	133	81	81.1	1.78	25.6	110	89	90	35.8
P010	24	F	107	73	58.9	1.64	21.9	96	84	58	36.5
P011	35	F	96	70	56	1.6	21.9	82	67	68	36.8
P012	33	M	121	72	98	1.86	28.3	115	105	69	36.6
P013	32	M	99	68	93.7	1.83	28.0	108	101	90	36.7

Abbreviations (Units): Age (years); BMI, body mass index (kg/m2); SBP, systolic blood pressure in (mmHg); DBP, diastolic blood pressure (mmHg); Weight (Kg); Height (meters); Hip, hip circumference (cm); waist, waist circumference (cm); HR, heart rate (per minute); Temp, body temperature (degree centigrade, ^{o}C)

3.3.2 The Pharmacokinetics of Amlamax® (see figure 3.2)

For the samples from the 6 individuals analysed, 5/6 samples had no detectable levels of ellagic acid at baseline (Time point 0 hour i.e. before administration of the polyphenol preparation). The presence of ellagic acid was observed over a 2-hour period, with no detectable levels observed at 4 or 8 hours post ingestion of the Amlamax® capsules (containing amla fruit extract and has the same size as a standard 500mg Paracetamol capsule). Thus, the maximum (or peak) serum

concentration (C_{max}) for Amlamax® was between 1-2 hour post ingestion, with elimination from the plasma by 4 hour. The C_{max} was estimated to be 339.8 nM and area under the curve (AUC) calculated to be 789.0 ± 526.9 nM per h. The data were used to generate the graph in figure 3.2 from the values in Table 3.3. Table 3.4 displays the calculated values for the AUC calculations for Amlamax®.

Table 3-2 Bioavailability data for Amlamax®.
Plasma concentrations, determined from plasma-spiked standard curves, are displayed in nM.

Study ID	Hours post-ingestion of preparation					
	0	0.5	1	2	4	8
001	0.00	0.00	696.11	335.57	0.00	0.00
002	0.00	0.00	0.00	585.46	0.00	0.00
003	0.00	0.00	923.52	666.64	0.00	0.00
005	0.00	0.00	92.41	172.65	0.00	0.00
008	178.51	244.24	106.08	112.64	0.00	0.00
009	0.00	0.00	193.55	166.03	0.00	0.00

Table 3-3 Calculation of AUC for Amlamax® using the trapezoid method.
Values are expressed in nM.h

Time-point bracket (h)	Volunteer ID					
	001	**002**	**003**	**005**	**008**	**009**
0-0.5	0.00	0.00	0.00	0.00	105.69	0.00
0.5-1	174.03	0.00	230.88	23.10	87.58	48.39
1-2	515.84	292.73	795.08	132.53	109.36	179.79
2-4	335.57	585.46	666.64	172.64	112.64	166.03
4-8	0.00	0.00	0.00	0.00	0.00	0.00
Total	1025.43	878.20	1692.60	382.28	415.27	394.21

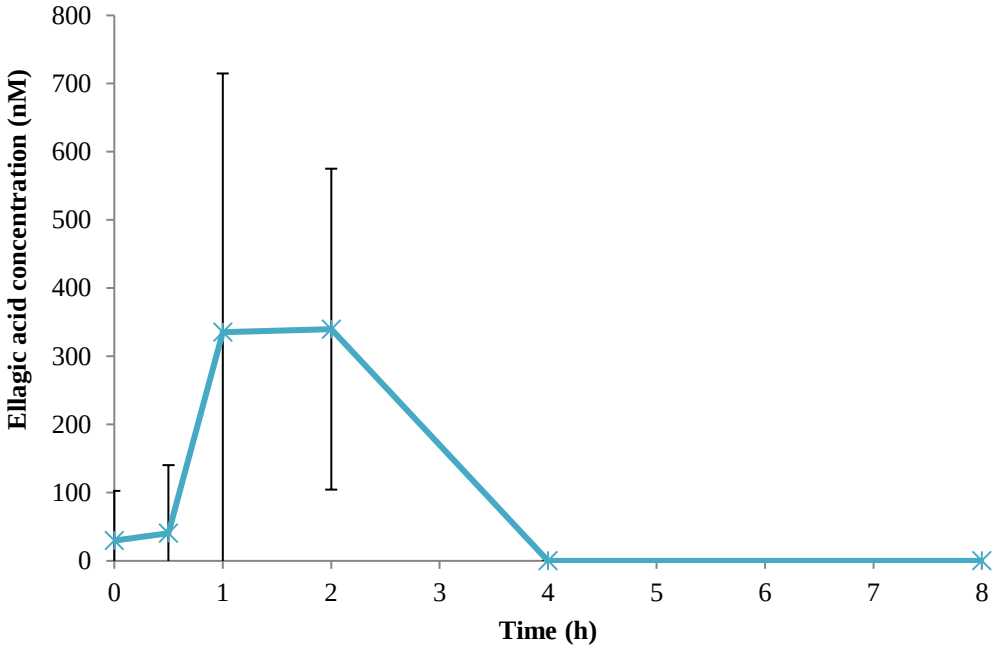


Figure 3-1 The pharmacokinetic properties of Amlamax®.
Data plotted as mean plasma concentration of ellagic acid (detected by LC-MS) for each time-point ± SD (n=6**).**

3.3.3 The Pharmacokinetics of Oligopin® (see figure 3.3)

For the six volunteer samples analysed, all had detectable levels of catechin at baseline (Time point 0 hour). Epicatechin was not detected in any samples. Catechin was detected over the full 8-hour time-course, with no evidence of C_{max} being reached or catechin being eliminated from the plasma. The AUC was calculated to be 2173.81 ± 1295.13 nM.h for Oligopin® in this study. The plasma concentrations of catechin detected for each individual are listed in Table 3.5, with the calculated AUC data displayed in Table 3.6.

Table 3-4 The bioavailability data for Oligopin®.
Plasma concentrations, determined from plasma-spiked standard curves, are displayed in nM. Note that P011 has a missing value for the 0.5 hour sample due to the failure of this sample to inject properly on the LC-MS system.

Study ID	Hours post-ingestion of preparation					
	0	**0.5**	**1**	**2**	**4**	**8**
006	481.36	277.72	656.68	391.12	328.39	888.17
007	147.59	230.77	173.34	278.85	407.42	349.02
008	214.20	173.09	267.52	234.78	238.33	330.36
009	327.92	157.67	173.89	118.29	54.51	367.84
010	115.33	116.48	218.95	140.82	56.19	40.05
011	403.62		712.62	514.59	739.76	900.19

Table 3-5 The calculation of AUC for Oligopin® using the trapezoid method.
Values are expressed in nM.h

Time-point bracket (h)	Volunteer ID					
	006	**007**	**008**	**009**	**010**	**011**
0-0.5	189.77	94.59	96.82	121.40	57.95	100.90
0.5-1	233.60	101.03	110.15	82.89	83.86	178.16
1-2	523.90	226.10	251.15	146.09	179.89	613.60
2-4	719.51	686.27	473.10	172.80	197.01	1254.36
4-8	2433.12	1512.88	1137.97	844.70	192.49	3279.91
Total	4099.91	2620.87	2069.20	1367.87	711.20	5426.93

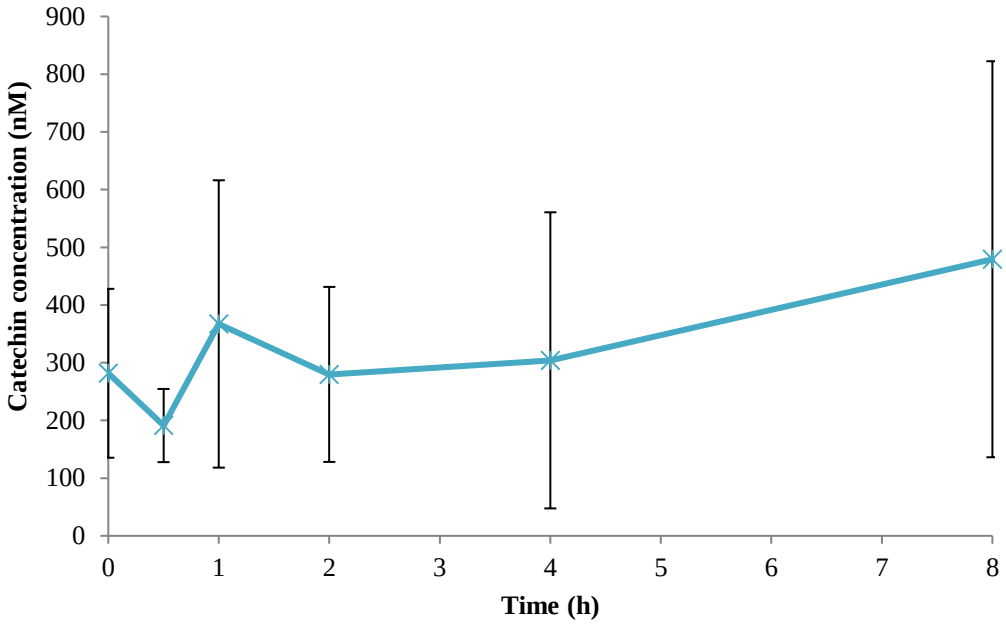


Figure 3-2 The pharmacokinetic properties of Oligopin®.
Data plotted as mean plasma concentration of catechin (detected by LC-MS) for each time-point ± SD (n=6).

3.3.4 The Pharmacokinetics of sporopollenin exine-encapsulated Oligopin® (see figure 3.4)

For the samples of the four volunteers analysed, all had detectable levels of catechin at baseline (0 hour). Epicatechin was not detected in any samples. Accumulation of catechin was observed over the full 8 hours, with no evidence of C_{max} being reached or elimination from the plasma. The AUC was calculated to be 5638.58 ± 2660.77 nM.h for sporopollenin exine-encapsulated Oligopin® in this study. The plasma concentrations of catechin detected for each individual assessed are listed in Table 3.7, with the calculated AUC data displayed in Table 3.8.

Direct comparison of Oligopin® and sporopollenin exine-encapsulated Oligopin® is difficult, given the small number of samples analysed leading to wide variability in the plasma levels of catechin. However, the current data, based on the comparison of AUC for Oligopin® versus sporopollenin exine-encapsulated Oligopin®, suggests that there is a 2.6 times increase in the bioavailability of

Oligopin® when encapsulated in sporopollenin exines, however this difference is not statistically significant.

Table 3-6 The bioavailability data for sporopollenin exine-encapsulated Oligopin®. Plasma concentrations, determined from plasma-spiked standard curves, are displayed in nM

Study ID	Hours post-ingestion of preparation					
	0	**0.5**	**1**	**2**	**4**	**8**
010	772.78	670.62	1025.40	852.68	892.98	954.00
011	309.60	1280.51	1272.82	1152.61	1015.51	1064.37
012	331.42	367.84	410.53	436.65	388.09	707.98
013	356.54	276.73	302.56	380.01	311.77	522.38

Table 3-7 The calculation of AUC for Oligopin® using the trapezoid method. Values are expressed in nM.h

Time-point bracket (h)	Volunteer ID			
	010	**011**	**012**	**013**
0-0.5	360.85	397.53	174.82	158.32
0.5-1	424.01	638.33	194.59	144.82
1-2	939.04	1212.71	423.59	341.28
2-4	1745.67	2168.12	824.74	691.77
4-8	3693.97	4159.74	2192.14	1668.30
Total	7163.54	8576.43	3809.87	3004.50

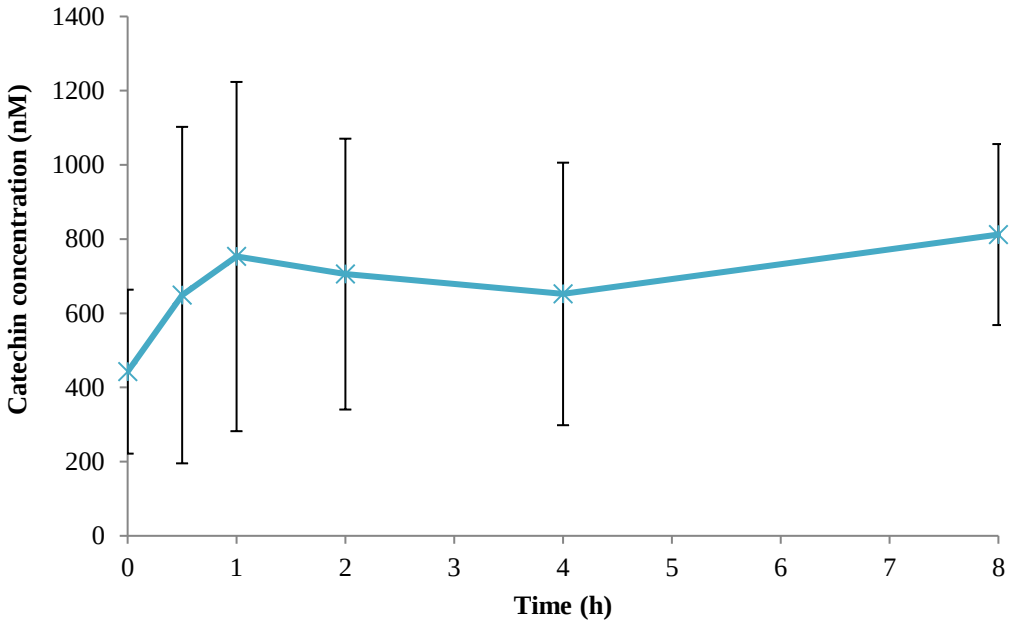


Figure 3-3 The pharmacokinetic properties of sporopollenin exine-encapsulated Oligopin®.
Data plotted as mean plasma concentration of catechin (detected by LC-MS) for each time-point ± SD (n=4).

3.3.5 Effects of Amlamax®, Oligopin®, and sporopollenin exine-encapsulated Oligopin® on nitrate and nitrite levels in plasma samples from healthy volunteers

For Amlamax® (n=11), the plasma levels of nitrate and nitrate at all-time points did not change significantly from baseline levels, with the nitrite plasma concentrations shown in Table 3.9 and Figure 3.5.

Table 3-8 Nitrite concentrations (µM) measured in plasma samples from volunteers. Corrected for baseline measures

Time (h)	Volunteer ID										
	001	**002**	**003**	**005**	**006**	**007**	**008**	**009**	**010**	**011**	**012**
0	0	0	0	0	0	0	0	0	0	0	0
2	0.37	-1.10	0.73	4.03	0	0	0	0	2.28	-3.32	3.79
4	0	-1.10	-0.73	0	0	0	0	0	-1.39	-8.03	-1.58
8	0.37	0.37	-0.73	0	0	0	1.91	4.40	-0.76	0.45	0.76

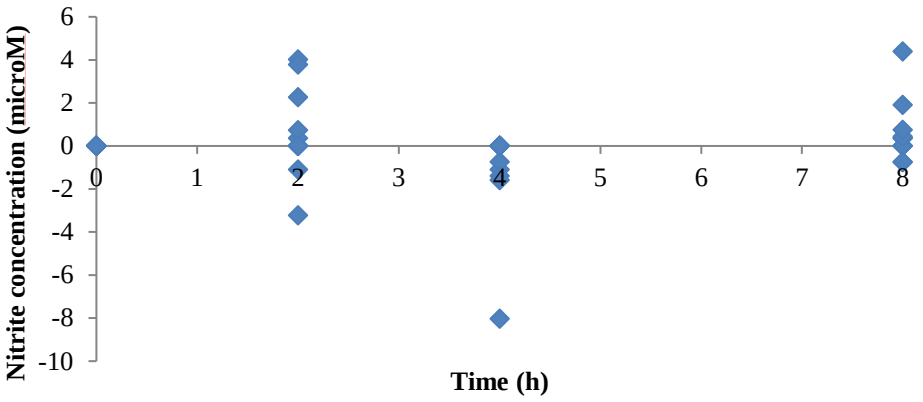


Figure 3-4 Amlamax® ingestion did not significantly alter plasma nitrite concentrations relative to baseline (n=11). Each point plotted on the above graph represents a single individual from the study.

For Oligopin® (n=11), the plasma levels of nitrate at all time points did not change significantly from baseline levels. However, the nitrite levels significantly changed by hour 8 compared to baseline levels ($p<0.05$) Table 3.10 and Figure 3.6.

Table 3-9 Nitrite concentrations (µM) measured in plasma samples from volunteers. Corrected for baseline measures

Time (h)	Volunteer ID										
	001	**002**	**003**	**005**	**006**	**007**	**008**	**009**	**010**	**011**	**012**
0	0	0	0	0	0	0	0	0	0	0	0
4	0	0	-10.25	0	-4.03	0	0	-0.96	-1.33	-3.86	-2.47
8	1.10	2.56	4.76	7.32	1.46	4.03	4.79	-0.19	0.38	1.71	0.38

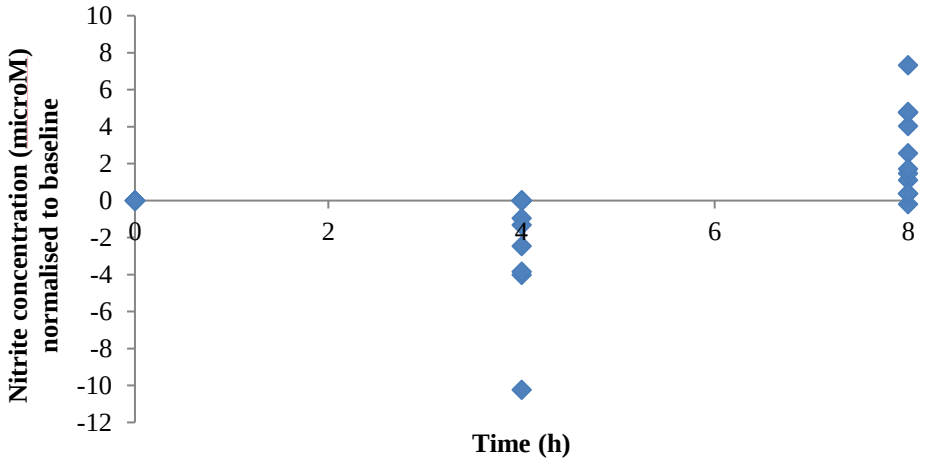


Figure 3-5 Oligopin ingestion significantly increased plasma nitrite concentrations (8 h post ingestion) relative to baseline ($p<0.05$, n=11). Each point plotted on the above graph represents a single individual from the study.

Sporopollenin exine-encapsulated Oligopin did not significantly alter nitrate levels in volunteer plasma post ingestion, compared to baseline levels (n=9). Interestingly, the observed trend of nitrite level increase for the Oligopin at 8 h post-ingestion was not observed for the sporopollenin exine-encapsulated Oligopin ($p>0.05$, n=10) Table 3.11 and Figure 3.7.

Table 3-10 Nitrite concentrations (µM) measured in plasma samples from volunteers. Corrected for baseline measures

Time (h)	Volunteer ID									
	001	**002**	**003**	**005**	**013**	**007**	**008**	**010**	**011**	**012**
0	0	0	0	0	0	0	0	0	0	0
4	1.39	5.38	0	4.17	0	-0.57	0	-0.96	0	0
8	10.88	0	0	2.28	-.077	-0.57	0.19	-0.96	0.19	1.53

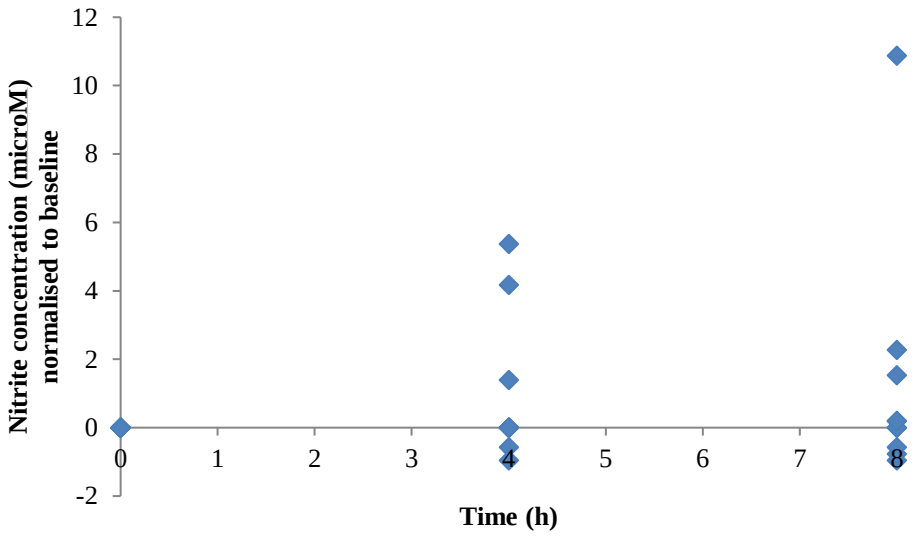


Figure 3-6 Sporopollenin exine-encapsulated Oligopin® did not significantly alter plasma nitrite concentrations relative to baseline levels (n=10). Each point plotted on the above graph represents a single individual from the study.

3.4 Discussion

This study has assessed the uptake and potential for three preparations (Amlamax®, Oligopin®, and sporopollenin exine-encapsulated Oligopin®) to modulate vascular function, using nitrate and nitrite plasma concentrations as a measure of nitric oxide availability. It has shown that although all three preparations are detectable in the plasma of healthy volunteers, at concentrations of several hundred Nano molar, only Oligopin® (when not encapsulated in sporopollenin exines) increases nitrite

concentrations (at 8 hours post ingestion) compared to baseline measurements. Sporopollenin exine encapsulation does not appear to improve the bioavailability of Oligopin® substantially (although this comparison should be interpreted with caution due to the low number of samples analysed) and encapsulation resulted in a loss of the effect of Oligopin® on plasma nitrite concentrations. The lack of complete bioavailability data does not however prevent the selection of a single preparation for the second clinical study, as PK data does not provide any information regarding the bioactivity of the preparation. This information is provided by the nitrate/nitrite measurements.

Although some data on the bioavailability of Amlamax® and Oligopin® has been generated, this data is incomplete. We have shown that both preparations are detectable in the blood plasma of healthy volunteers after the ingestion of a single dose, and Amlamax® was observed to peak and then undergo clearance from blood plasma over 4 hours. The pharmacokinetics of Oligopin® are substantially more complicated. No plasma maximum or clearance was observed over the 8 hour period assessed, and catechin was also detected in baseline samples. As a result, it is difficult to draw any conclusions regarding the pharmacokinetics of Oligopin® from this study. With regard to assessing the effect, if any, of sporopollenin exine encapsulation on the bioavailability of Oligopin®, the low number of samples analysed (n=6 individuals for non-encapsulated Oligopin® and n=4 individuals for encapsulated Oligopin®) combined with the complicated pharmacokinetics of Oligopin®, means that drawing any robust conclusion from this data is not possible.

The measurement of plasma nitrate and nitrite as indicators of nitric oxide levels (and thus potential biological effects of the tested preparations on the vasculature), were completed for each preparation for the majority of samples (n=11 for Amlamax® and Oligopin®, n=10 for sporopollenin exine-encapsulated Oligopin®) at baseline, 2 hours (Amlamax® only), 4 hours and 8 hours. For the sporopollenin exine-encapsulated Oligopin® samples, only 10 individuals were analysed. The nitrate and nitrite measurements showed that only Oligopin® significantly altered nitrite levels above those of baseline samples, and sporopollenin exine encapsulation of Oligopin® appeared to prevent this effect. This data suggests that

Oligopin® may increase nitric oxide levels and thus have bioactive effects on the vasculature in healthy volunteers, meeting the secondary objective of this study.

Overall, despite the incomplete bioavailability data, there was sufficient information produced by this study to select Oligopin® as the candidate preparation to take forward for a proof of principle study.

Although this study has provided evidence that all of the tested preparations can be detected in blood plasma after a single dose, there are several limitations of this study. Firstly, equipment failure resulted in incomplete analysis of all the samples taken. Although this does not prevent the calculation and estimation of pharmacokinetic parameters, it does mean that it is difficult to determine the effect, if any, that sporopollenin exine encapsulation has on the bioavailability of Oligopin®. Another limitation of this study is the detection of catechin in the baseline samples of volunteers during this study, and the lack of any observable clearance of Oligopin® over the 8 hours of this study. It has been reported for another French maritime pine bark extract (Pycnogenol®) that its constituents can be detected in plasma up to 14 hours post ingestion, and this study also failed to determine complete clearance from blood plasma samples in healthy volunteers (19). As both these extracts contain procyanidins (complex branched multimer structures made from catechin and epicatechin monomer units), it is likely that these complex structures result in longer half-life in the human body than that of catechin and epicatechin monomers alone.

Based upon these data it was determined that Oligopin® should be taken forward to the second clinical study for proof of principle assessment for beneficial effects upon vascular function and tone, based on the measurement of plasma nitrate and nitrite levels, and non-invasive measurement of vascular reactivity.

4 Effects of polyphenols treatment on endothelial (HUVEC) cells in in vitro model

4.1 Introduction

Oligopin® (DRT) accumulated over time in healthy volunteers. Plasma samples analysis for nitrate and nitrite levels (which are indicative of nitric oxide bioavailability – a key regulator of vascular tone and function) indicated that the pine bark extract (Oligopin®) could improve nitric oxide bioavailability. In order to investigate this further, the effects of Oligopin® (DRT) on NO bioavailability were assessed in vitro by treating Human umbilical vein endothelial cells (HUVECs) with a dose in the physiological range obtained from the bioavailability study (see **Chapter 3** for details) of the same preparation in healthy volunteers.

The primary aim of the study was to confirm and evaluate further, whether Oligopin® (in a physiologically relevant dose) has any effects on nitric oxide bioavailability and thus on endothelial function.

4.2 Methods

HUVECs were used as an *in vitro* model for this study and the methods used included Nitric oxide assay, Superoxide assay and eNOS activity assay. The cell culture conditions and the protocols used for each of the above methods are detailed in **Chapter 2**.

4.3 Results

4.3.1 Effects of Oligopin® on Nitric oxide production

Oligopin® treatment of overnight serum starved HUVEC cells for 8 hours significantly improved nitric oxide availability at all physiologically relevant concentrations used (ranging from 1nM to 1000 nM). The percentage change in nitric oxide production for all the concentrations used is shown in Figure 4-1.

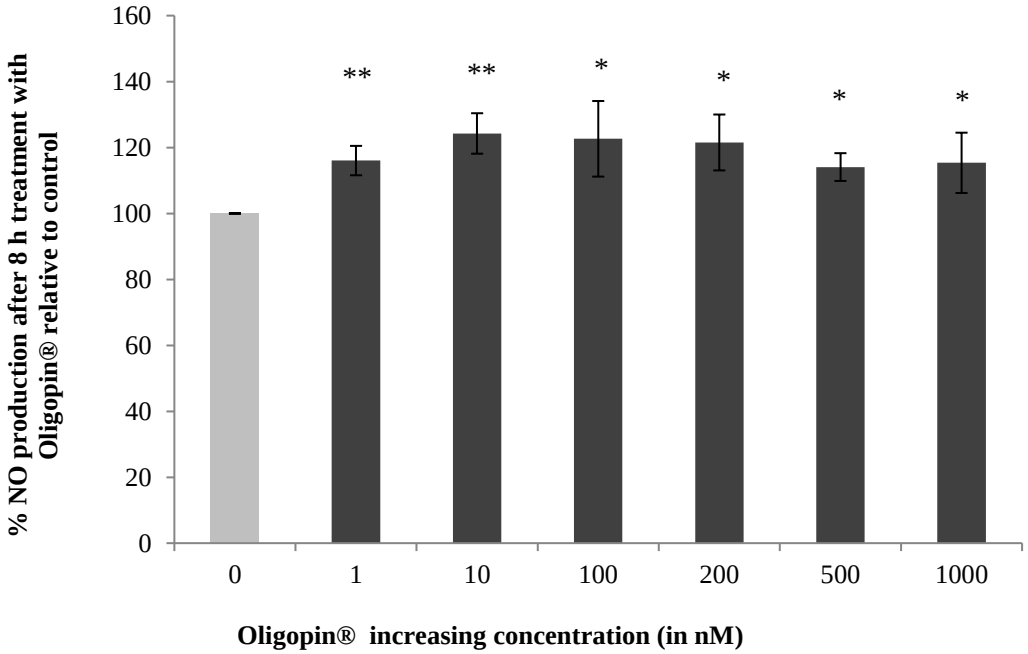


Figure 4-1 Percent change in Nitric oxide production in HUVEC cells after 8 hours treatment with different concentrations (in Nano moles) of Oligopin® relative to controls (with no treatment).
Transformed means % ± SE (t-test). Number of individual experiments = 4. Significance: a value of < 0.05 was considered as significant. *, p-value ≤ 0.05; **, p-value ≤ 0.005; nM, nanoMole; NO, Nitric oxide; HUVEC, Human umbilical vein endothelial cells.

4.3.2 Effects of Oligopin® on Superoxide production

When overnight serum starved HUVEC cells were incubated for 8 hours with 500nM (a dose obtained from earlier work on Oligopin® absorption and bioavailability in healthy volunteers) Oligopin® it resulted in a significant decrease in superoxide production (p-value = 0.0361). The percent change in superoxide production is presented in Figure 4-2.

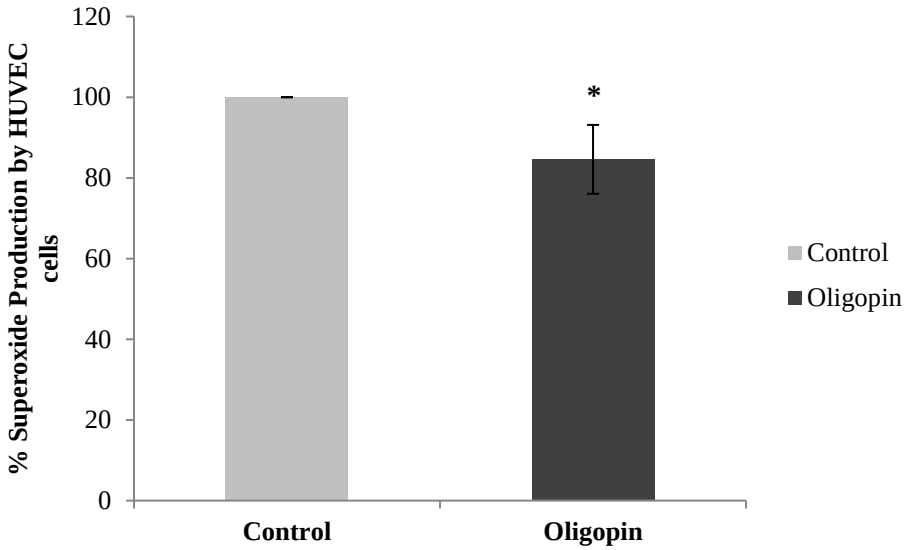


Figure 4-2 Percent change in Superoxide production in HUVEC cells after 8 hours treatment with 500 nM Oligopin® relative to controls (with no treatment).
Transformed means % SE (t-test). Number of individual experiments = 4. Significance: a value of ≤ 0.05 was considered as significant. *, p-value ≤ 0.05; nM, nanoMole; NO, Nitric oxide; HUVEC, Human umbilical vein endothelial cells.

4.3.3 Effects of Oligopin® on endothelial nitric oxide synthase (eNOS) activity

Liquid Chromatography-Mass Spectrometric (LC-MS) analysis of the cell lysate (obtained from treatment of overnight serum starved HUVEC cells with 500nM Oligopin® for 8 hours) containing a heavy isotope (^{13}C labelled) of the L-arginine amino acid showed a significant increase in the product L-citrulline (^{13}C labelled). (P-value = 0.0066) and thus suggested an increased eNOS activity Figure 4-3.

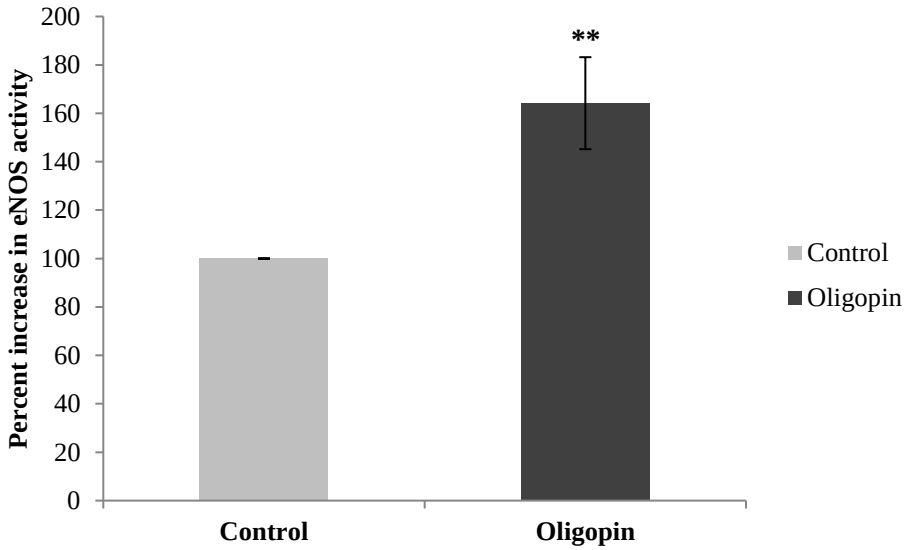


Figure 4-3 Percent change in eNOS activity in HUVEC cells after 8 hours treatment with 500 nM Oligopin relative to controls (with no treatment). Transformed means % SE (t-test). Number of individual experiments = 4. Significance: a value of ≤ 0.05 was considered as significant. *, p-value ≤ 0.05; nM, nanoMole; eNOS, endothelial Nitric oxide synthase; HUVEC, Human umbilical vein endothelial cells.

4.4 Discussion

Polyphenols have attracted increasing attention in the past few decades for their reported health benefits (182, 183). The commercially available polyphenol preparations such as the Oligopin® used in this study are a mixture of a diverse group of chemicals including flavonoids mainly procyanidins and phenolic acids and there is no surprise that these mixtures have different modes of action including effects such as an antioxidant, anti-inflammatory and through eNOS stimulation. Although there is an increasing evidence in support of the fact that polyphenols modulate the risk of cardiovascular diseases (2, 15, 17, 184-187) however, the molecular mechanisms underlying these effects are still unclear. One suggested mechanism is that polyphenols modulate endothelial nitric oxide synthase (eNOS) activity and thus NO bioavailability to endothelium (188-191).

Nitric oxide (NO) is produced in the endothelial cells by an enzyme endothelial nitric oxide synthase (eNOS) from L-arginine in the presence of molecular oxygen and other co-factors such as tetrahydrobiopterin (BH_4) and calmodulin (CaM) (54).

The NO acts directly on vascular smooth muscles and causes relaxation which results in vasodilation. Availability of NO in the vasculature also inhibits nuclear factor- kB dependent expression of chemoattractant and adhesion molecules, inhibits platelet activation and tissue factor expression and is responsible for suppression of abnormal smooth muscles proliferation (57).

NO is recognized as an important anti-atherogenic factor and its bioavailability may serve as a marker for cardiovascular risk (71). Our results show that Oligopin® significantly improves nitric oxide bioavailability in *in vitro* HUVEC cell model with a range of physiologically relevant doses (1nM to 1000nM) and thus improves endothelial function.

Endothelial dysfunction can result from either a decreased production by eNOS in the L-arginine/NO pathway and or by an increased deactivation of NO by reactive oxygen species such as superoxide. Endothelial NOS dysfunction also results in increased superoxide production at the expense of Nitric oxide. Increased superoxide production by any mechanism may ultimately result in eNOS uncoupling and thus more superoxide production (192-194). The presence of superoxide in the vasculature greatly alters the bioavailability of NO, and increased reactive oxygen species (ROS) is a hallmark of cardiovascular diseases (57).

Our results suggest that Oligopin® significantly increases eNOS activity and decreases superoxide oxide production in *in vitro* cell model. This action can result in an increased NO bioavailability to the endothelial cells and thus an improved functioning endothelium.

In conclusion, the results of this *in vitro* study may suggest that Oligopin® in a physiologically relevant dose improves endothelial function. However translating these effects *in vivo* may not necessarily yield the same results due to the complex nature of the metabolic processes these chemicals have to undergo before reaching the circulation so the next step would be to investigate further the effects of this preparation *in vivo* in healthy volunteers.

5 Development of *in vitro* model for hypertension for investigating the effects of polyphenols on HUVECs in a hypertensive state

5.1 Introduction

There is increasing interest in the pharmacological role of dietary polyphenols in modulating the risk of cardiovascular disease in humans. Evidence suggests that protection from vascular endothelial dysfunction is at the core of this potential role, which may be characterised physiologically by modulation of hypertension. However, the mechanisms underlying this possible relationship are not fully understood because the literature lack consistency and reproducibility, mainly due to the absence of a standardised, easily reproducible, stable *in vitro* model of hypertension.

The present study therefore aimed to develop and validate a standardized, easily reproducible *in vitro*, model of angiotensin II (Ang II)-induced hypertension designed specifically to study the effects of dietary polyphenols on endothelial function in a hypertensive setting.

5.2 Methods

HUVECs were used as the in vitro model for this study, followed by protein extraction and measurement and Western blotting. The cell culture conditions and the protocols used for each of these methods are described in **Chapter 2** and representative blots for P47phox, gp91phox, AT1 and their respective loading control b-actin are shown in figure 5.1.

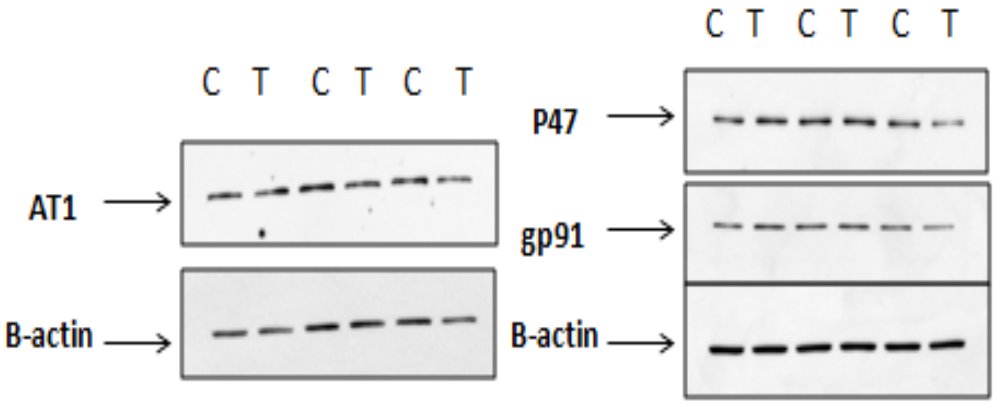


Figure 5-1 Representative blots for P47phox, gp91^{phox}, AT_1 and their respective loading control b-actin.
Protein from HUVEC culture cells with either no treatment(C) or treatment (T) with angiotensin II (100 nM) for 8 h before extraction. AT_1, angiotensin receptor type 1; C, untreated; T, treated.

5.3 Results

5.3.1 P47^{phox} Expression after Angiotensin II treatment

P47^{phox} protein expression tended to increase in angiotensin II treated samples from all the three experimental days (2, 3 and 4 after seeding) in comparison to untreated controls. However, the trends failed to reach statistical significance for any of the assessed time points (p-values for day 2=0.077 (n=4), day 3 =0.279 (n=7) and day 4=0.111 (n=5)) as shown in Figure 5-2. For p47 at 2 days post seeding, each separate independent experiment showed an increase in p47 levels in the angiotensin-treated cells compared to controls, however due to the variation between the different independent experiments in the p47: b-actin ratio, differences were not statistically significant. The data shows that there is a significant difference in p47^{phox} protein expression on the different experimental days (p-value = 0.002) in both angiotensin II treated and control samples, as shown in Figure 5-3.

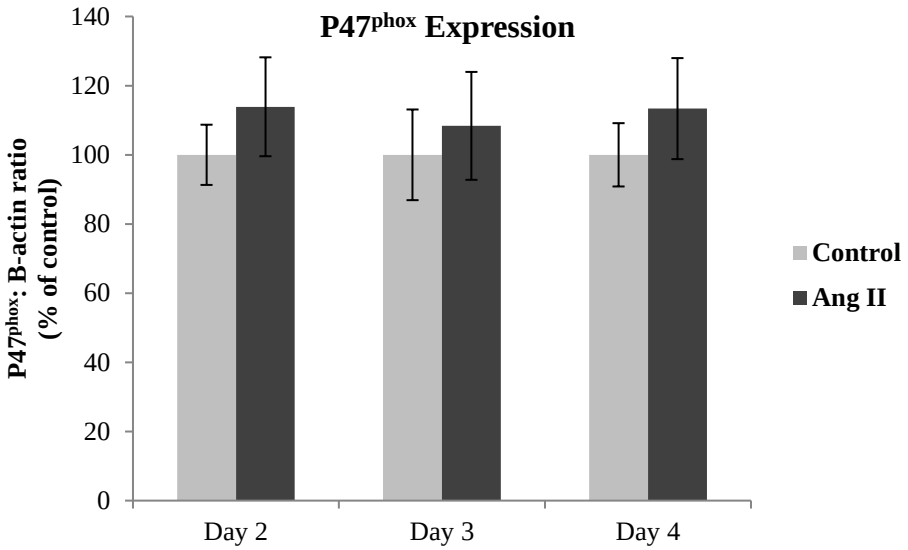


Figure 5-2 The % change in expression of P47^{phox} in HUVEC cells on days 2, 3 and 4 after seeding following angiotensin II treatment for 8 h before extraction.
Transformed means % ± SE (t-test). Significance: * $p < 0.05$, Obtained p-values for day 2=0.077 (n=4), day 3 =0.279 (n=7) and day 4=0.111 (n=5) a value of < 0.05 was considered as significant. Ang II, angiotensin II; n, number of individual experiments.

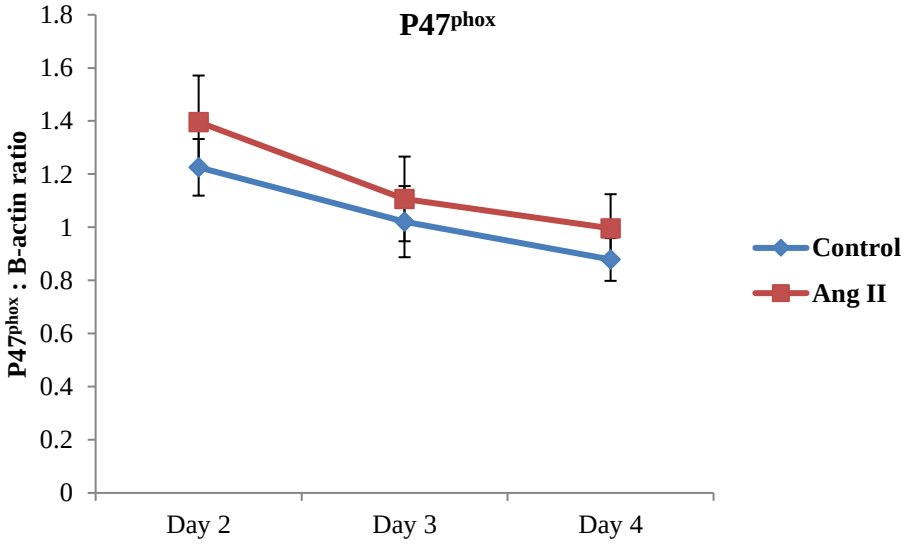


Figure 5-3 The change in expression of P47^{phox} in HUVEC cells on days 2, 3 and 4 after seeding in angiotensin II treatment and untreated samples.
Ratios ± SE (one-way ANOVA with Holm-Sidak method post hoc test). P-value=0.002 for control and treated groups. Ang II, angiotensin II; n, number of individual experiments.

5.3.2 Gp91^{phox} Expression after Angiotensin II treatment

The data show no change in gp91^{phox} protein expression when cells were treated with angiotensin II (100 nM, 8 h) in comparison with controls on the three experimental days (day 2, 3 and 4) with p-values for day 2=0.616 (n=4), day 3 =0.121 (n=7) and day 4=0.973 (n=4) as shown in Figure 5-4. There was no change in expression with increase in confluence of the cells as shown in Figure 5-5.

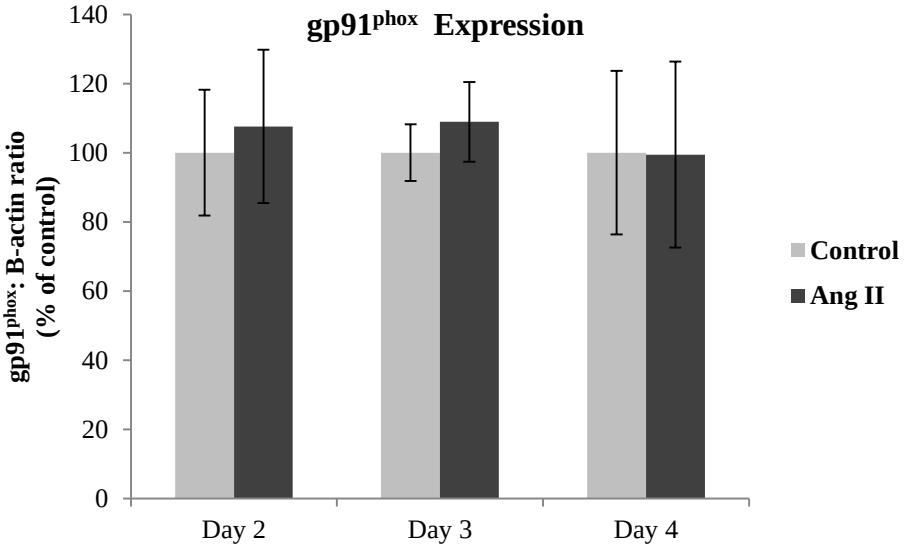


Figure 5-4 The proportional change in expression of gp91^{phox} in HUVEC cells on days 2, 3 and 4 after seeding following angiotensin II treatment for 8 h before extraction.
Transformed means % ± SE (t-test). Significance: * $p < 0.05$, Obtained p-values for day 2=0.616 (n=4), day 3 =0.121 (n=7) and day 4=0.973 (n=4) a value of ≤ 0.05 was considered as significant. Ang II, angiotensin II; n, number of individual experiments.

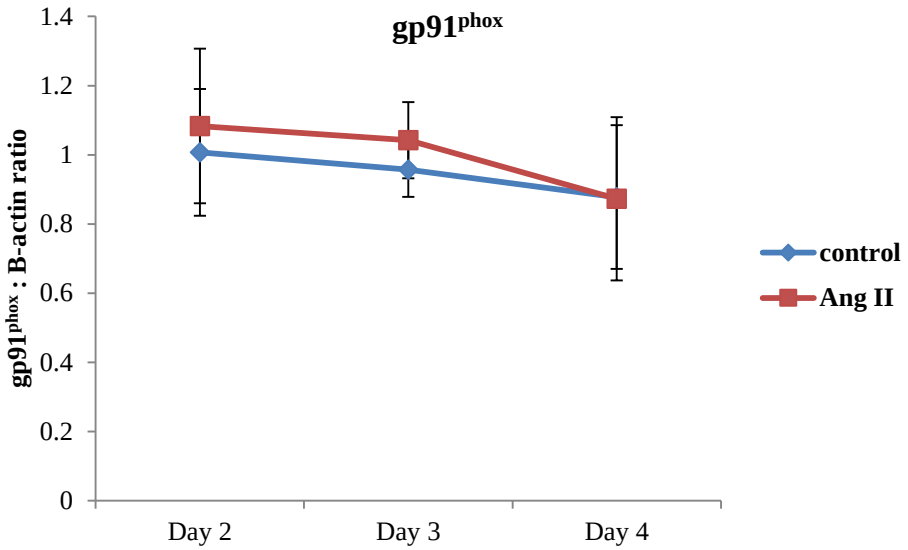


Figure 5-5 The change in expression of gp91^{phox} in HUVEC cells on days 2, 3 and 4 after seeding in angiotensin II treatment and untreated samples.
Ratios ± SE (one-way ANOVA with Holm-Sidak method post hoc test). P-value=0.528 for control and p=0.168 for treated groups. Ang II, angiotensin II; n, number of individual experiments.

5.3.3 AT_1 Expression after Angiotensin II treatment

Although there was no change in AT_1 protein expression in the angiotensin treated cells in comparison to their controls on the three experimental days (p-values for day 2=0.458 (n=4), day 3 =0.255 (n=5) and day 4=0.403 (n=4)) as shown in Figure 5-6. However, there were significant differences in protein expression on the different days within the control and treated groups where AT_1 protein expression tended to increase with increase in confluence of the cells (Figure 5-7).

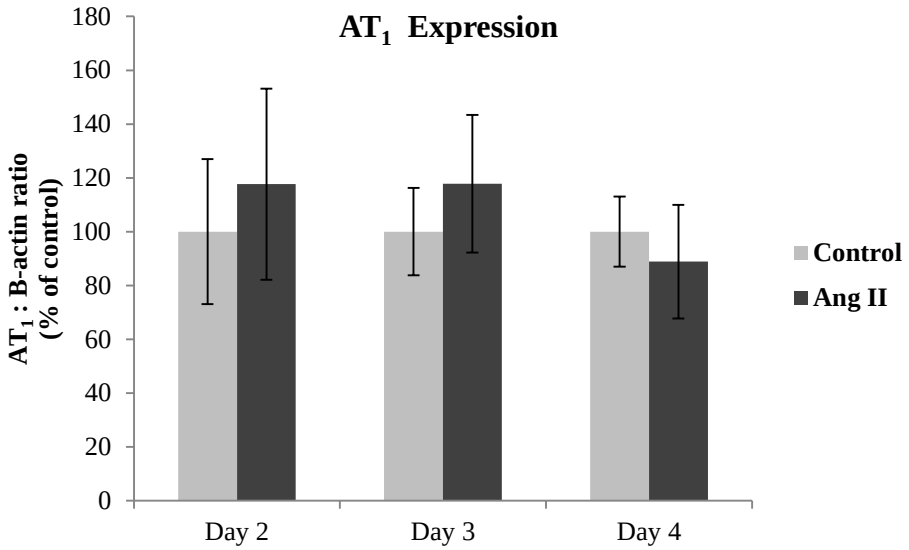


Figure 5-6 The proportional change in expression of AT_1 in HUVEC cells on days 2, 3 and 4 after seeding following angiotensin II treatment for 8 h before extraction.
Transformed means % ± SE (t-test). Significance: * $p < 0.05$, Obtained p-values for day 2=0.458 (n=4), day 3 =0.255 (n=5) and day 4=0.403 (n=4) a value of ≤ 0.05 was considered as significant. Ang II, angiotensin II; n, number of individual experiments.

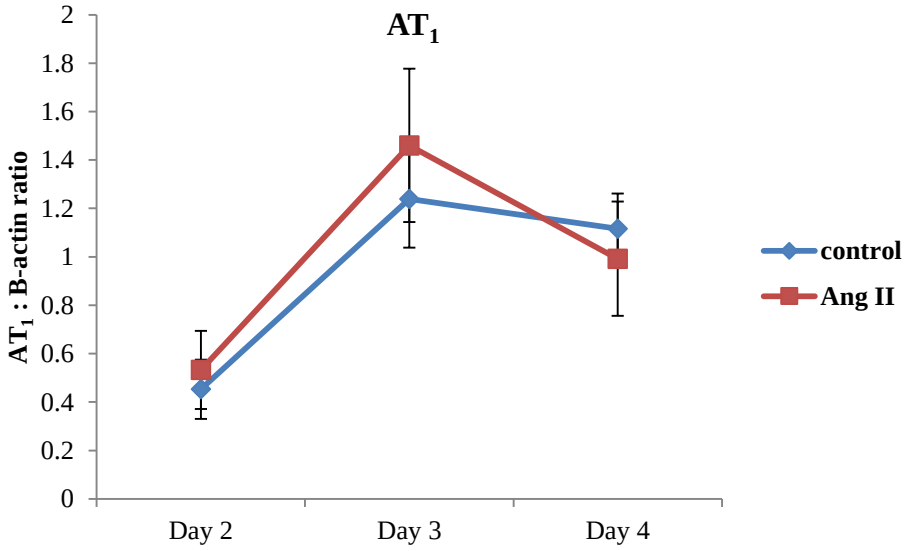


Figure 5-7 The change in expression of AT_1 in HUVEC cells on days 2, 3 and 4 after seeding in angiotensin II treatment and untreated samples.
Ratios ± SE (one-way ANOVA with Holm-Sidak method post hoc test). P-value=0.001 for control and treated groups. Ang II, angiotensin II; n, number of individual experiments.

5.4 Discussion

Angiotensin II plays an important role in the development and progression of cardiovascular diseases including hypertension, atherosclerosis, myocardial infarction and vascular hypertrophy (75-77). The molecular mechanisms through which angiotensin II mediates its action are not fully understood but it has been suggested that NADPH oxidase plays a central role given angiotensin II has been shown to increase NADPH oxidase activity and overexpression of its subunits such as P47 and $gp91^{phox}$ (93).

Angiotensin II has been suggested to mediate its actions through AT_1 receptor resulting in activation of NADPH oxidases and increased production of ROS (79), well supported by the anti-atherosclerotic effects of AT_1 inhibitors in experimental studies (195) and improvement of endothelial function in coronary artery disease patients (196). Knowing this, the expression of AT_1 will be expected to increase in HUVEC cells after angiotensin II treatment but the data suggest that there was no difference in AT_1 protein expression in angiotensin II treated and untreated cells. Similar results have been shown by Salisch et al (197) where HUVECs were treated with 100nM angiotensin II for either 24 or 48 hours.

Although the levels of $P47^{phox}$ tend to increase in all the samples from the three experimental days, the data suggest that there were no significant differences in $P47^{phox}$ in angiotensin II treated compared with untreated cells. The data also suggest that with increasing cell confluence there is a significant decrease in $P47^{phox}$ expression. A similar effect of cell confluence has been shown in NO assays (Jones et al., unpublished data) where there was a blunted NO response in HUVECs with increasing cell confluence. While Peshavariya et al (198) showed that more confluent endothelial cells decreased the expression of NADPH oxidase subunit (Nox4) but there was no change in $gp91^{phox}$ (Nox2) expression.

Angiotensin II has also been found to increase $gp91^{phox}$ expression in endothelial cells (199, 200) and in atherosclerotic arteries from patients (201). However, the present study fails to show any differences in the expression of $gp91^{phox}$ in HUVECs after angiotensin II treatment due to considerable variability within the

individual experiments. This variability can be a result of technical difficulties in performing the experiments or from shortcomings in the model itself. One reason can be that NADPH oxidase subunit expression is regulated at different levels (transcriptional and translational). The serum deprivation used for arresting the growth of endothelial cells may also have an effect as there is evidence in literature where serum deprived endothelial cells show increase in NADPH oxidase isoform (Nox4) gene expression while its protein expression decreased (202).

Dietary Polyphenols such as quercetin (found mostly in fruits, grains and vegetables) have been suggested to modulate NADPH activity *in vitro* in human endothelial cells (92) and aortic rings (93) where polyphenols administration resulted in a decrease in NADPH oxidase activity and selective reduction in its subunit expression. The dietary polyphenols have been shown to modulate cardiovascular disease risk and improve endothelial function (2, 4, 203). The molecular mechanisms underlying the beneficial effects of polyphenols are not known. The primary aim of the study was to attain a standardised and easily reproducible model for hypertension and thus be able to study the effects of dietary polyphenols on the endothelium *in vitro*.

Endothelial dysfunction has been suggested to be an early sign of atherosclerotic diseases (68, 204) and can predict future cardiovascular events. Assessment of endothelial function can identify individuals at risk of developing cardiovascular disease (69, 70). Targeting this at risk population with interventions such as administration of dietary polyphenols can potentially improve future outcome and reduce cardiovascular events. Non-invasive techniques such as Flow mediated dilation (FMD) assessed by brachial artery ultrasound scanning and reactive hyperaemia peripheral arterial tonometry (RH-PAT) can prove helpful in risk stratification and also assessing the effects on endothelial function (164, 166, 167).

6 Intra-Individual correlation between Flow Mediated Dilation (FMD) and Reactive Hyperaemia Peripheral Arterial Tonometry (RH-PAT) in PCOS

DOI:10.1530/endoabs.38. P27010.1530/endoabs.38. P270.

6.1 Introduction

A limitation of the bioavailability study (**Chapter 3**) was that the measurement of nitrate and nitrite as markers of nitric oxide availability were merely indicative. In order to overcome this, non-invasive measures of vascular function such as flow-mediated dilation or EndoPAT® measurements should be used. Although there are studies in healthy volunteers, where both the techniques have been compared (205) there are limited data for diseases such as polycystic ovary syndrome.

Assessing endothelial function in women with Polycystic Ovary Syndrome (PCOS) has provided evidence of cardiovascular disease in the absence of a clinically evident disorder. Flow mediated dilation and Reactive hyperaemia peripheral arterial tonometry are frequently used non-invasive techniques for assessing endothelial function and both techniques have been used in PCOS patients. However, there are few results on the two techniques being used simultaneously in the same individual and recent research suggests the underlying physiological mechanisms may differ since the two techniques examine different set of arterial vessels.

6.2 Methods

Flow mediated dilation and Reactive hyperaemia peripheral arterial tonometry were performed simultaneously on thirty apparently healthy normotensive women (15 PCOS and 15 controls, with a mean age of 31.5 ± 7.5 and 32 ± 7.8 years respectively), who underwent 5 minutes of suprasystolic cuff-induced ischemia followed by post ischemic measurements. Representative images for the FMD and

EndoPAT test results are shown in Figures 6.1 and 6.2 respectively and the details of the methods used are given in **Chapter 2.**

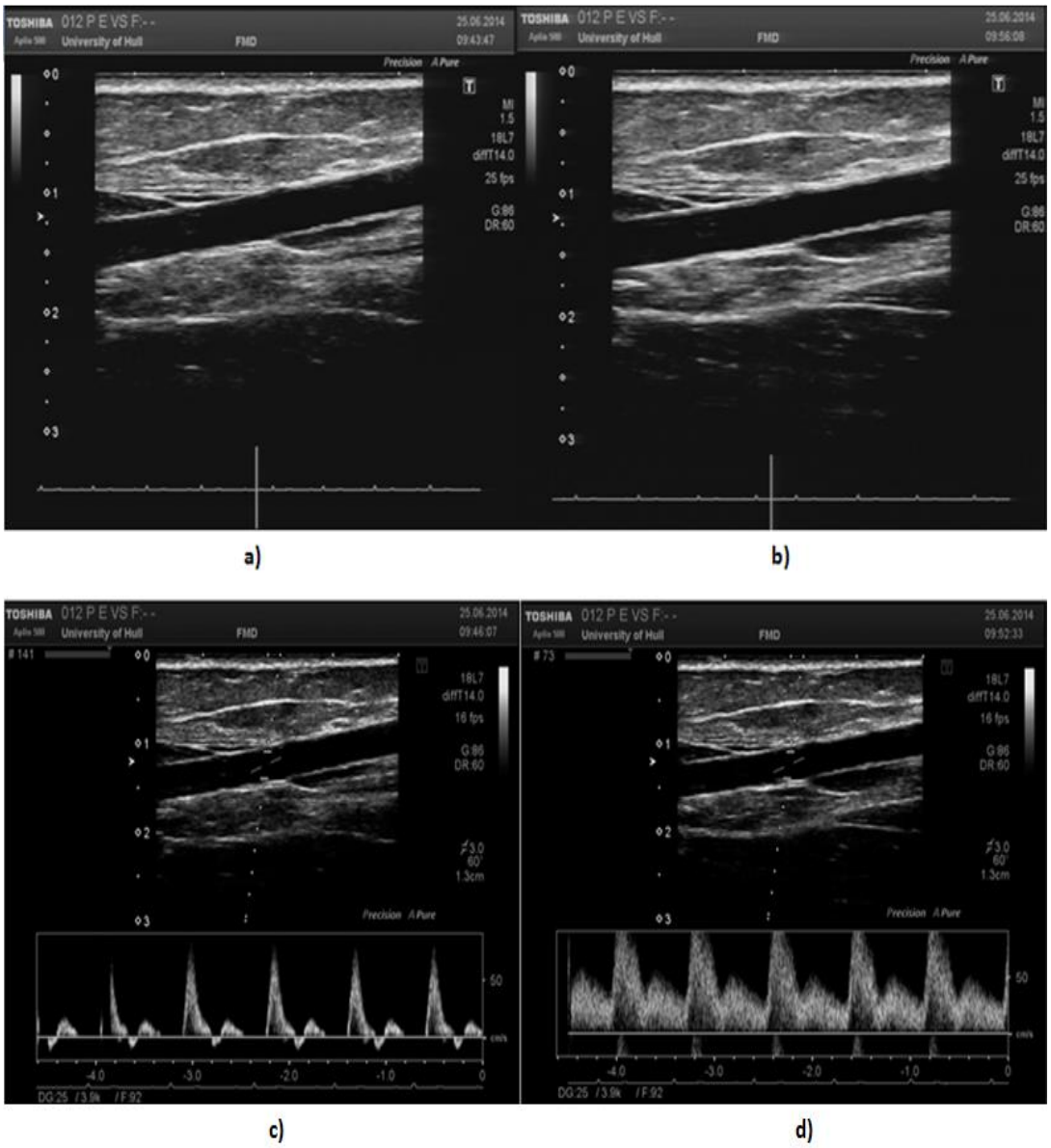


Figure 6-1 B-mode ultrasound images of brachial artery
a) and b) showing images for diameter measurement at baseline and after cuff deflation respectively. While image c) and d) shows blood flow images in pulse wave view at baseline and immediately after cuff release respectively used for shear rate calculation.

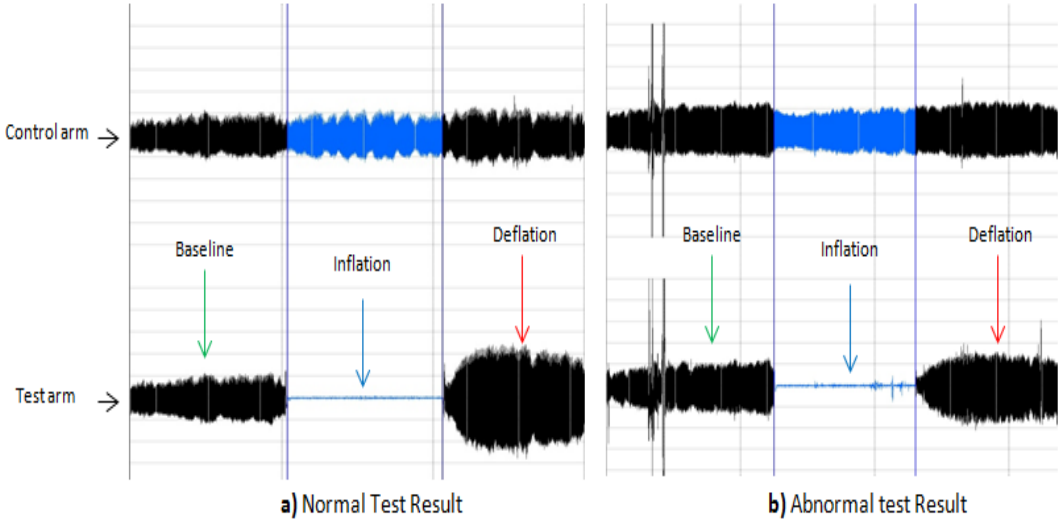


Figure 6-2 Representative images for EndoPAT® test Results
a) Normal and b) Abnormal showing the recordings for baseline, cuff inflation and after cuff deflation (5 minutes each) for both the control and test arms.

6.3 Results

The participants were young and healthy as demonstrated by mean blood pressure and body mass index values (Table 6-1).

Table 6-1 Demographics and baseline characteristics for PCOS and control groups

	PCOS (n=15)	Control (n=15)	P-Value
Age (year)	29.1 ± 6	32 ± 7	0.25
BMI (Kg/m^2)	26.1 ± 4.1	23.7 ± 2.8	0.08
Systolic BP(mmHg)	105 ± 13.2	102.5 ± 8.7	0.55
Diastolic BP (mmHg)	64.5 ± 7.7	63.7 ± 6.6	0.76
Heart Rate (bpm)	66.3 ± 9.8	63.3 ± 12.8	0.48

There were no differences in endothelial function measurements between PCOS and Control groups for either of the techniques used, Reactive hyperaemic Index (RHI)

(2.0 ± 0.7 vs 2.2 ± 0.7 (p-value = 0.51) Figure 6-3 and FMD (6.9 ± 3.1 vs 5.7 ± 3.1 % (p-value = 0.14) Figure 6-4 respectively. The Mean values of peripheral vascular reactivity measures are shown in Table 6-2

There was also no significant association between FMD and RHI (r = 0.326, p-value = 0.079) as shown in Figure 6-5.

Table 6-2 Clinical markers for Endothelial function

	PCOS (n=15)	Control (n=15)	P-Value
RHI	2.1 ± 0.7	2.3 ± 0.7	0.66
FMD %	7.11 ± 2.8	5.2 ± 3.5	0.18
AI@75	-2.9 ± 10.9	4.4 ± 15.4	0.14

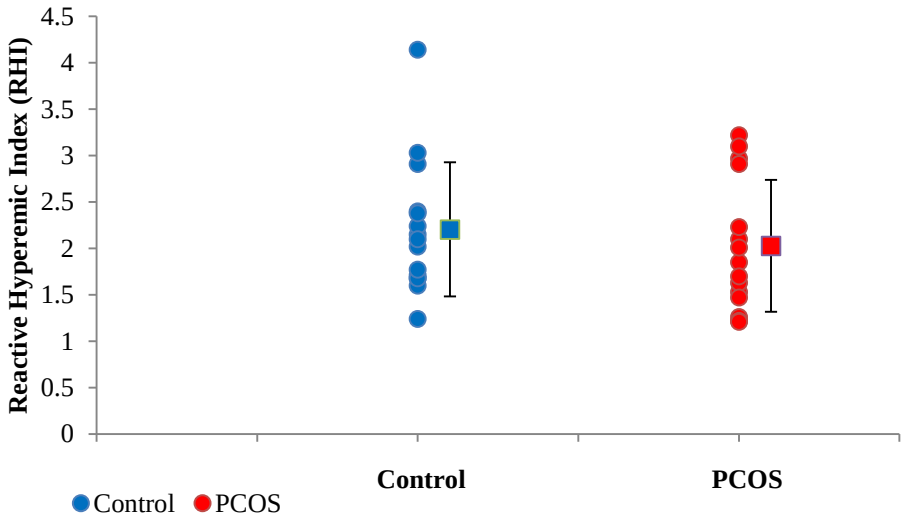


Figure 6-3 Reactive hyperaemic index (RHI) in both PCOS and control showing no significant differences between the two groups (p-value 0.51). Means ± SE. PCOS polycystic ovary syndrome**.**

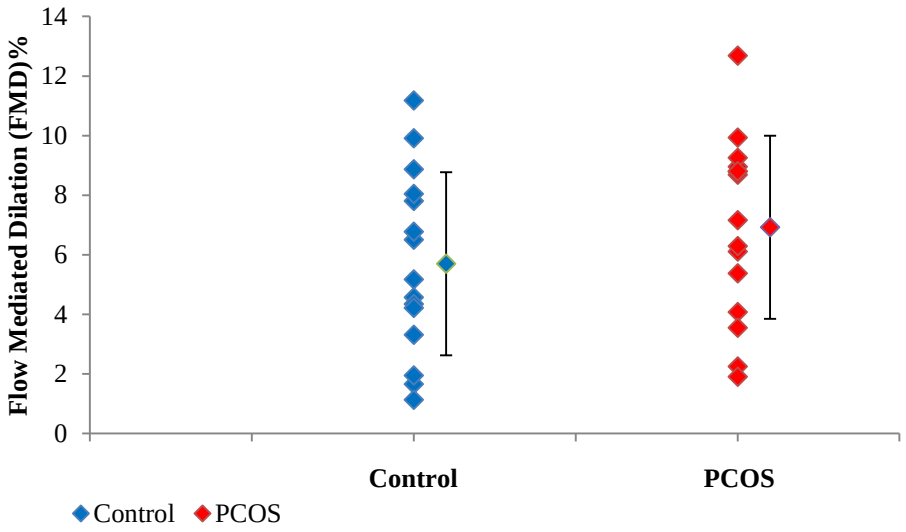


Figure 6-4 Flow Mediated Dilation (FMD) in both PCOS and control showing no significant differences between the two groups (p-value = 0.14). Means ± SE. PCOS polycystic ovary syndrome**.**

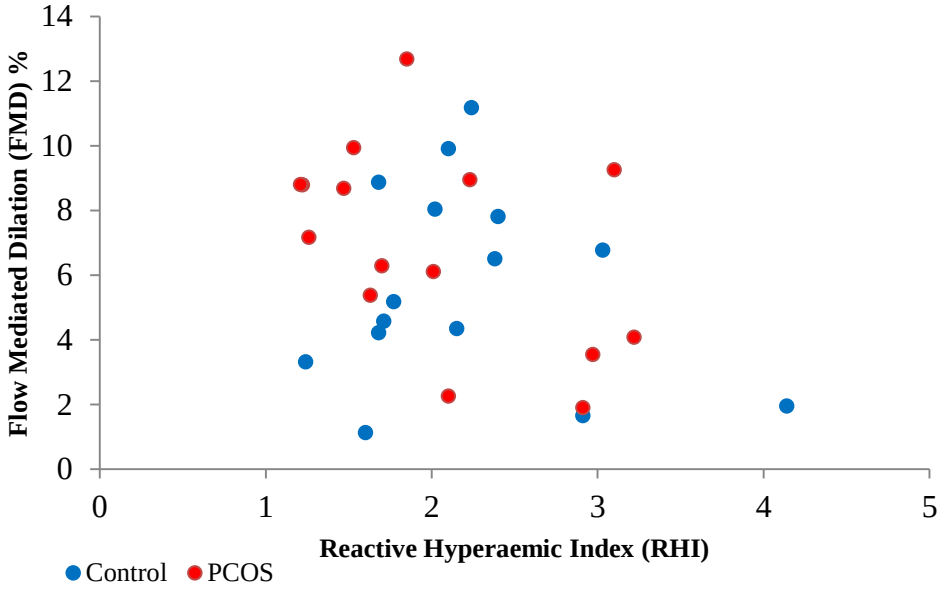


Figure 6-5 Association between FMD and RHI in both PCOS and control showing no significant association between the two (r = 0.326, p-value = 0.079). Means ± SE. PCOS polycystic ovary syndrome.

6.4 Discussion

This is one of the few studies to date to compare FMD and RH-PAT in healthy volunteers and individuals with PCOS and is the first one in our knowledge to investigate the association between the two techniques in women with PCOS. In the present study, there was no correlation between the two techniques in neither of the groups i.e. healthy and PCOS separately or when they were taken together as a single group. There is very limited data available on the correlation between FMD and RH-PAT in the same individual and that the available literature on the subject is contradictory, with a few studies reporting at best a modest but significant correlation (104, 205-208) while others reporting lack of any correlation between the two techniques (209-212). This difference of view may be a result of the differences in technical as well as in the study population selected and therefore these issues need to be considered while interpreting and/or comparing the results from these studies.

Endothelial dysfunction is an early sign of atherosclerotic disease progression (60, 68, 213) and with the increased risk of cardiovascular disease in PCOS (159, 161, 162, 164, 165) one would expect to find an impaired endothelial function in these individuals. However, our study results showed no impairment in endothelial function in PCOS as assessed by the two different non-invasive techniques namely FMD and RH-PAT.

In conclusion, there was no difference in endothelial function assessment by the two Techniques FMD and EndoPAT® in PCOS. Both the techniques have been previously shown to correlate with coronary endothelial function (111) and thus both can be used alternatively. The lack of correlation between the two techniques here may be a result of the different underlying physiological mechanisms (suggested in recent literature on the subject) (205) as the two techniques look at different sets of vessels with the FMD measuring larger arterial while RH-PAT assessing small arterial vessel reactivity. EndoPAT® is advantageous because it is easy to set-up, is automated and non-user dependent and can be used to identify individuals at risk of developing cardiovascular disease.

7 Double blind, randomized, placebo-controlled, crossover study of the effects of a French maritime pine bark extract (Oligopin®) on endothelial function

(ClinicalTrails.gov Identifier: NCT02116816)

7.1 Introduction

The initial study of polyphenol preparations (detailed in **Chapter 3**) on healthy volunteers, which assessed the pharmacokinetics of Oligopin® in a small number of healthy volunteers, and suggested that Oligopin® can improve nitric oxide bioavailability that is a key regulator of vascular tone and function. The results from the *in vitro* study (see **Chapter 4** for details) also suggested that Oligopin® in a physiologically relevant dose improves endothelial function. However translating these effects *in vivo* may not necessarily yield the same results due to the complex nature of the metabolic processes these chemicals have to undergo before reaching the circulation so the need for a clinical trial.

The present study aimed to investigate that Oligopin® modulates vascular function by using vascular tone and function assessment in healthy volunteers employing a non-invasive, direct measurement of vasodilatory capacity (using EndoPAT, detailed in chapter 6). Nitrate and nitrite measurements as well as blood pressure measurements were also recorded to provide a clear clinical assessment of the efficacy of this preparation upon vascular function. The effects of Oligopin® were compared to a placebo (methylcellulose-filled capsules) in a randomised, crossover design to allow a robust assessment of any clinical effects.

7.2 Subjects and Methods

Twenty-four healthy volunteers (11 males and 13 females) were screened initially after identification from diabetes research database of healthy volunteers (Michael White Diabetes Centre, Hull Royal Infirmary). Three subjects withdrew the consent, one before entering the clinical phase and remaining two during the clinical phase without giving any reasons. Another three healthy volunteers from

the dataset of healthy volunteers replaced withdrawn participants. Twenty-four subjects (11 males and 13 females) entered the clinical phase and successfully completed all study related procedures. The study was conducted in accordance with Good Clinical Practice (Guideline for GCP). National Research Ethics Services Committee, Yorkshire and the Humber approved all the study procedures. Written informed consent was obtained from all the participants after discussing the details of study with a study doctor. At the time of recruitment, the participants had a meeting with dietician who provided them a list of foods that are rich in polyphenols that should be avoided. The participants were instructed to follow a diet that is low in polyphenols for 1 week before starting the trial and for the duration of the trial. In addition, subjects were required to avoid alcohol for 24 hours before each visit. An independent person (a research doctor) not involved in the study did the randomisation using a computer generated randomisation list, and they un-blinded the samples at the end of the study.

7.3 Results

Twenty-four healthy volunteers (11 male and 13 female with a mean age of 36 ± 14 years) completed the study. The characteristics of the study participants are shown in Table 7-1.

Table 7-1 Study participant's characteristics

Variable	Value
N	24
Men/Women	11/13
Age (years)	36 ± 14
Height (cm)	171 ± 10
Weight (kg)	77 ± 11
BMI (kg/m²)	26 ± 3

Values are given as means ± Standard Deviation. BMI, body mass Index

7.3.1 Assessment of the effects of Oligopin and placebo upon indices of vascular function in healthy volunteers

Reactive hyperaemic index (RHI) and augmentation index (AI) were assessed as markers of endothelial function in volunteers at 0, 4, 8 and 24 h post ingestion of

either placebo or Oligopin capsules. There were no differences observed between these groups for either marker of endothelial function, suggesting that there is no functional effect of Oligopin in healthy volunteers (Table 7-2). The RHI data is represented graphically in Figure 7-1 and Figure 7-2, and AI in Figure 7-3 and Figure 7-4. There was also no effect of Oligopin treatment upon blood pressure (diastolic and systolic) in healthy volunteers at any of the time-points assessed (Table 7-2). Plasma nitrate and nitrite concentrations were also quantified, with no differences observed after Oligopin ingestion compared with placebo (Figure 7-5 and Figure 7-6, raw data is tabulated in the appendix).

Table 7-2 Clinical markers for endothelial function and blood pressure at each time point

	Time Point (hours)	Control (mean ± SD)	Treated (mean ± SD)	Between groups difference 95% CI	P-Value
RHI	0	1.9 ± 0.5	2.1 ± 0.8	1.7-2.4	0.93
	4	2.3 ± 0.6	2.5 ± 0.7	2.1-2.8	0.36
	8	2.0 ± 0.6	2.2 ± 0.7	1.8-2.5	0.46
	24	2.0 ± 0.4	2.0 ± 0.7	1.8-2.4	0.48
AI@75	0	-0.1 ± 20.5	3.0 ± 22.4	-8.3-11.9	0.89
	4	4.1 ± 26.8	4.6 ± 22.2	-4.3-14.8	0.78
	8	-2.6 ± 16.1	-4.0 ± 19.4	-11.7-3.8	0.39
	24	-0.9 ± 19.4	2.6 ± 22.2	-6.3-11.5	0.78
Systolic BP	0	120 ± 14	121 ± 13	114-126	0.43
	4	119 ± 13	122 ± 15	114-128	0.59
	8	116 ± 9	118 ± 12	112-123	0.65
	24	117 ± 12	119 ± 11	112-123	0.44
Diastolic BP	0	71 ± 11	72 ± 10	67-76	0.75
	4	72 ± 10	72 ± 11	68-76	0.89
	8	69 ± 11	70 ± 10	65-74	0.86
	24	70 ± 10	69 ± 10	65-74	0.97

RHI: Reactive Hyperaemic Index, AI@75: Augmentation Index normalized for Heart rate 75 beats per minute, Control: Given Placebo, Treated: given Oligopin. BP, blood pressure (mean of 3 measurements for each participant at a single time point)

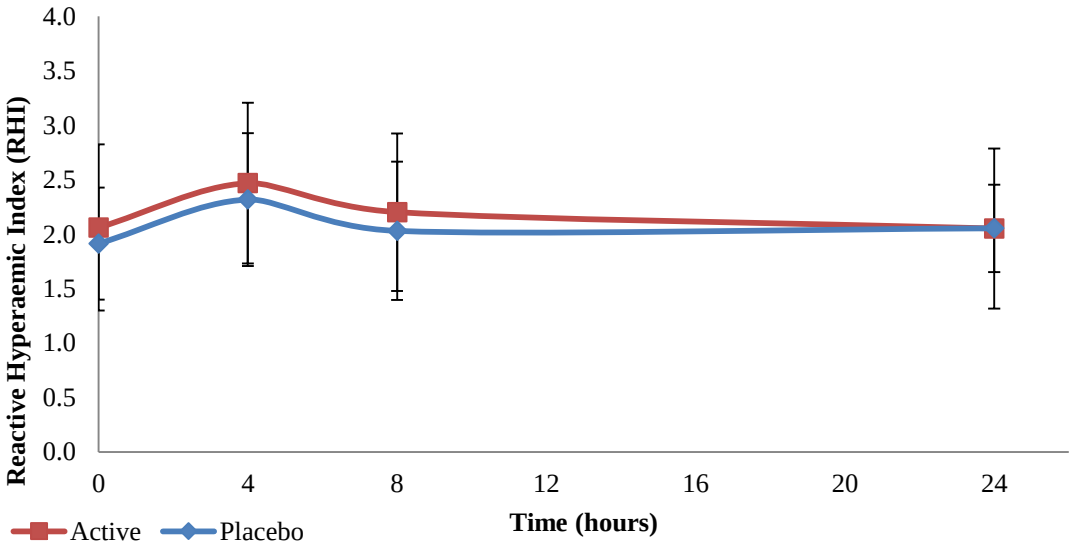
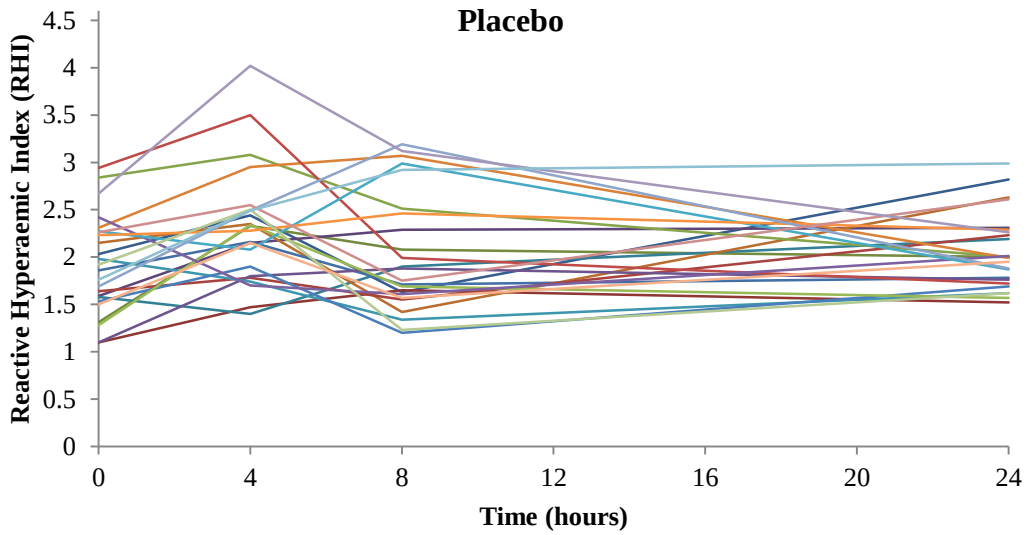


Figure 7-1 Graphical presentation of Reactive hyperaemic Index (RHI)
Values for the study Participants (n=24) at time points 0, 4, 8 and 24 hours after ingestion of placebo (Methylcellulose) or active (Oligopin – French maritime pine bark extract). Data shown as mean ± Standard Deviation.



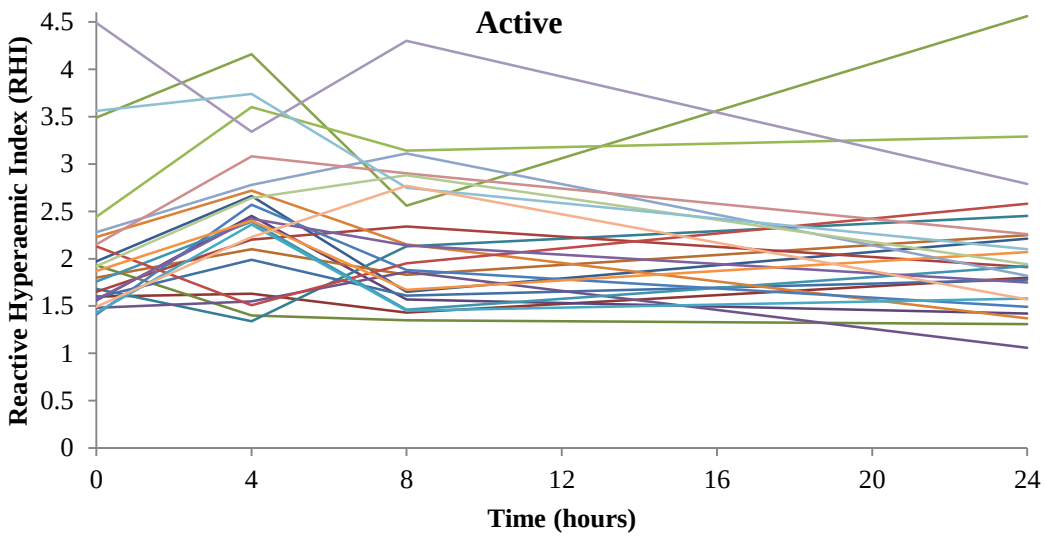


Figure 7-2 Individual plots for reactive hyperaemic index values for both the placebo (methylcellulose) and active (Oligopin) group.

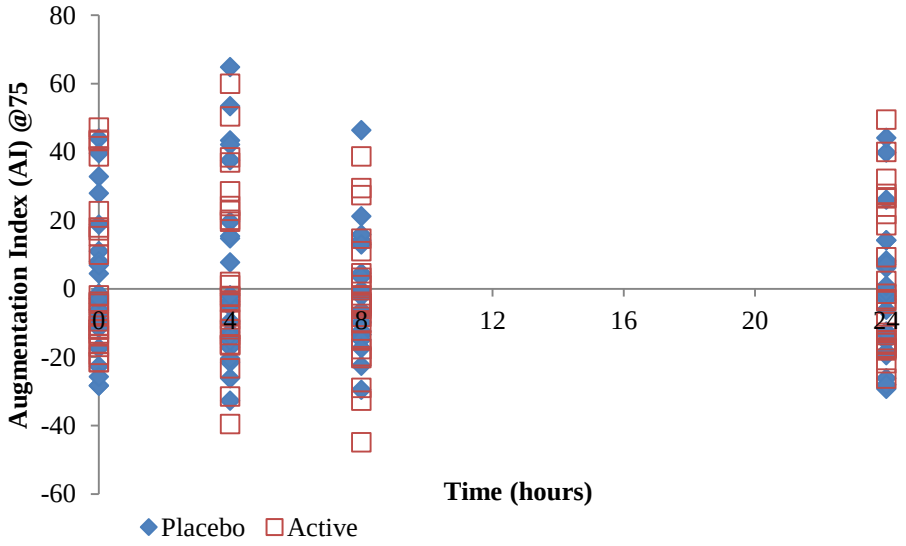
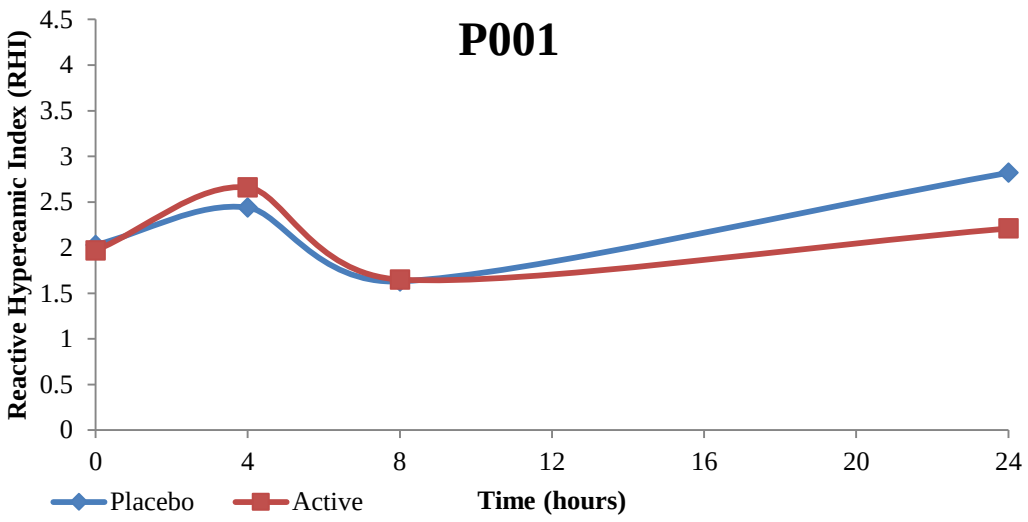
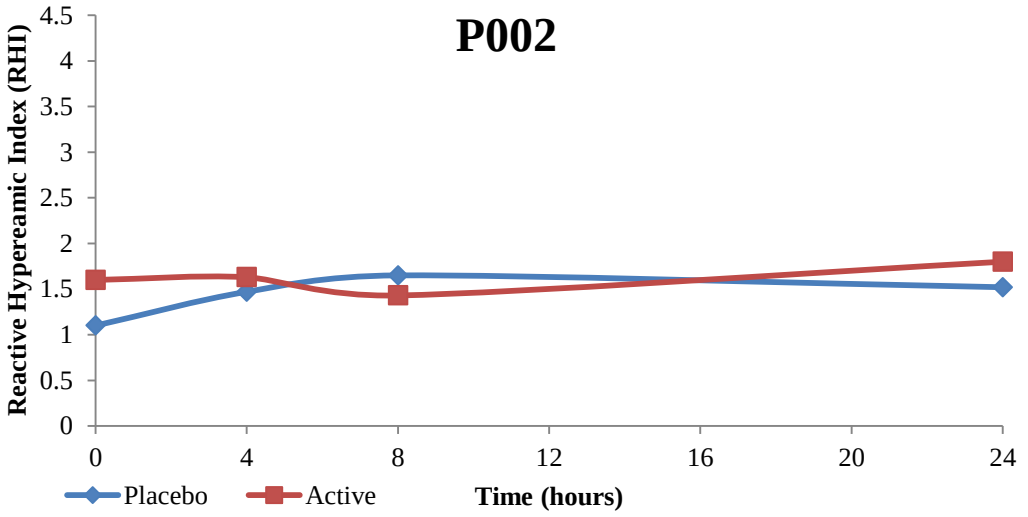
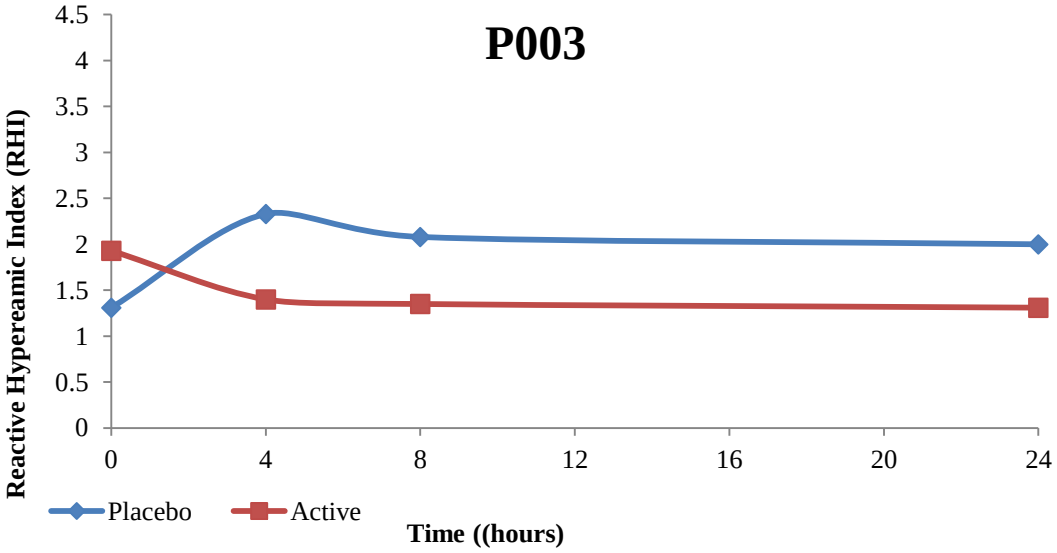
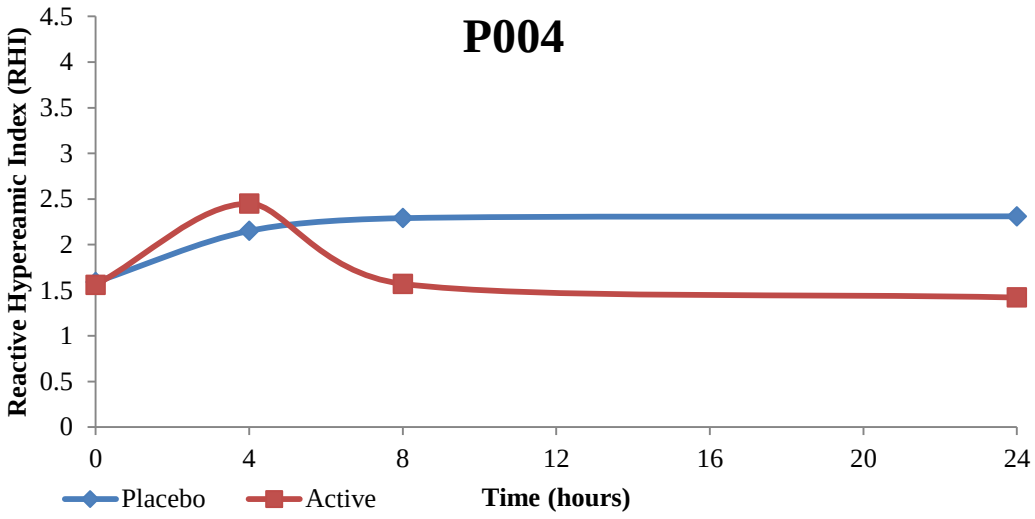


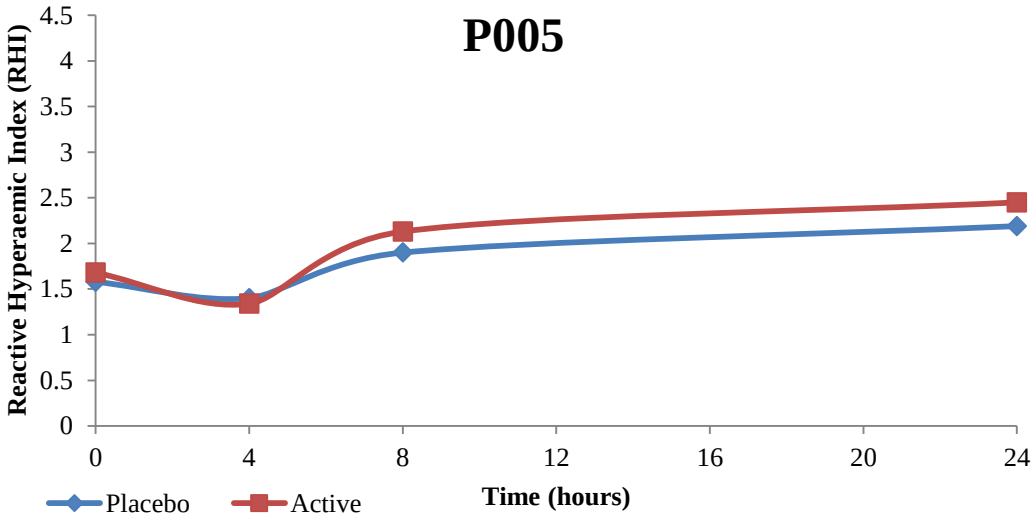
Figure 7-3 Augmentation Index normalized for heart rate at 75 bpm.
Data presented as mean ± Standard Deviation. Placebo, Methylcellulose. Active, Oligopin – French maritime pine bark extract

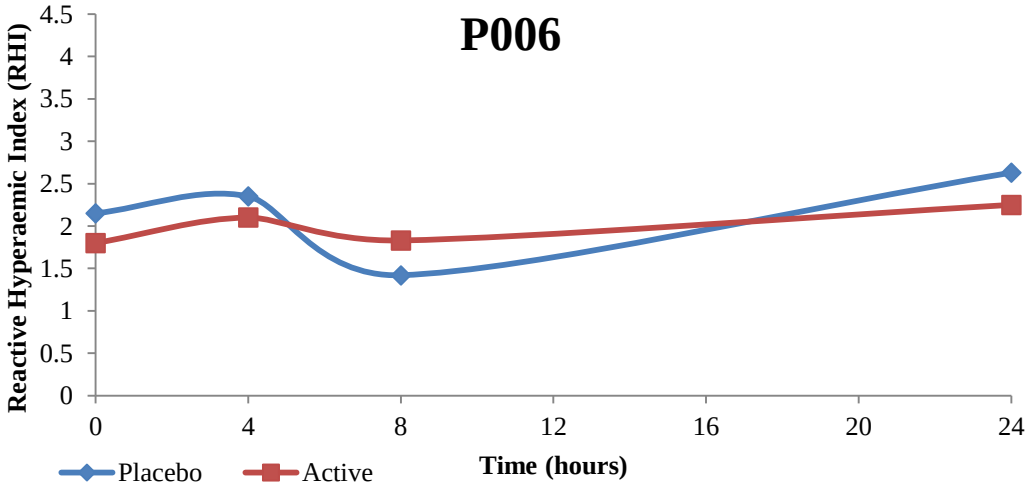

P001
Reactive Hypereamic Index (RHI)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
4
8
12
16
20
24
Placebo
Active
Time (hours)

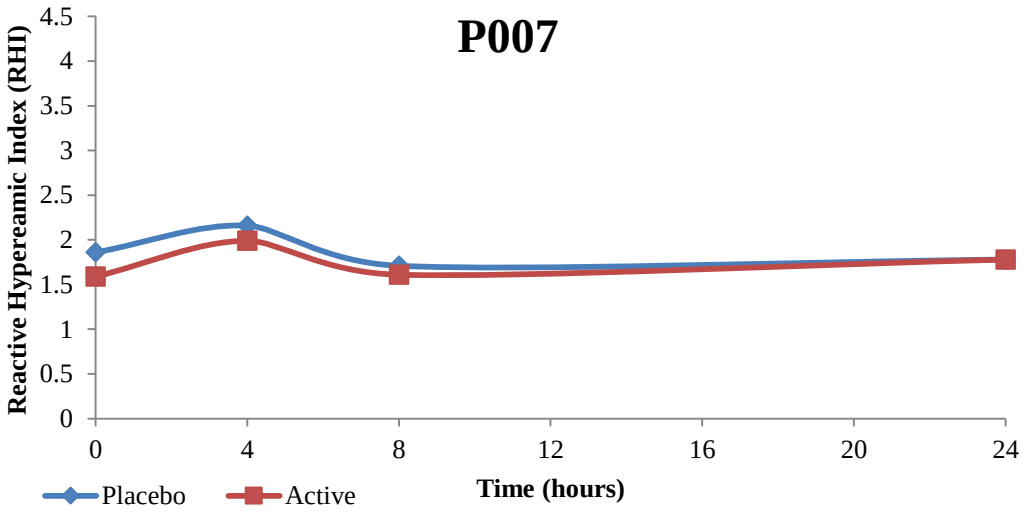

P002
Reactive Hypereamic Index (RHI)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
4
8
12
16
20
24
Placebo
Active
Time (hours)

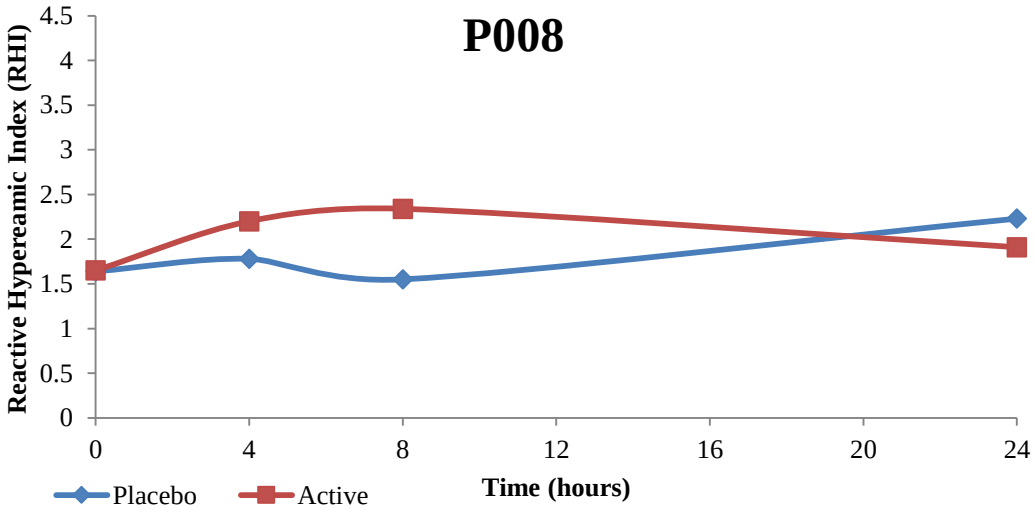

P003
Reactive Hypereamic Index (RHI)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
4
8
12
16
20
24
Placebo
Active
Time ((hours)

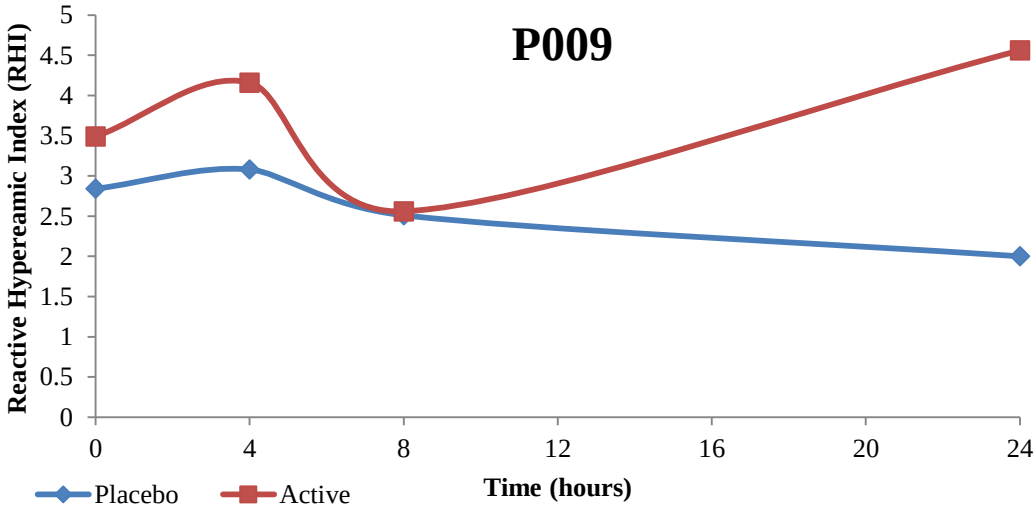

P004
Reactive Hypereamic Index (RHI)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
4
8
12
16
20
24
Placebo
Active
Time (hours)

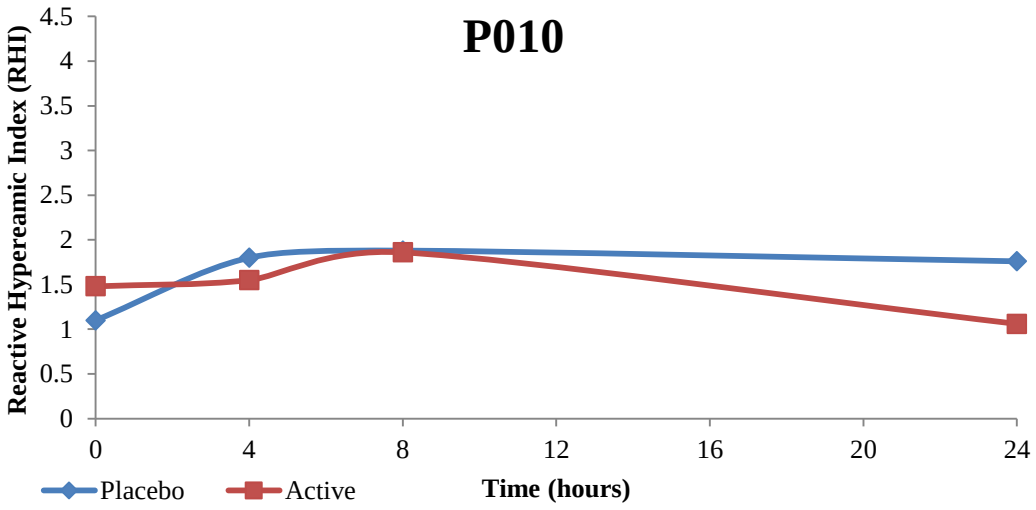

P005
Reactive Hyperaemic Index (RHI)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
4
8
12
16
20
24
Placebo
Active
Time (hours)

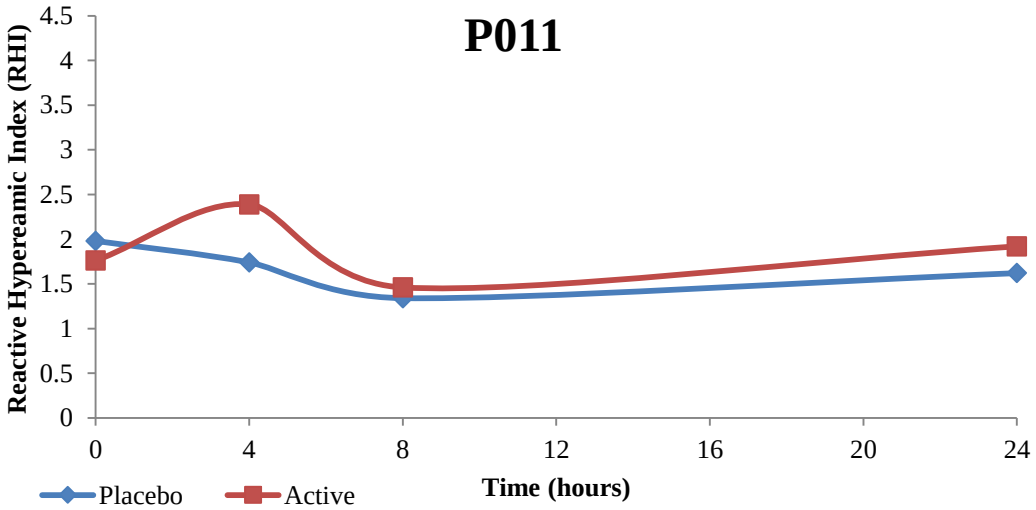

P006
Reactive Hyperaemic Index (RHI)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
4
8
12
16
20
24
Placebo
Active
Time (hours)

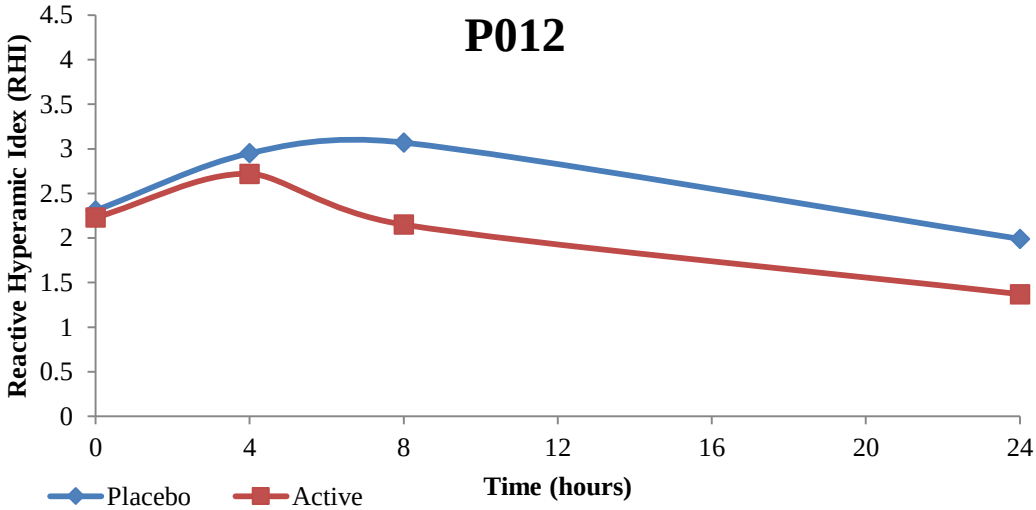

P007
Reactive Hypereamic Index (RHI)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
4
8
12
16
20
24
Time (hours)
Placebo
Active

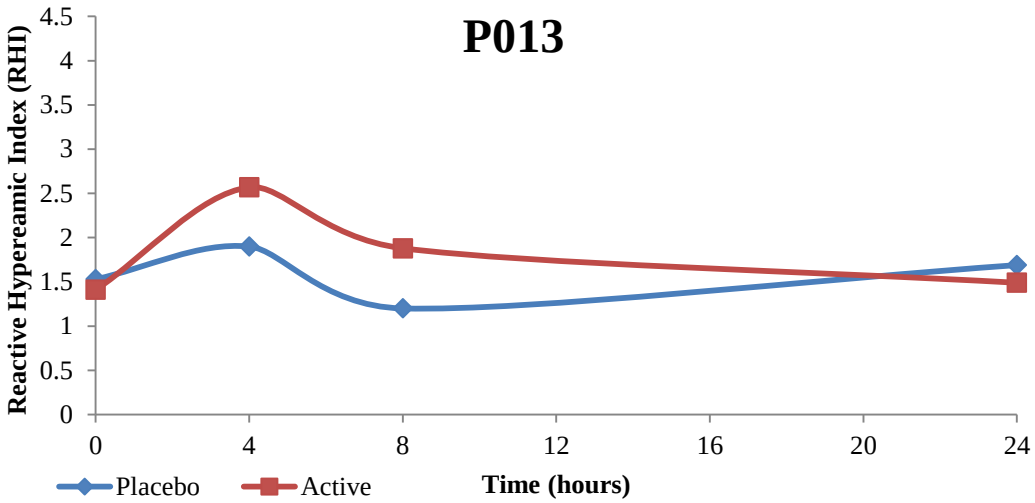

P008
Reactive Hypereamic Index (RHI)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
4
8
12
16
20
24
Time (hours)
Placebo
Active

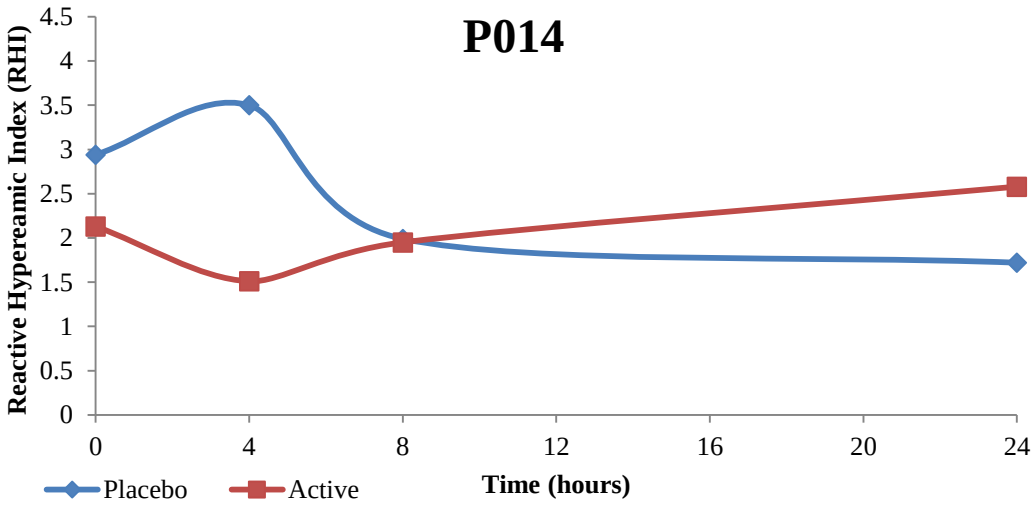

P009
Reactive Hypereamic Index (RHI)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
4
8
12
16
20
24
Time (hours)
Placebo
Active

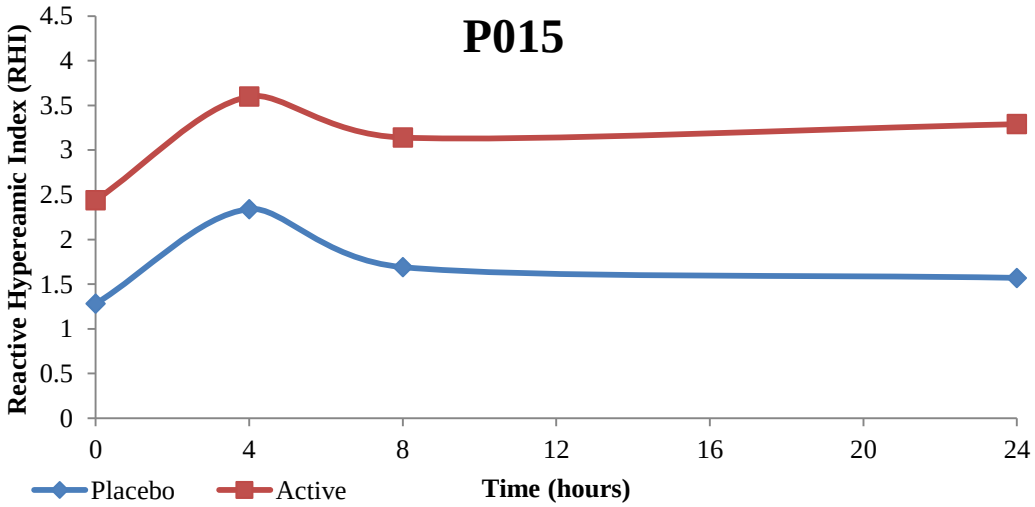

P010
Reactive Hypereamic Index (RHI)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
4
8
12
16
20
24
Time (hours)
Placebo
Active

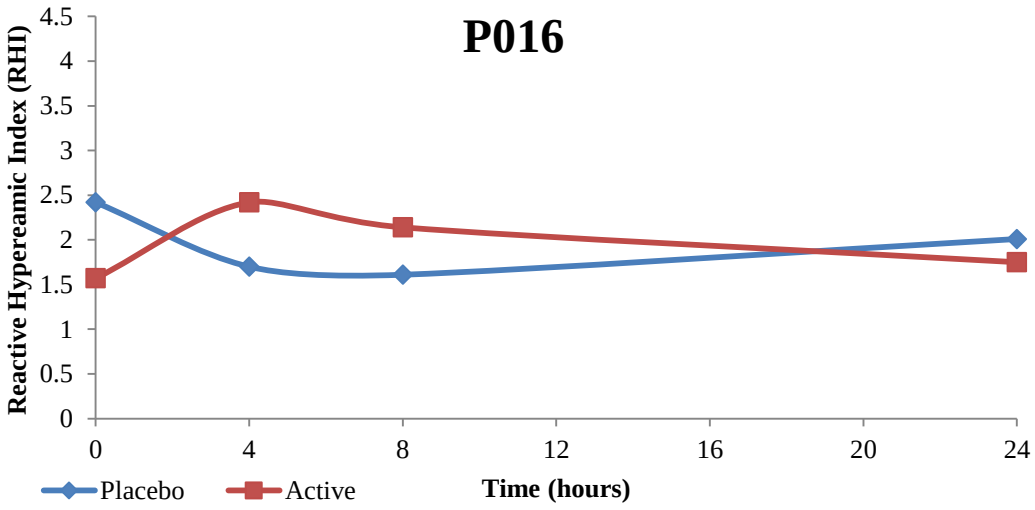

P011
Reactive Hypereamic Index (RHI)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
4
8
12
16
20
24
Time (hours)
Placebo
Active

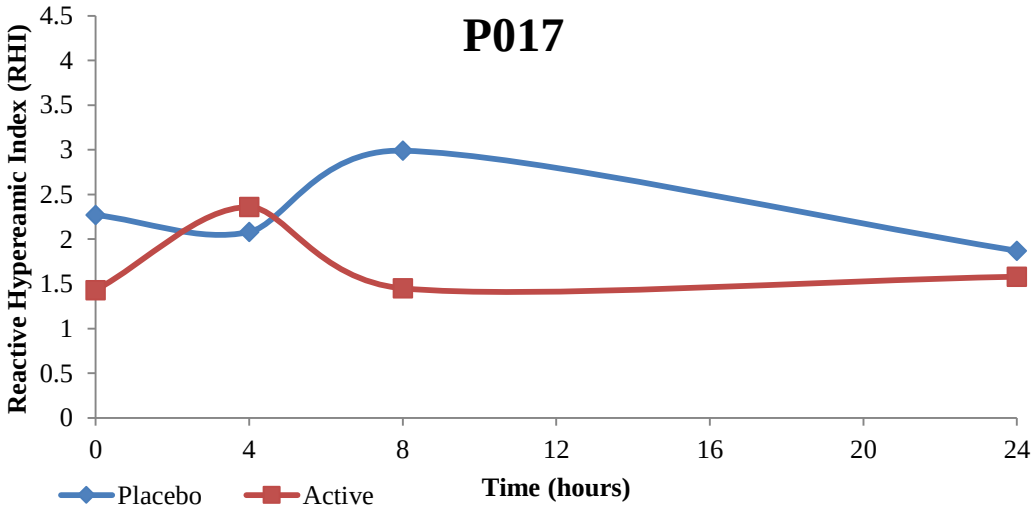

P012
Reactive Hyperamic Idex (RHI)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
4
8
12
16
20
24
Time (hours)
Placebo
Active

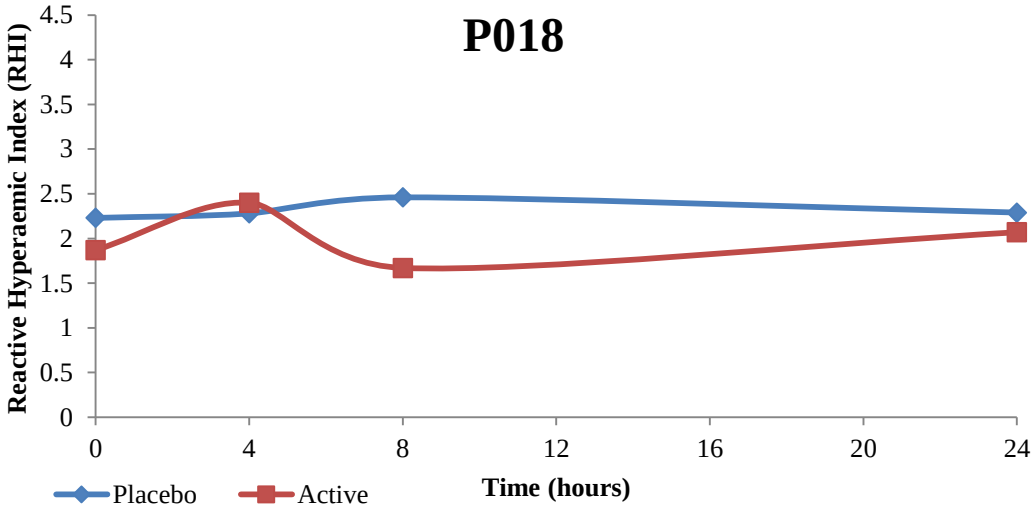

P013
Reactive Hypereamic Index (RHI)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
4
8
12
16
20
24
Placebo
Active
Time (hours)

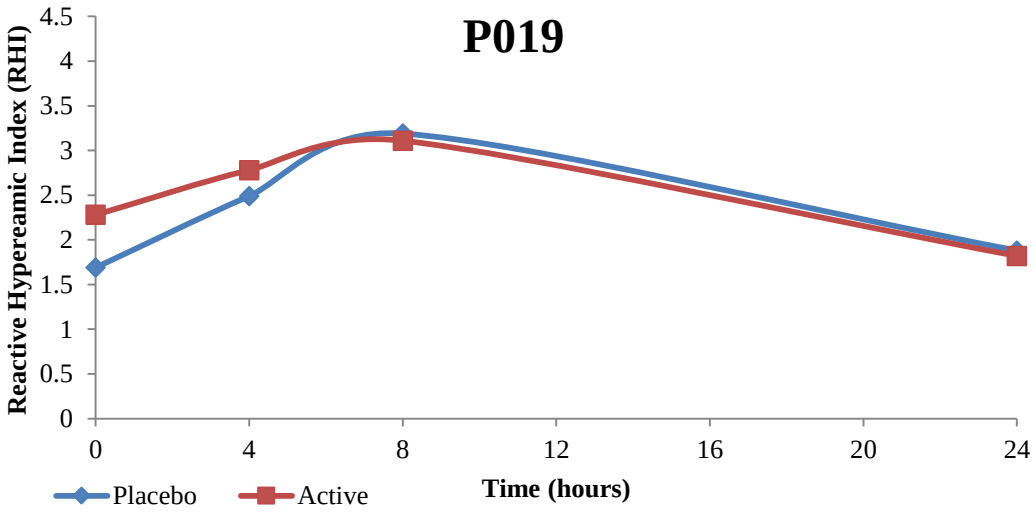

P014
Reactive Hypereamic Index (RHI)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
4
8
12
16
20
24
Placebo
Active
Time (hours)

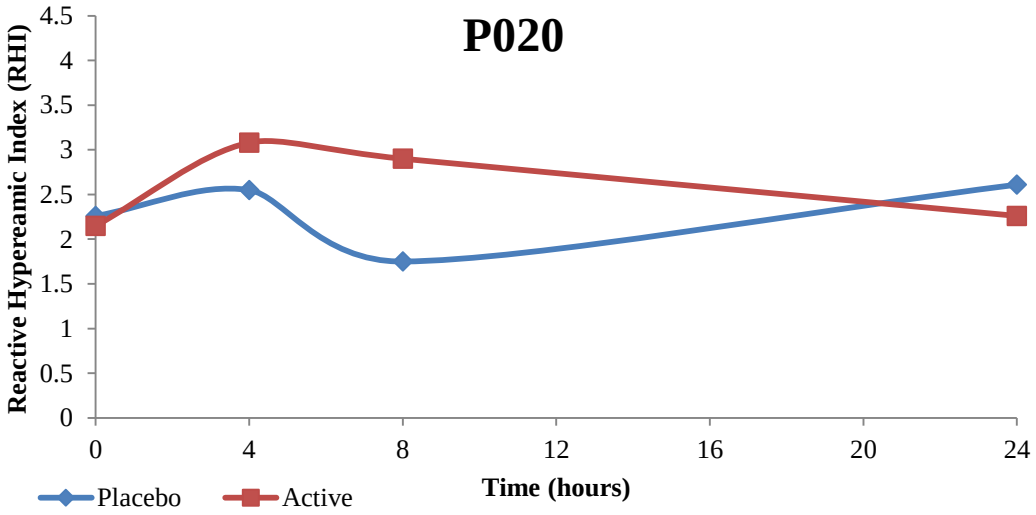

P015
Reactive Hypereamic Index (RHI)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
4
8
12
16
20
24
Placebo
Active
Time (hours)

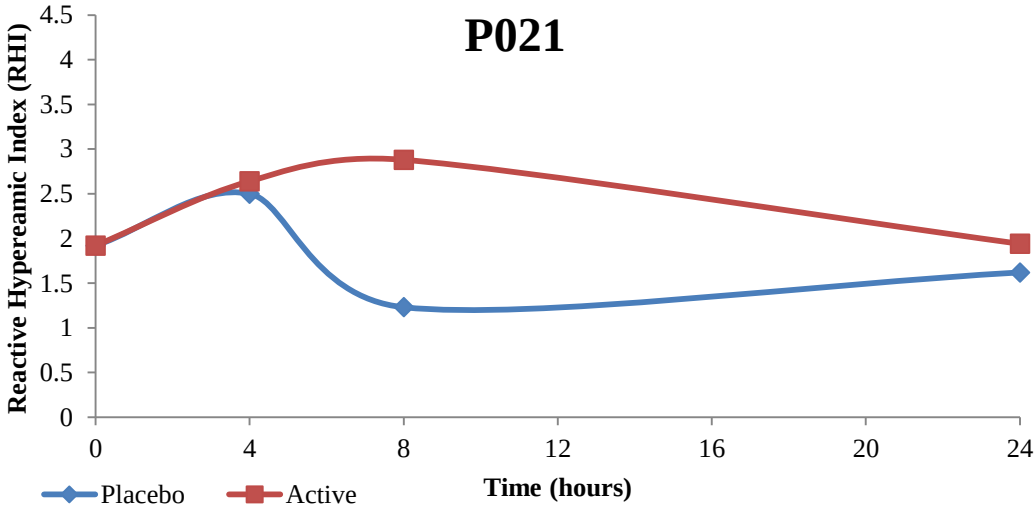

P016
Reactive Hypereamic Index (RHI)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
4
8
12
16
20
24
Placebo
Active
Time (hours)


P017
Reactive Hypereamic Index (RHI)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
4
8
12
16
20
24
Placebo
Active
Time (hours)


P018
Reactive Hypereamic Index (RHI)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
4
8
12
16
20
24
Placebo
Active
Time (hours)


P019
Reactive Hypereamic Index (RHI)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
4
8
12
16
20
24
Time (hours)
Placebo
Active


P020
Reactive Hypereamic Index (RHI)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
4
8
12
16
20
24
Time (hours)
Placebo
Active


P021
Reactive Hypereamic Index (RHI)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
4
8
12
16
20
24
Time (hours)
Placebo
Active

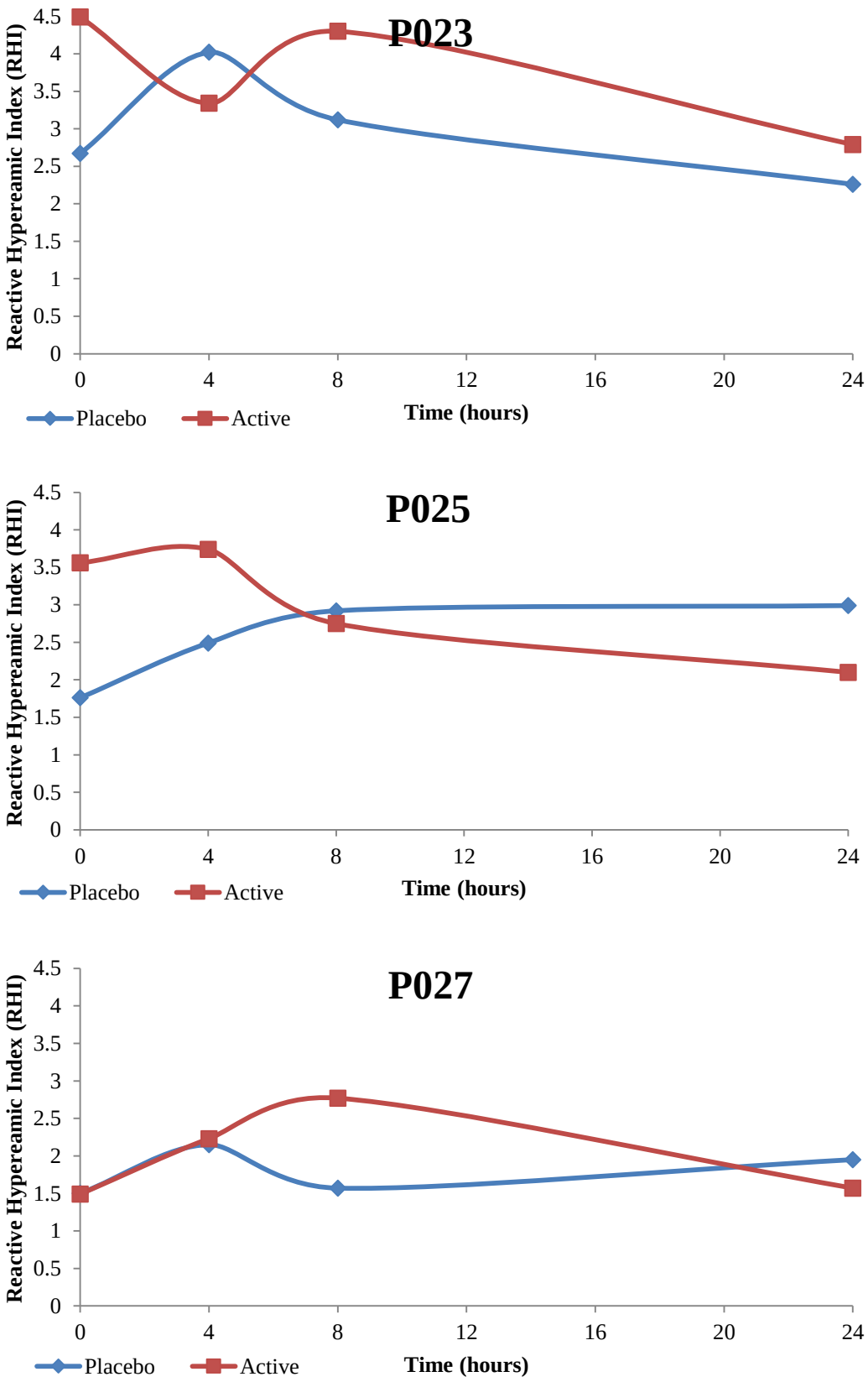
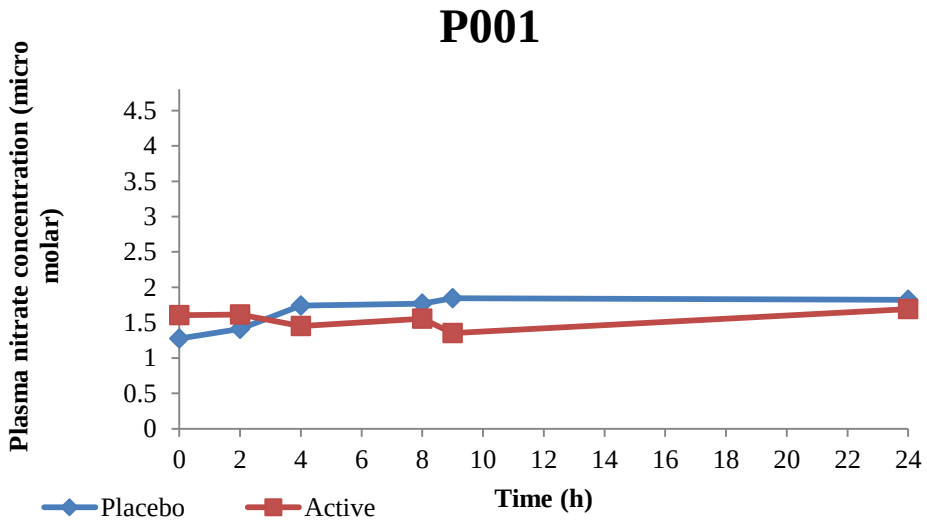
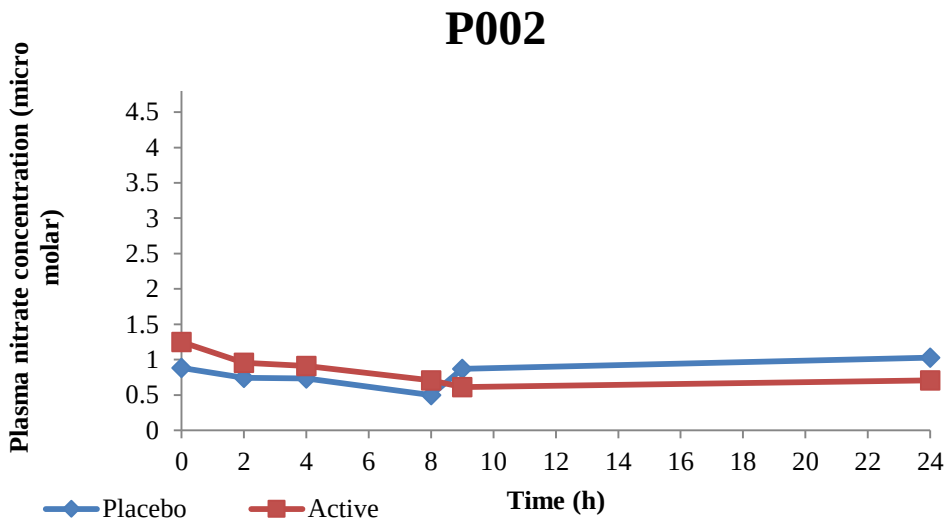
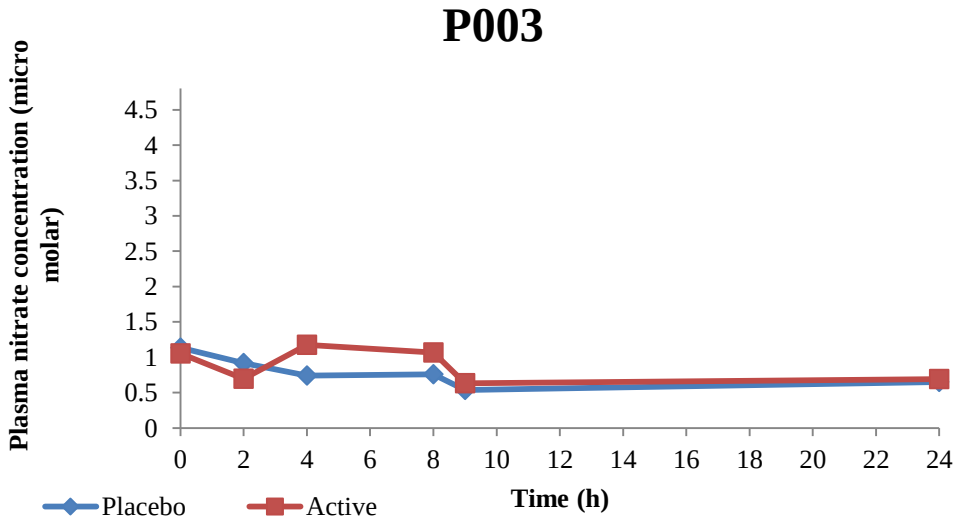
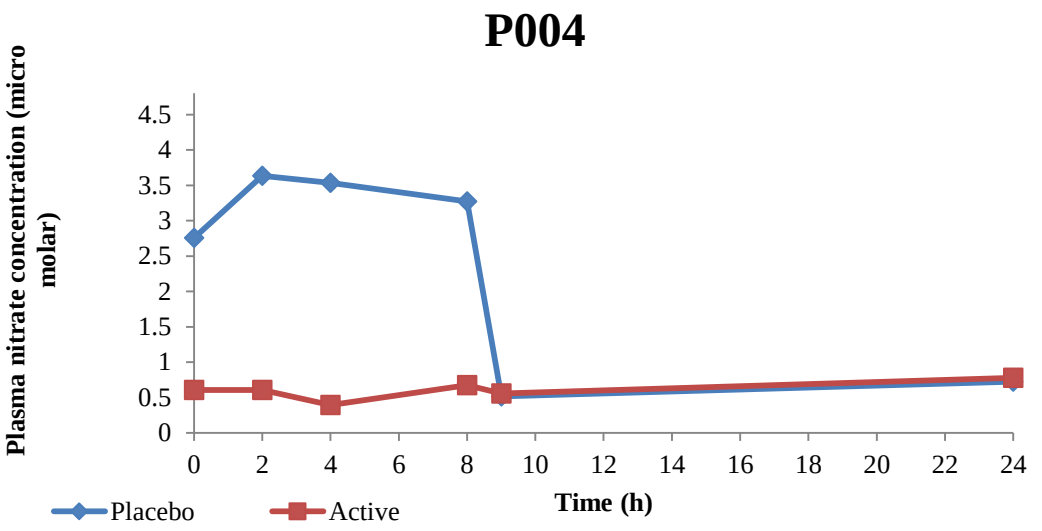


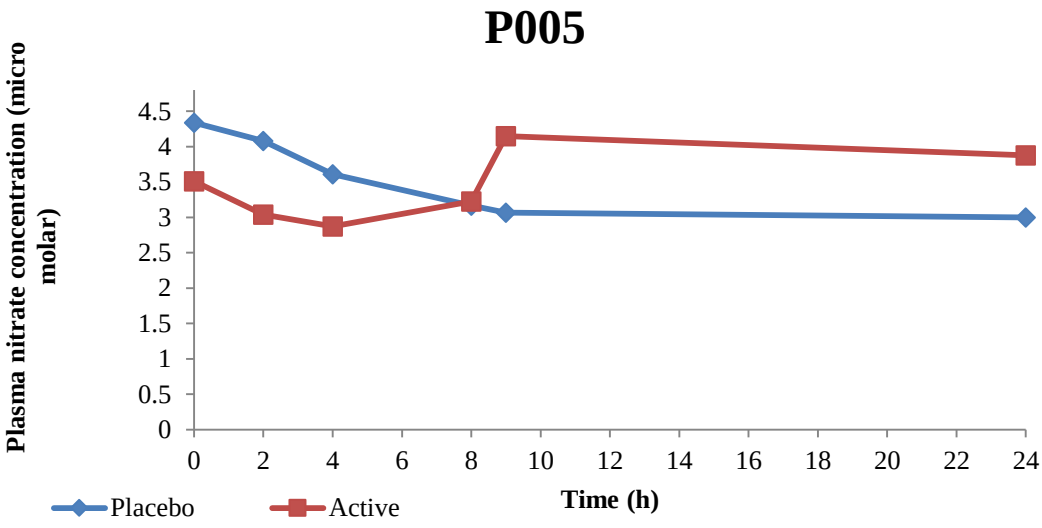
Figure 7-4 Individual Plots of reactive hyperaemic index values against time for all the participants (n=24)

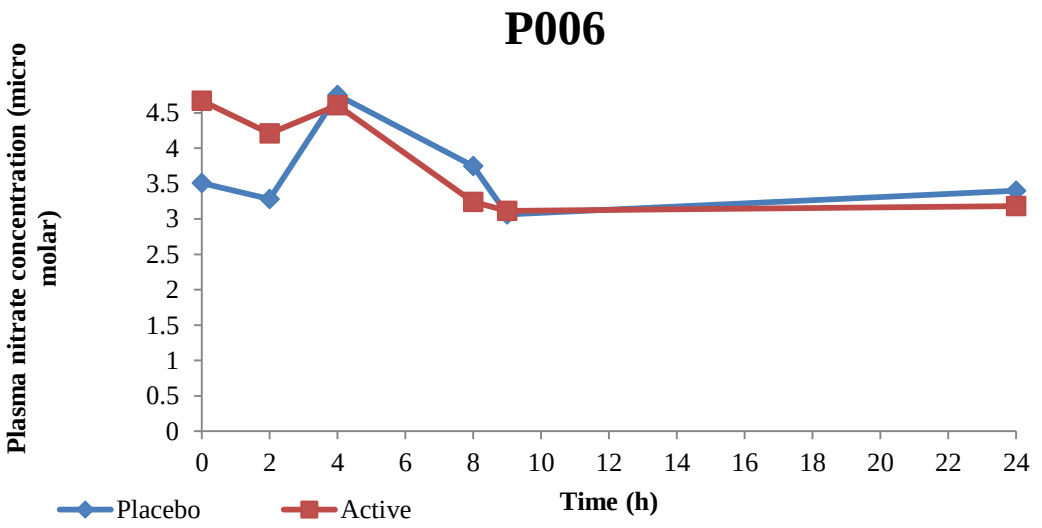

P001
Plasma nitrate concentration (micro molar)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (h)
Placebo
Active

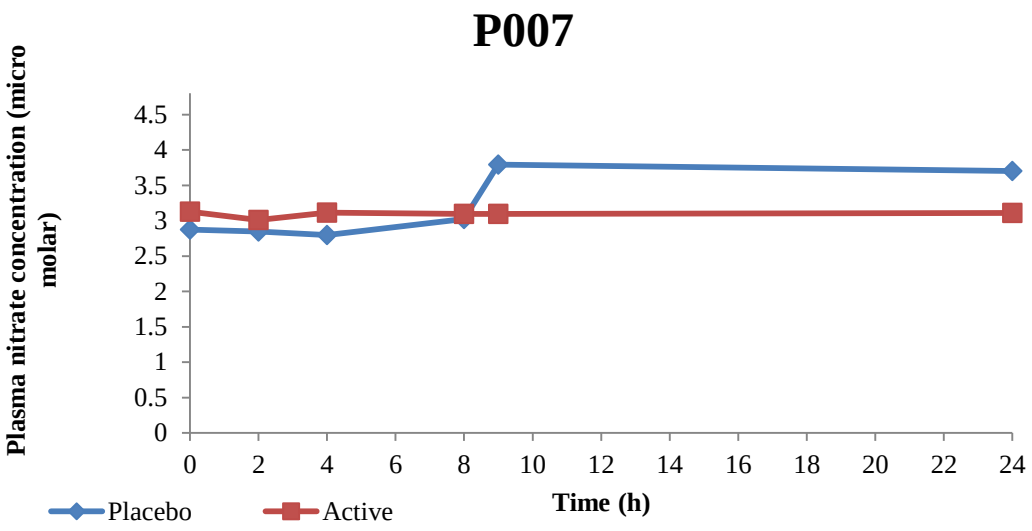

P002
Plasma nitrate concentration (micro molar)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (h)
Placebo
Active

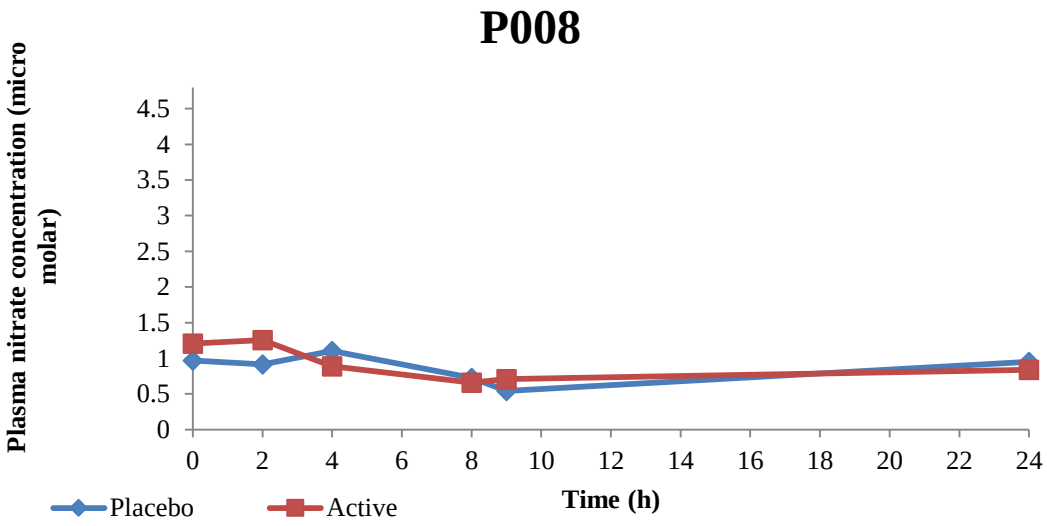

P003
Plasma nitrate concentration (micro molar)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (h)
Placebo
Active

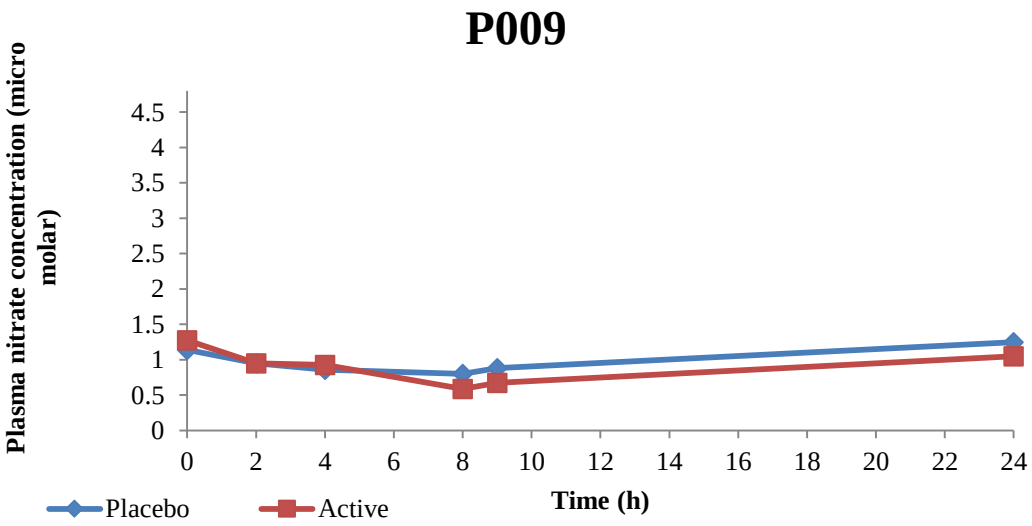

P004
Plasma nitrate concentration (micro molar)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (h)
Placebo
Active

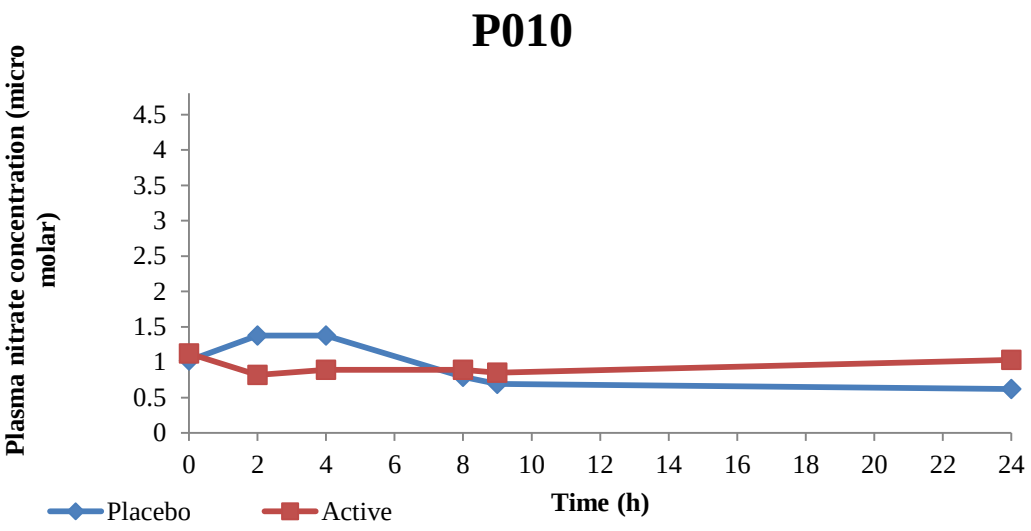

P005
Plasma nitrate concentration (micro molar)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (h)
Placebo
Active

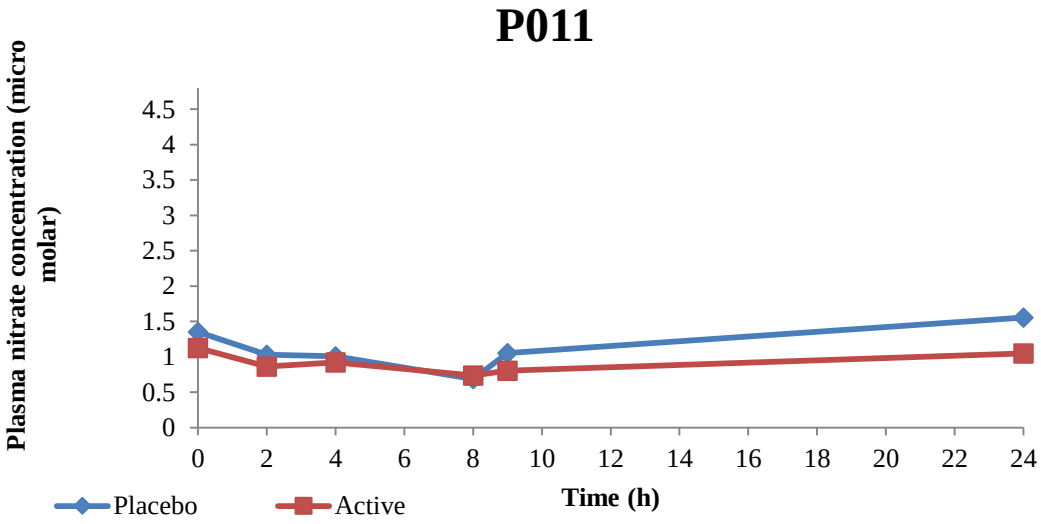

P006
Plasma nitrate concentration (micro molar)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (h)
Placebo
Active


P007
Plasma nitrate concentration (micro molar)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (h)
Placebo
Active


P008
Plasma nitrate concentration (micro molar)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (h)
Placebo
Active


P009
Plasma nitrate concentration (micro molar)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (h)
Placebo
Active


P010
Plasma nitrate concentration (micro molar)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (h)
Placebo
Active


P011
Plasma nitrate concentration (micro molar)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
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Time (h)
Placebo
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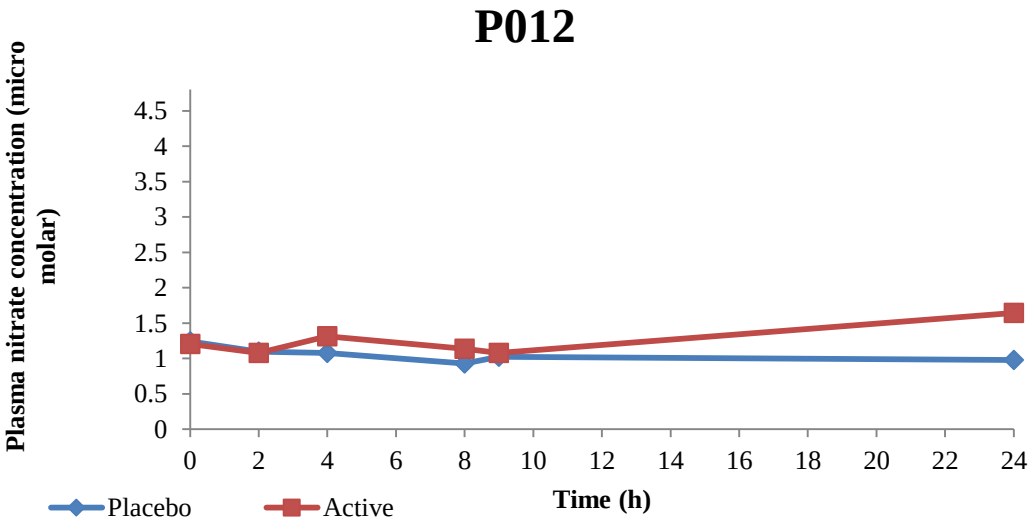
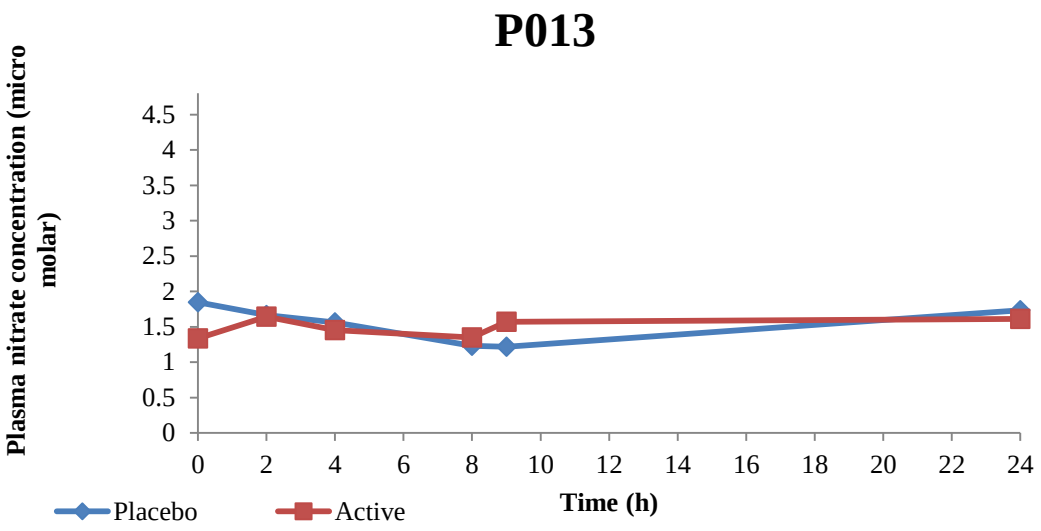
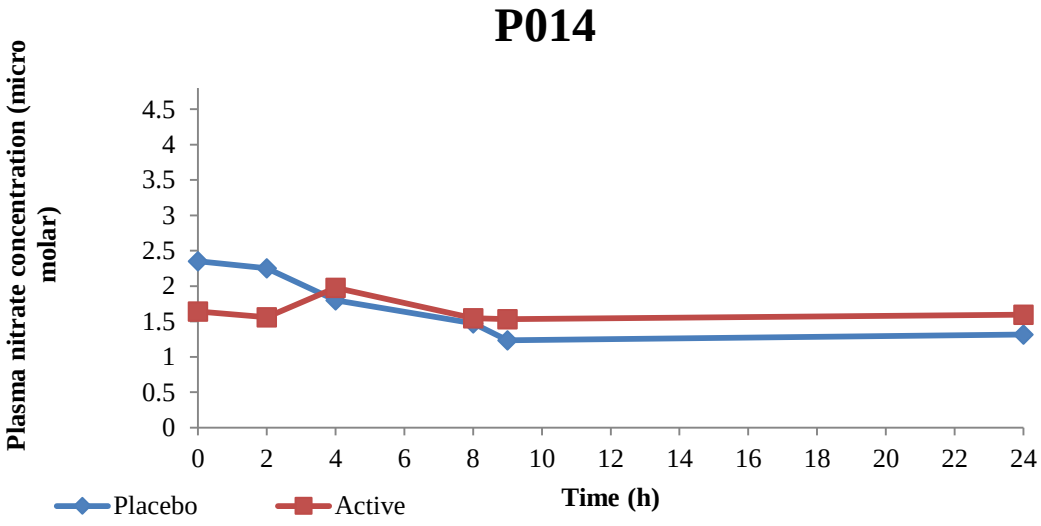
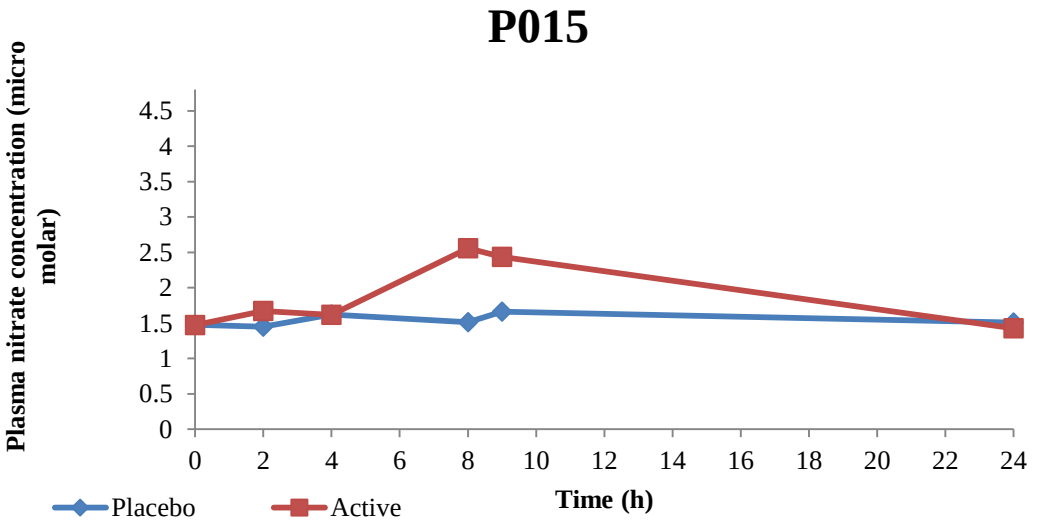
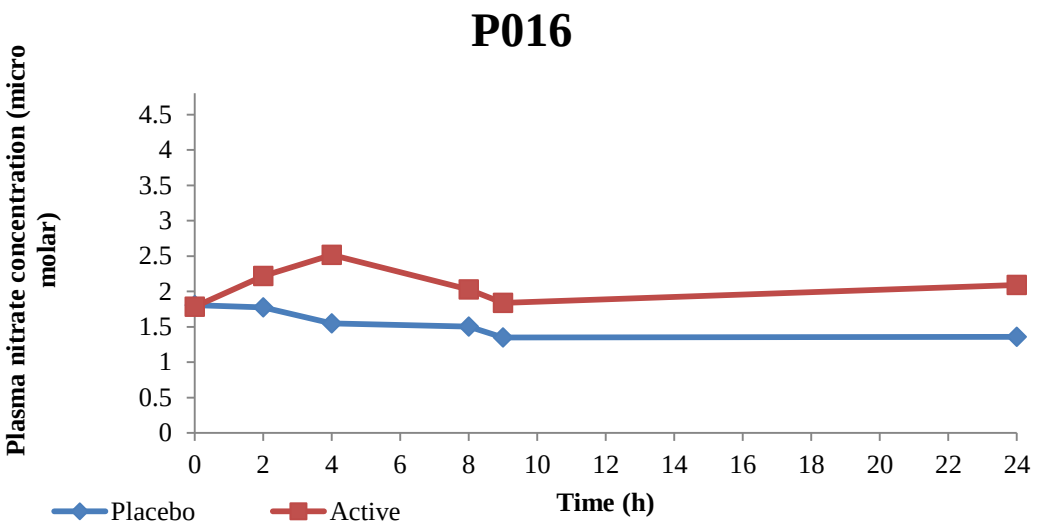
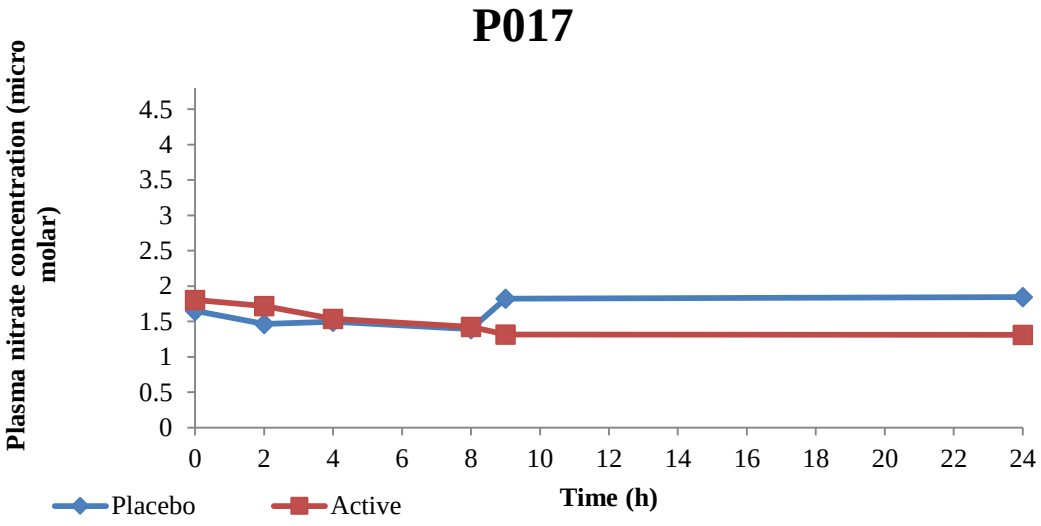

P012
Plasma nitrate concentration (micro molar)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (h)
Placebo
Active

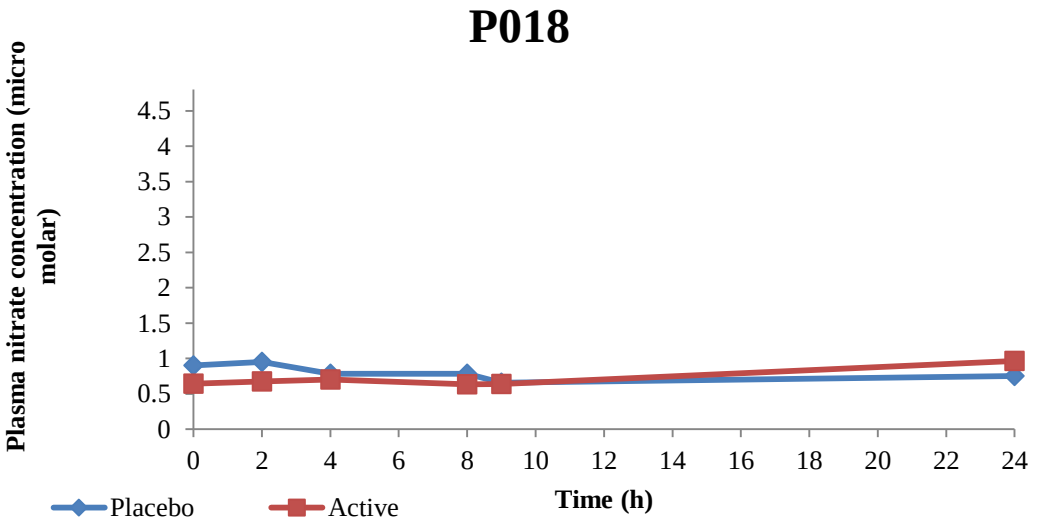

P013
Plasma nitrate concentration (micro molar)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (h)
Placebo
Active


P014
Plasma nitrate concentration (micro molar)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (h)
Placebo
Active

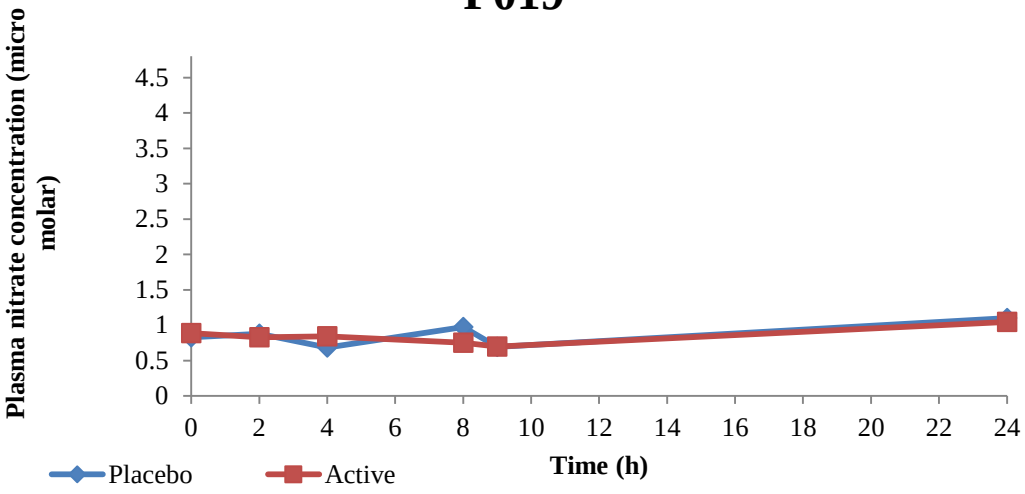

P015
Plasma nitrate concentration (micro molar)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (h)
Placebo
Active


P016
Plasma nitrate concentration (micro molar)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (h)
Placebo
Active


P017
Plasma nitrate concentration (micro molar)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (h)
Placebo
Active


P018
Plasma nitrate concentration (micro molar)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (h)
Placebo
Active

P019



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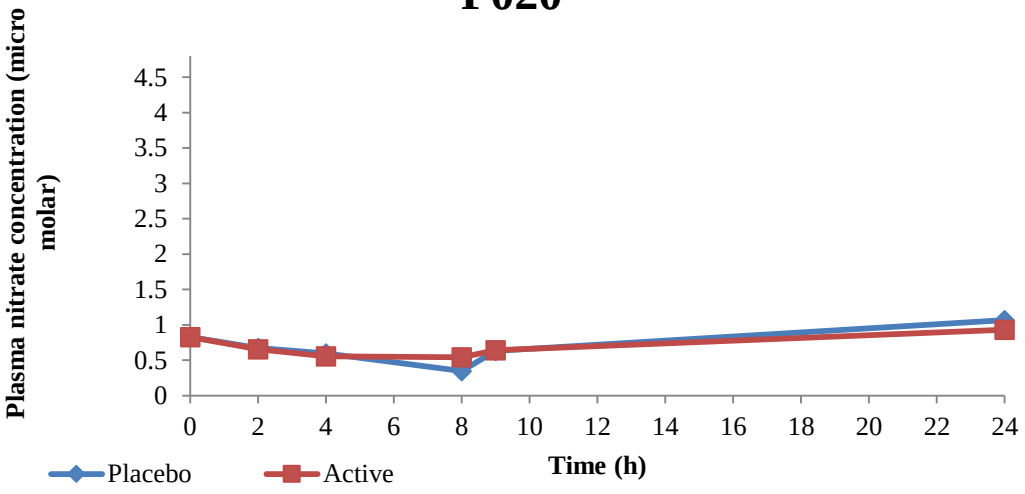

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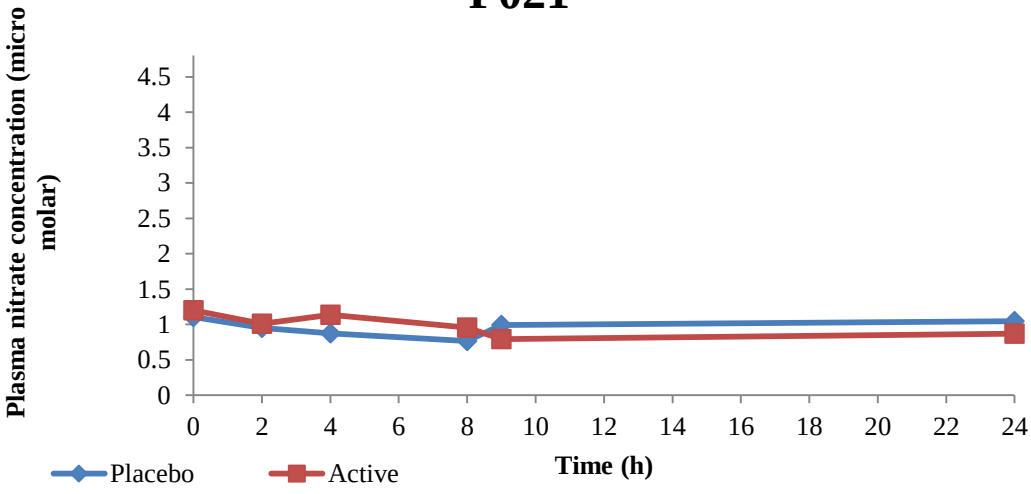


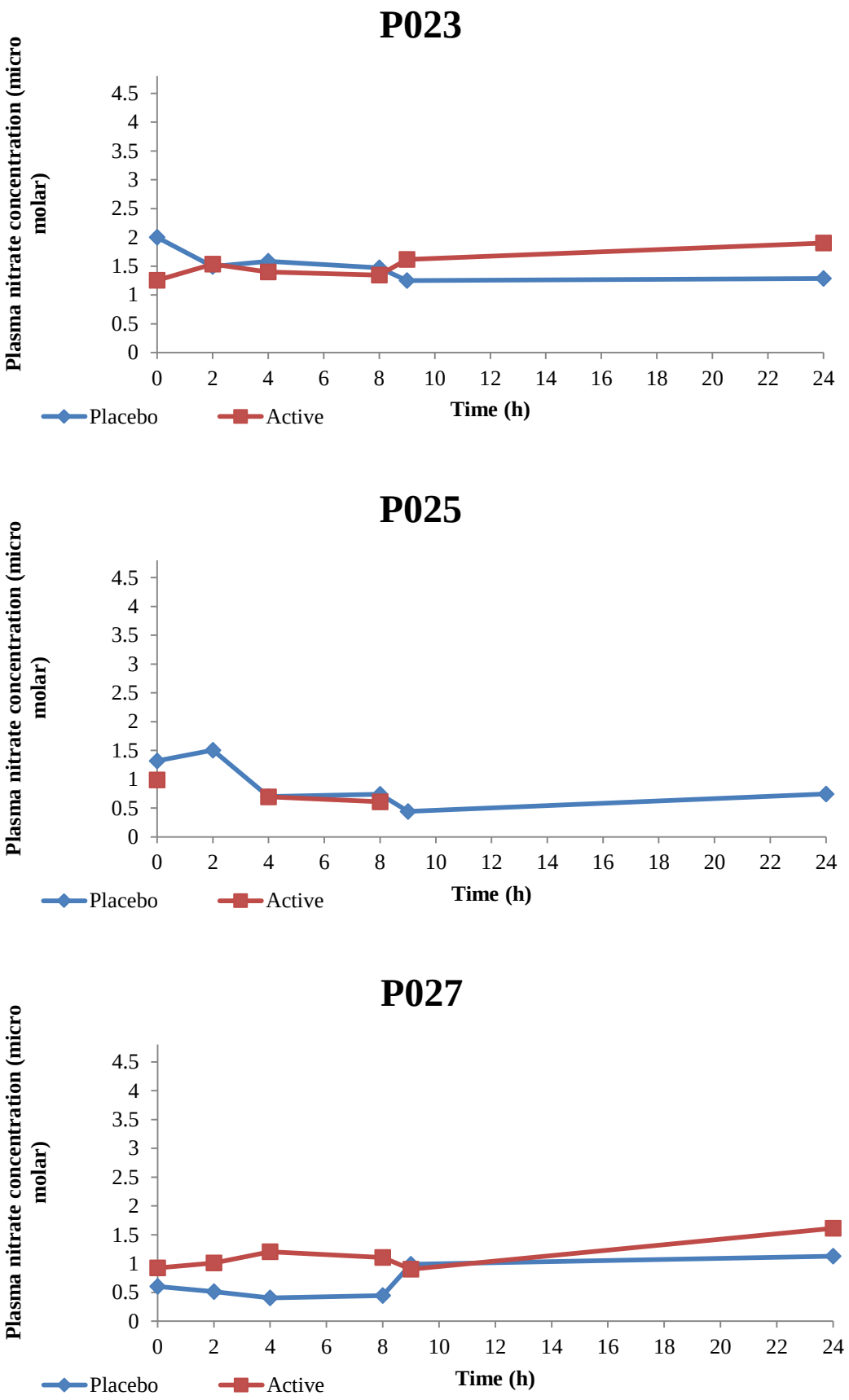
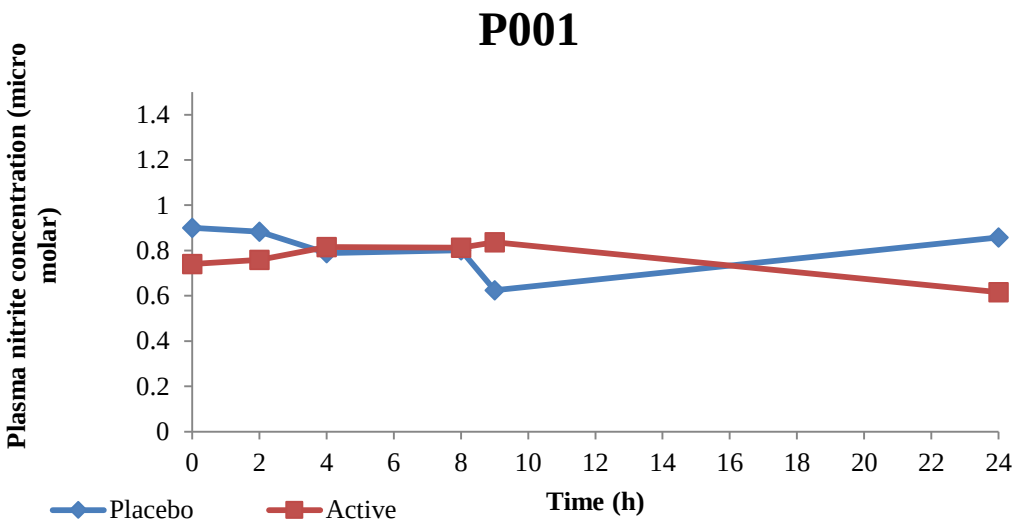
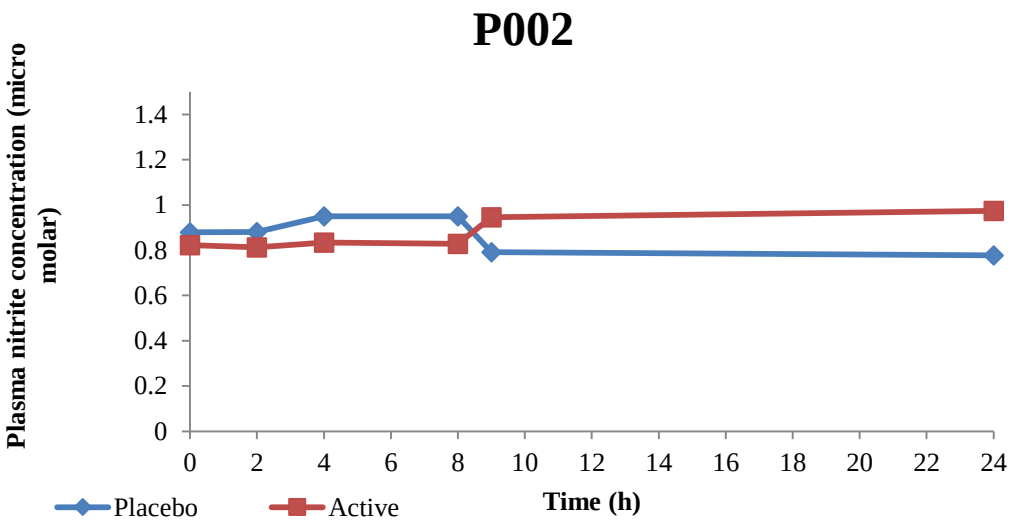


Figure 7-5 Individual plots of plasma nitrate concentrations for healthy volunteers post ingestion of either placebo or Oligopin®.


P001
Plasma nitrite concentration (micro molar)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (h)
Placebo
Active


P002
Plasma nitrite concentration (micro molar)
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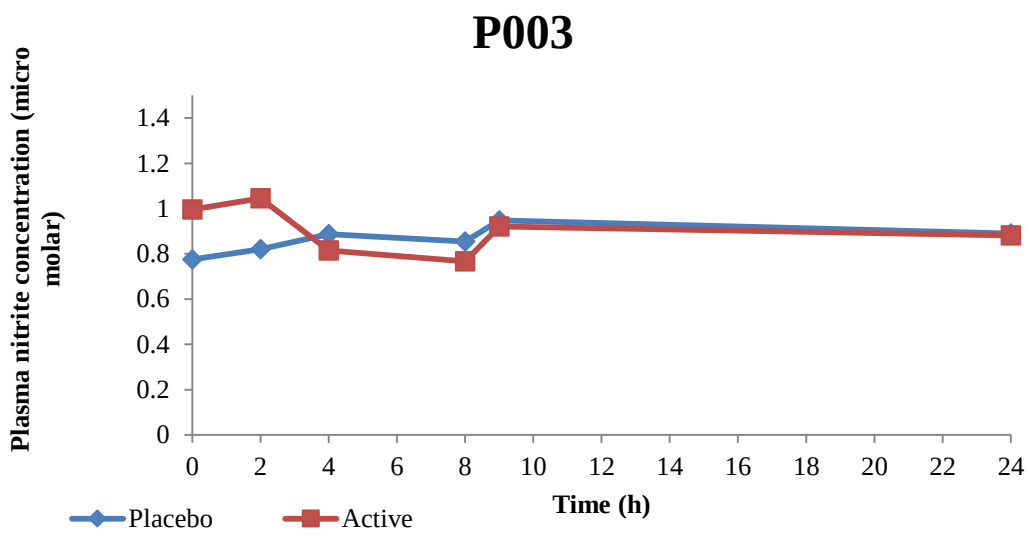
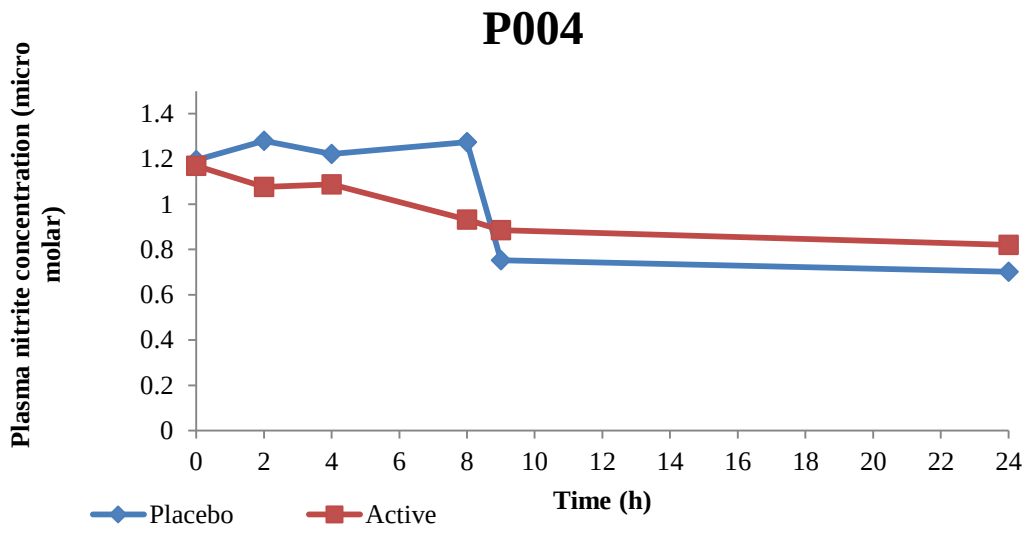
0.6
0.4
0.2
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (h)
Placebo
Active


P003
Plasma nitrite concentration (micro molar)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (h)
Placebo
Active


P004
Plasma nitrite concentration (micro molar)
1.4
1.2
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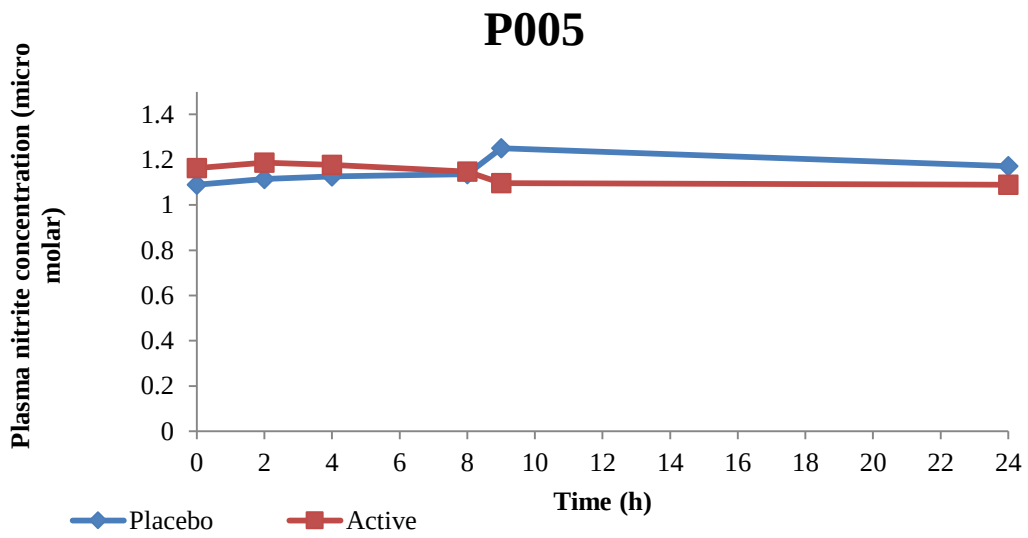
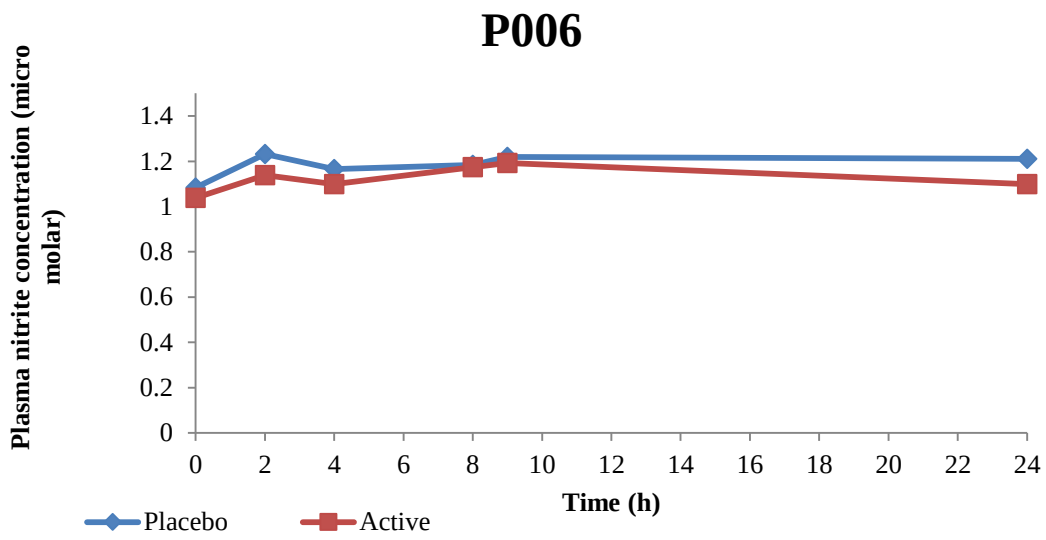
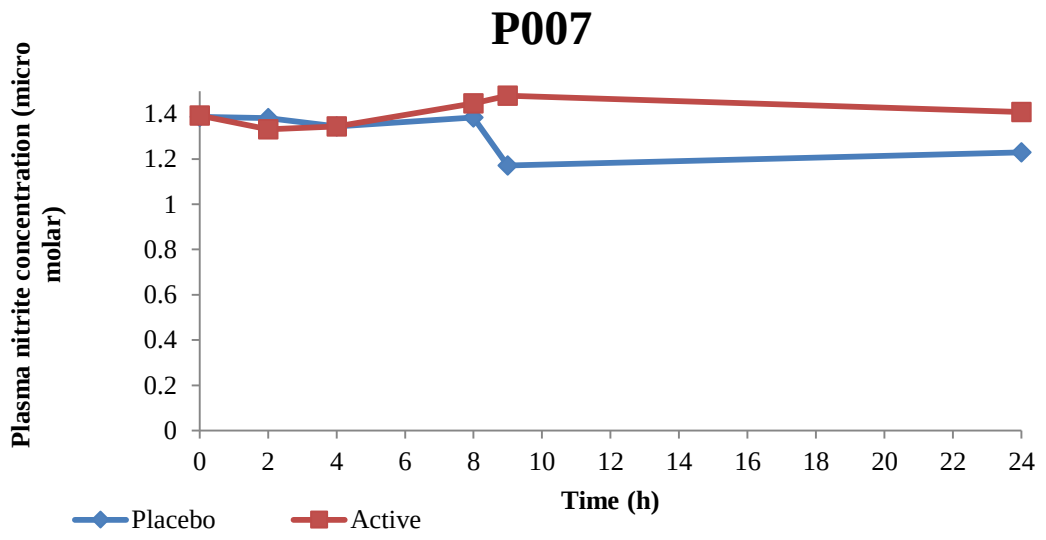
0.8
0.6
0.4
0.2
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (h)
Placebo
Active


P005
Plasma nitrite concentration (micro molar)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (h)
Placebo
Active


P006
Plasma nitrite concentration (micro molar)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (h)
Placebo
Active


P007
Plasma nitrite concentration (micro molar)
1.4
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0.8
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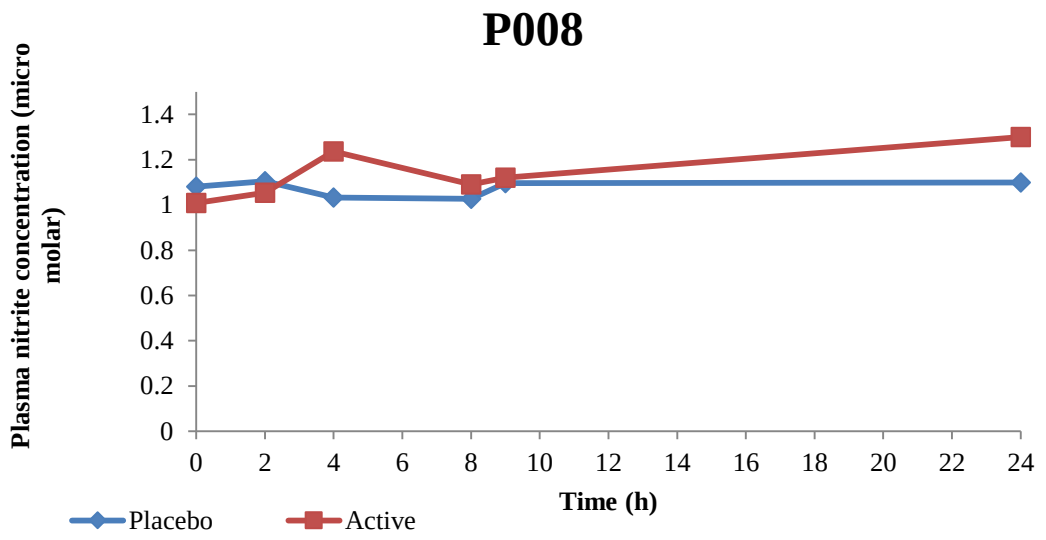
Time (h)
Placebo
Active


P008
Plasma nitrite concentration (micro molar)
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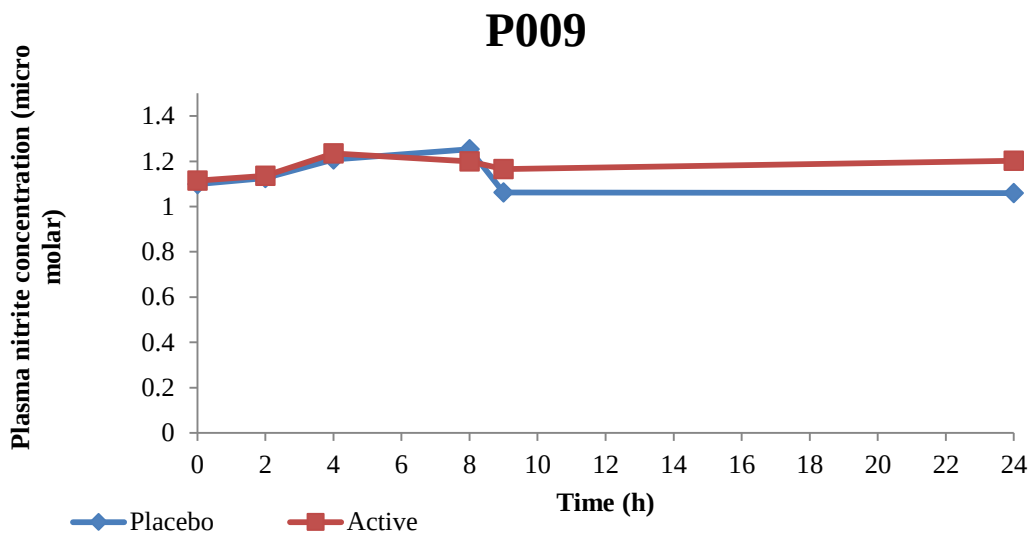
Time (h)
Placebo
Active


P009
Plasma nitrite concentration (micro molar)
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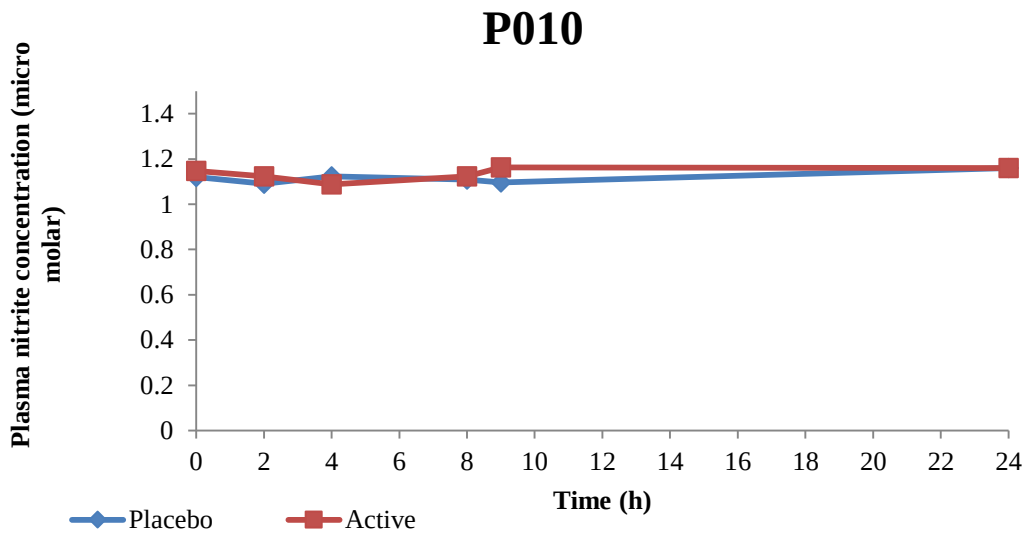
0.8
0.6
0.4
0.2
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (h)
Placebo
Active


P010
Plasma nitrite concentration (micro molar)
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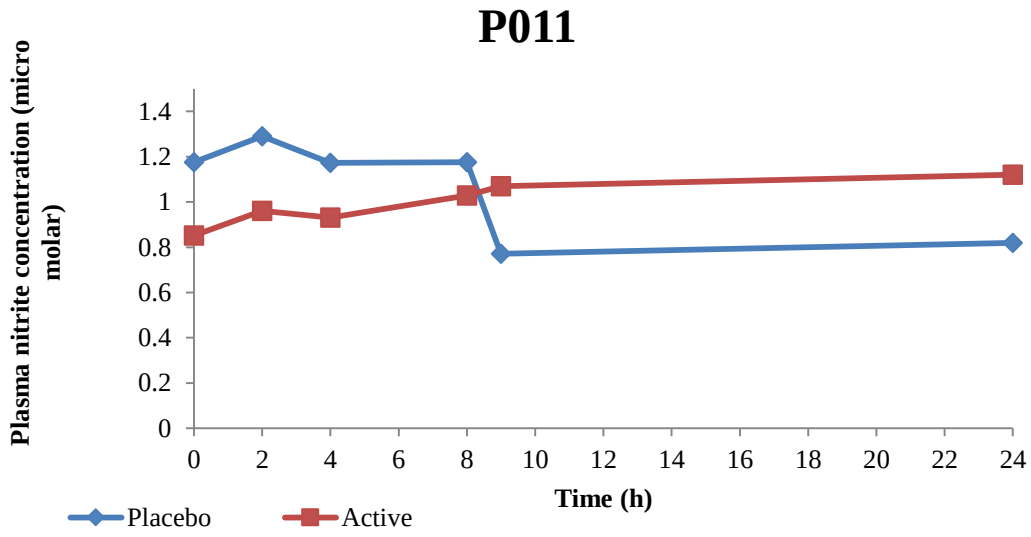
0.8
0.6
0.4
0.2
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (h)
Placebo
Active


P011
Plasma nitrite concentration (micro molar)
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0.8
0.6
0.4
0.2
0
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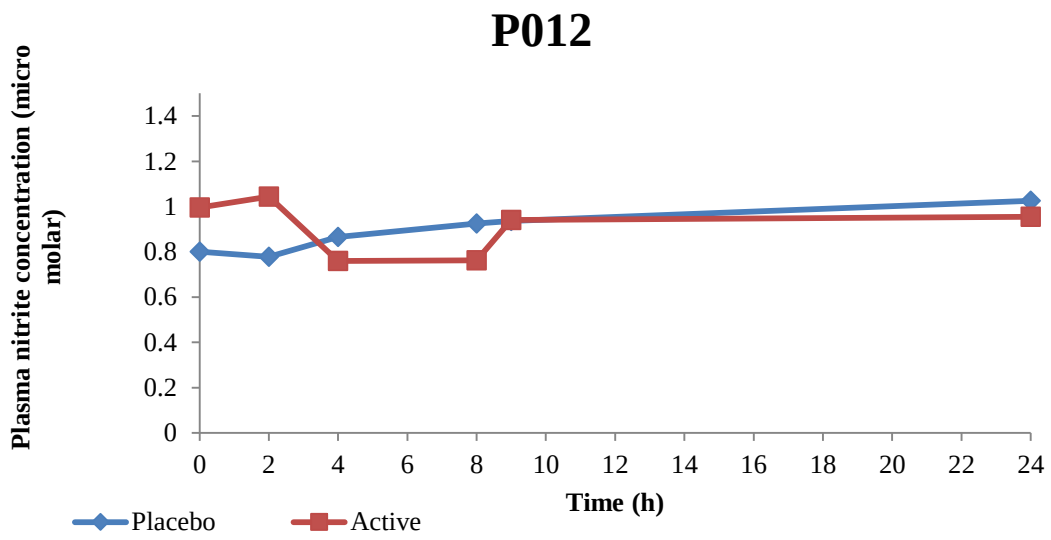
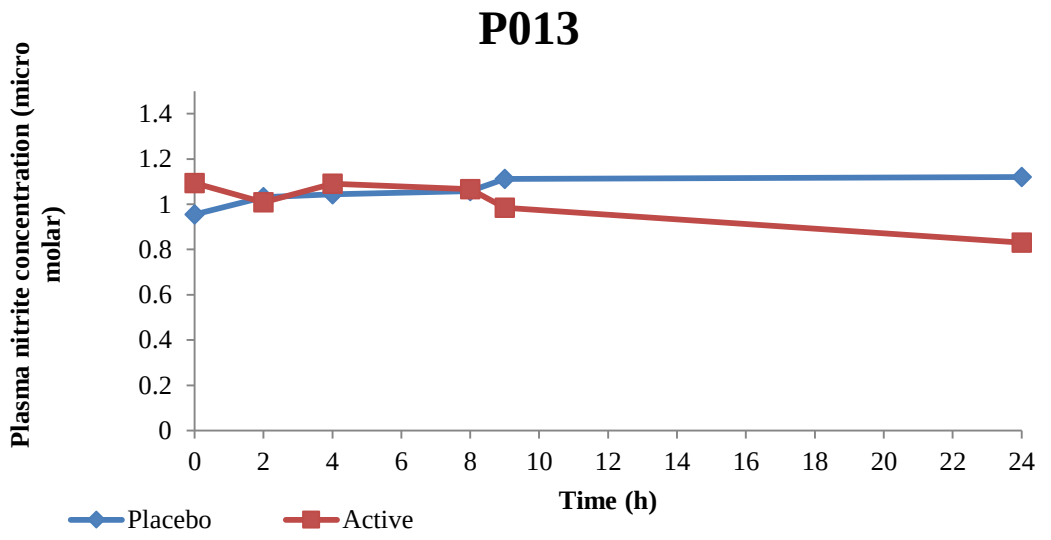
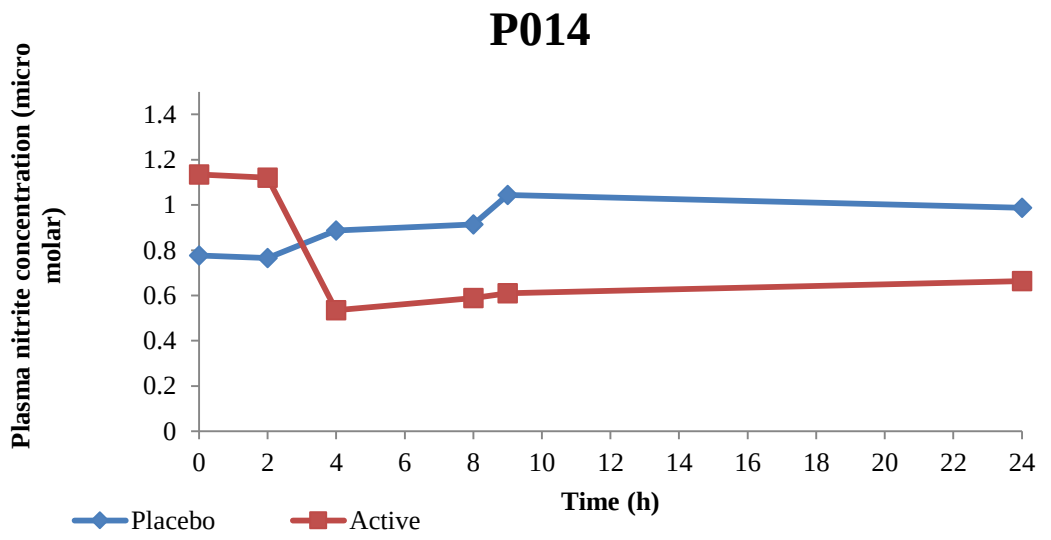
Time (h)
Placebo
Active


P012
Plasma nitrite concentration (micro molar)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (h)
Placebo
Active


P013
Plasma nitrite concentration (micro molar)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (h)
Placebo
Active


P014
Plasma nitrite concentration (micro molar)
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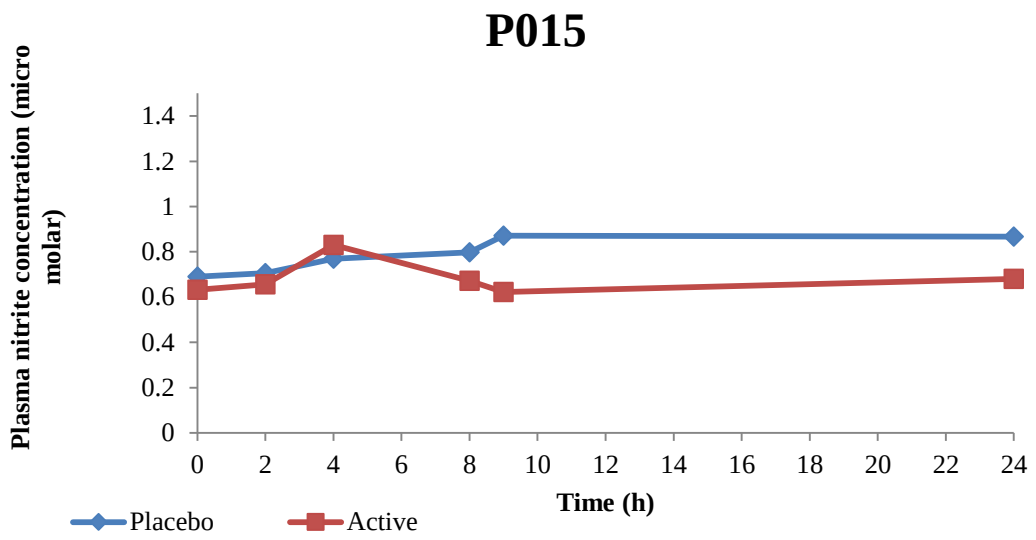
0.8
0.6
0.4
0.2
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (h)
Placebo
Active


P015
Plasma nitrite concentration (micro molar)
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0.6
0.4
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Time (h)
Placebo
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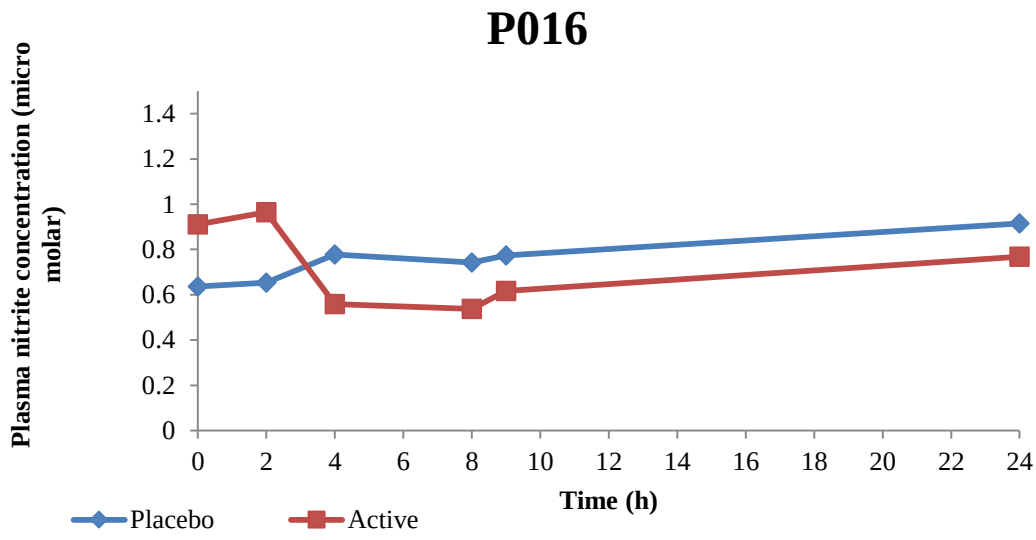
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Plasma nitrite concentration (micro molar)
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Time (h)
Placebo
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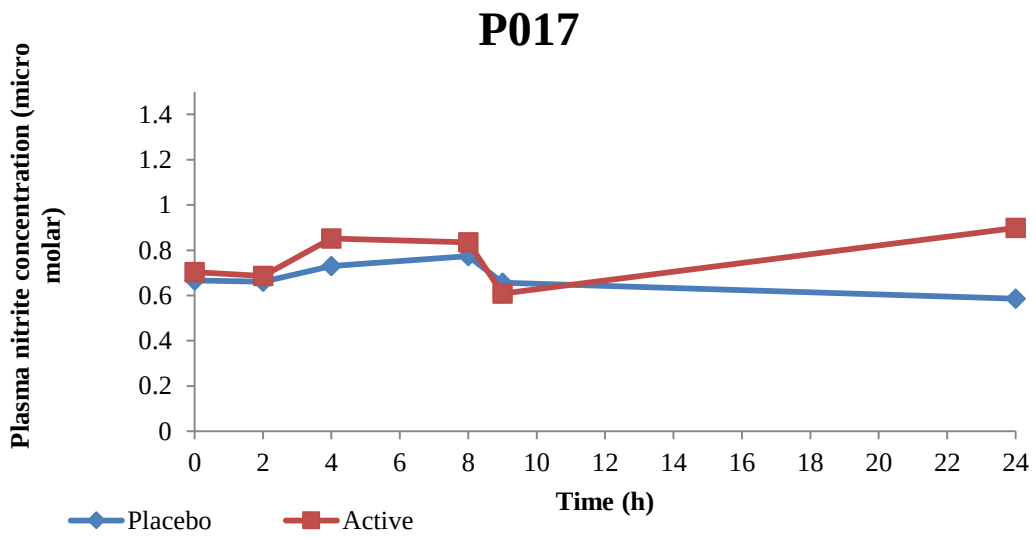
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Plasma nitrite concentration (micro molar)
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0.4
0.2
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Time (h)
Placebo
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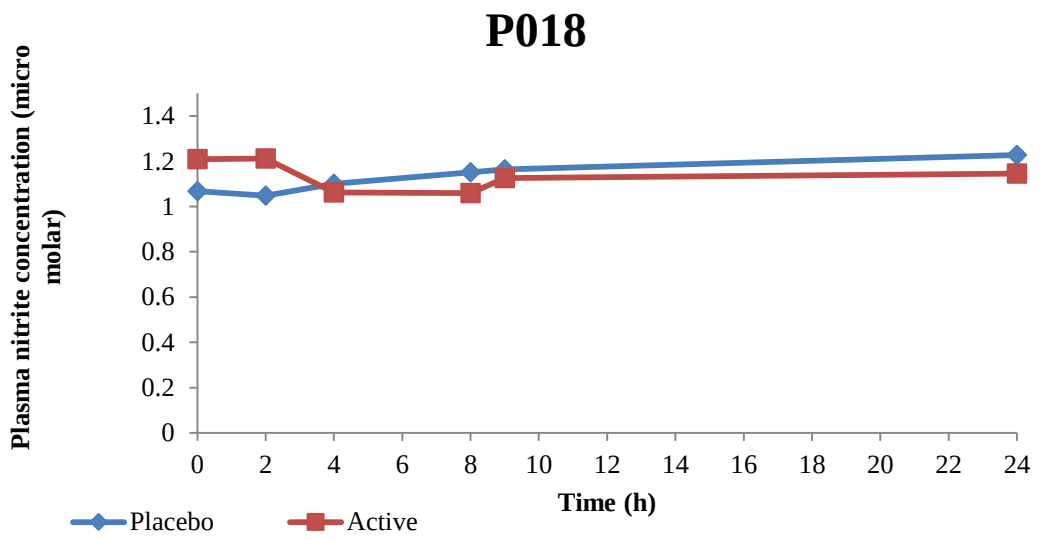
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Plasma nitrite concentration (micro molar)
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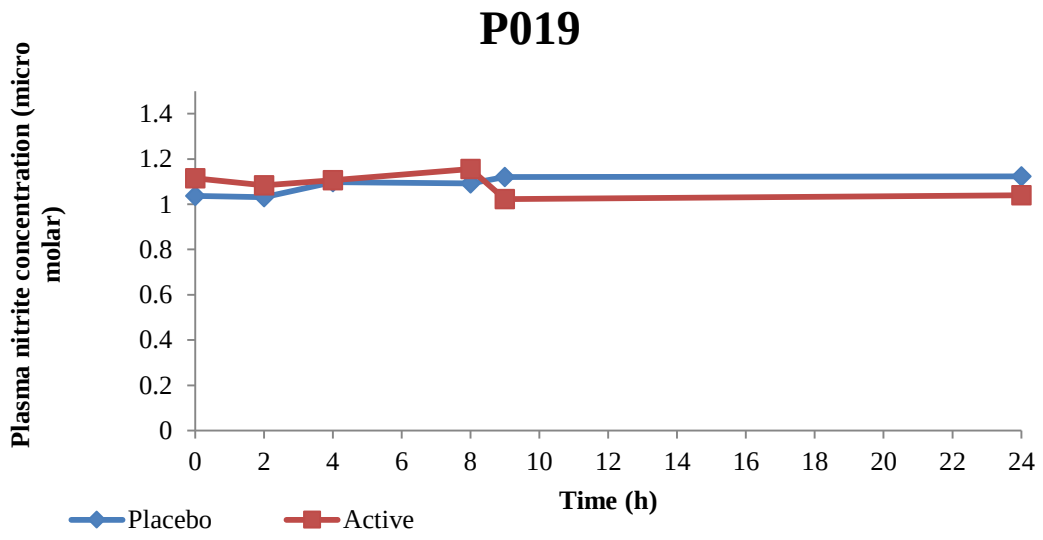
0.8
0.6
0.4
0.2
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (h)
Placebo
Active


P019
Plasma nitrite concentration (micro molar)
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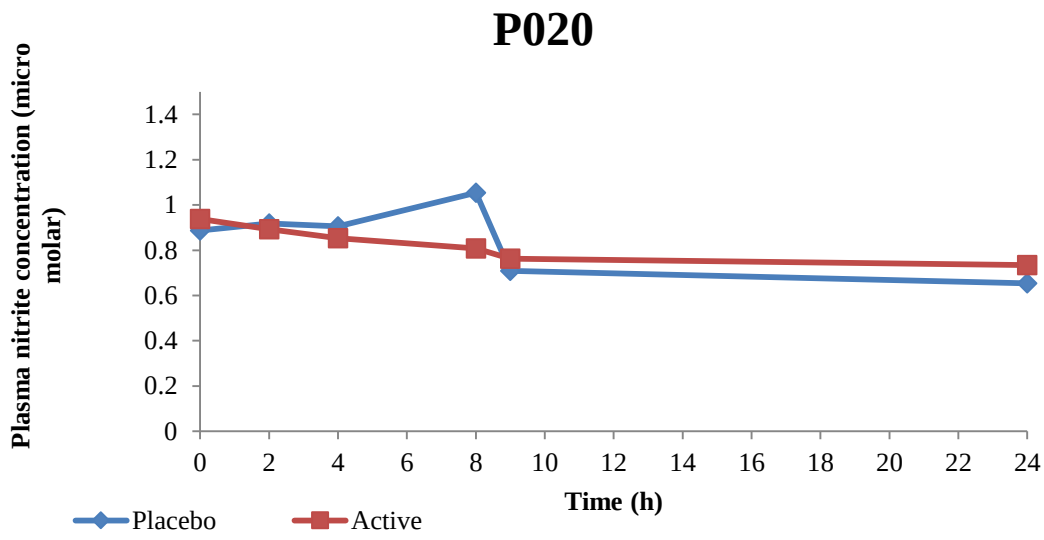
0.8
0.6
0.4
0.2
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (h)
Placebo
Active


P020
Plasma nitrite concentration (micro molar)
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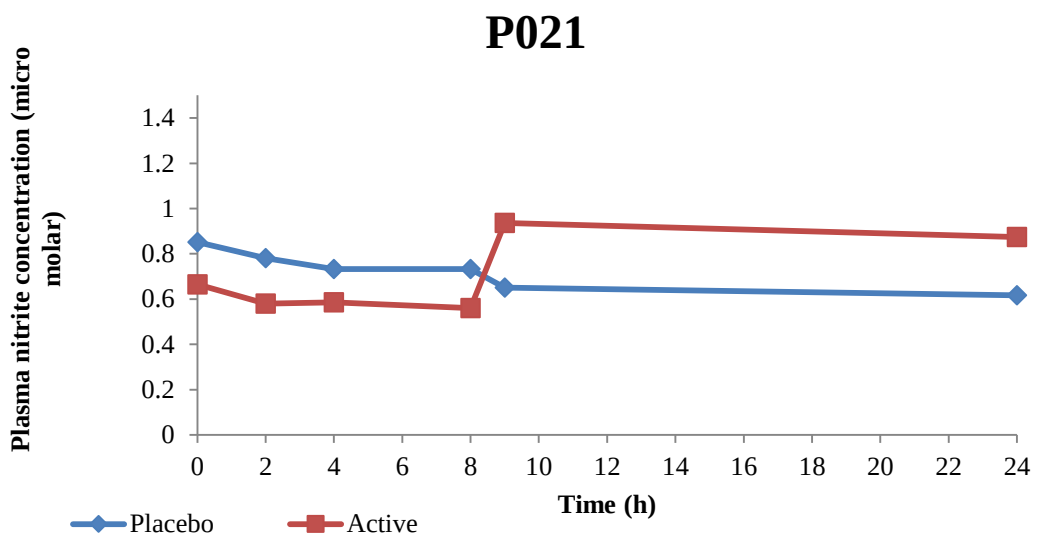
0.8
0.6
0.4
0.2
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (h)
Placebo
Active


P021
Plasma nitrite concentration (micro molar)
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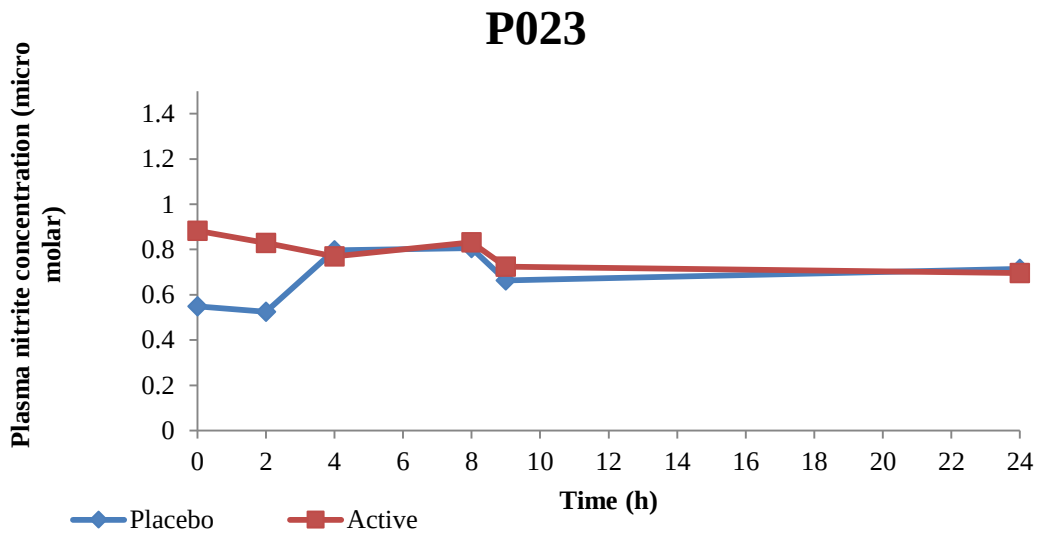
0.6
0.4
0.2
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (h)
Placebo
Active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Plasma nitrite concentration (micro molar)
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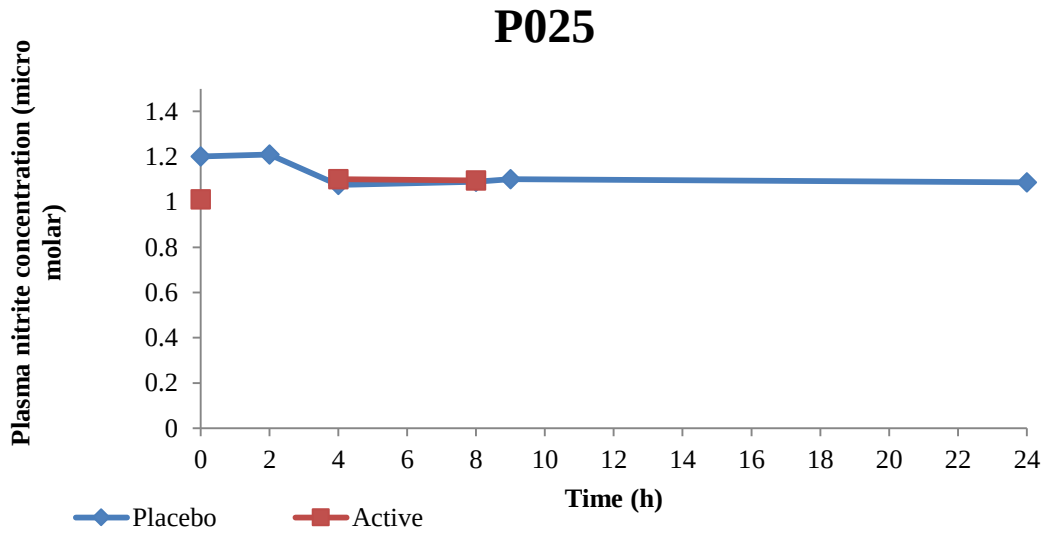
0.6
0.4
0.2
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (h)
Placebo
Active


P025
Plasma nitrite concentration (micro molar)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (h)
Placebo
Active

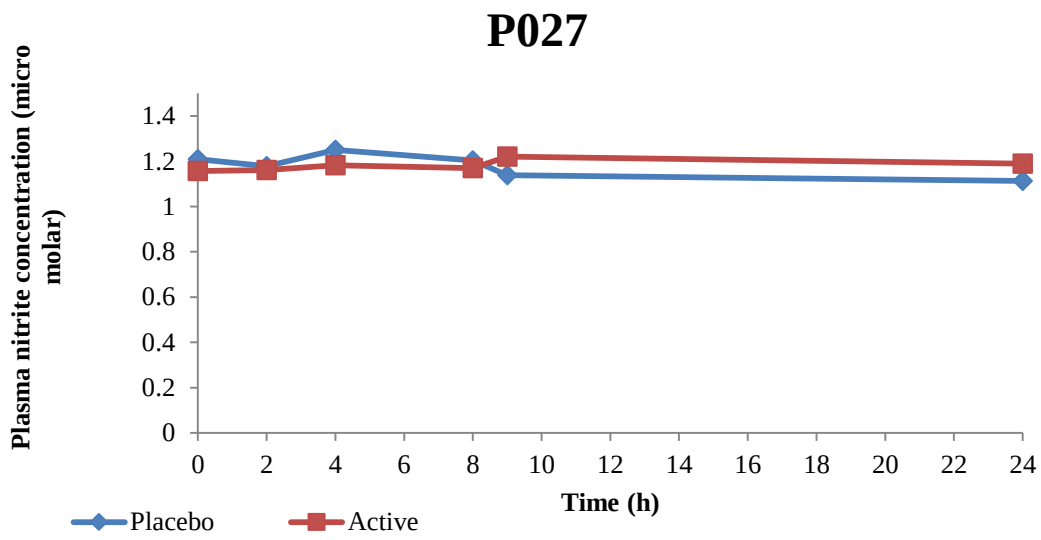


Figure 7-6 Individual plots of plasma nitrite concentrations for healthy volunteers post ingestion of either placebo or Oligopin®.

7.3.2 The Pharmacokinetics of Oligopin® (see figure 7.7)

Almost all samples (22 out of 24) had detectable levels of catechin at baseline (0 hours). Epicatechin was only detected in one out of the 24 samples analysed. Catechin was detected at 8 hours post ingestion of Oligopin®, with a significant rise noted from baseline (P- value 0.0003). The plasma concentrations of catechin detected for each individual assessed are listed in Table 7.4.

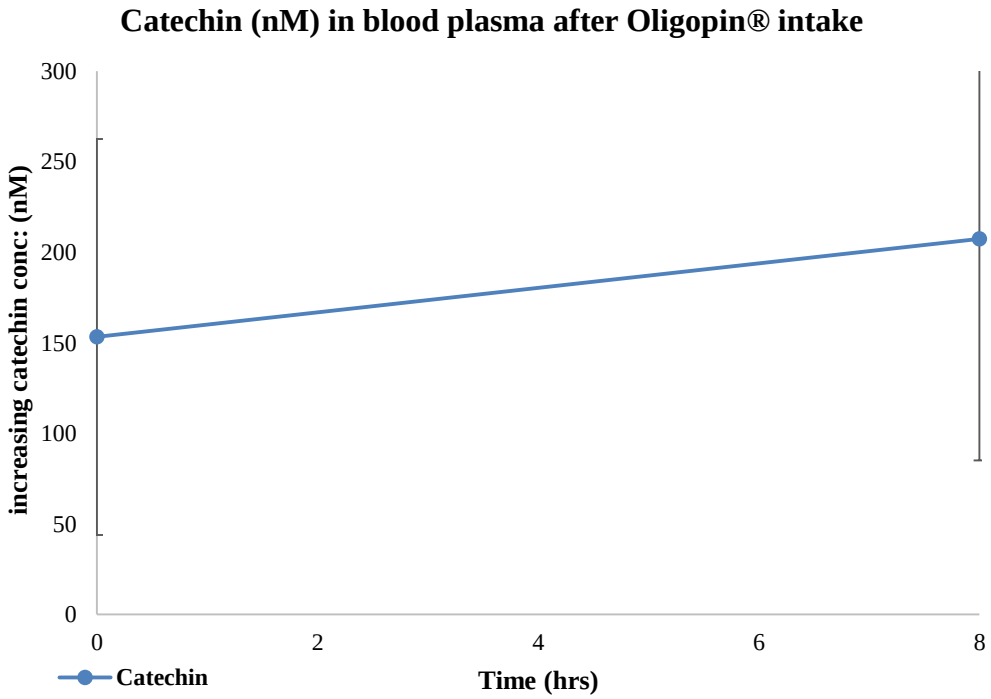


Figure 7-7 The pharmacokinetic properties of Oligopin. Data plotted as mean plasma concentration of catechin (detected by LC-MS) for each time-point ± SD (n=24).

7.4 Discussion

The main findings from this study are that reactive hyperaemia indices (RHI) measured at various time points (0, 4, 8 and 24 hours) did not differ significantly between Oligopin® and placebo treatments. There was also no discernible effect of Oligopin® compared to placebo on blood pressure or plasma nitrate and nitrite concentrations. Taken together, these data suggest that Oligopin® has no effect on vascular function in healthy volunteers after a single administration.

The results of plasma nitrate and nitrite analysis from the absorption and bioavailability study (**Chapter 3**) suggested, due to an increase in nitrite concentrations at 8 h post ingestion of a matched dose of Oligopin® to this current trial, that Oligopin® may improve nitric oxide bioavailability and thus vascular tone in healthy volunteers. This finding is not supported by this study. It became

apparent after this study was completed that different batches of Oligopin® had been used for the two studies.

As complex plant extracts are known to vary substantially between batches it was postulated that this might be an underlying reason why there was no effect observed in this study when one had been indicated by the earlier study (chapter 3). Using the *in vitro* screening assay developed as part of this project, the effect of each batch of Oligopin® on nitric oxide availability in human endothelial cells was assessed. The batch used for this present clinical study was shown not to improve nitric oxide availability in this assay (Table 7-3), which is in contrast to the previous screening data that resulted in the selection of Oligopin® for clinical assessment.

A second possible reason for the observed differences in the efficacy of the different batches in healthy volunteers is that the different batches have differing pharmacokinetic characteristics. We assessed the plasma concentrations of Catechin and Epicatechin in plasma samples at baseline and 8 h post-ingestion as a biomarker of Oligopin® bioavailability in plasma samples from volunteers who had ingested the Oligopin® preparation. Our findings showed that there were no significant differences in plasma Catechin concentrations at 8 h post-ingestion for volunteers from the earlier study (**Chapter 3**) and this study (Table 7-4).

Table 7-3 Rate of nitric oxide production (measured by DAF assay) in primary human endothelial cells incubated with different concentrations of the Oligopin® batch used for this clinical study.

Concentration (nM)	**0**	**1**	**10**	**100**	**200**	**500**	**1000**
Experiment 1	100	89.85	91.46	87.64	93.58	81.85	81.55
Experiment 2	100	104.83	103	102.57	104.06	100.18	93.26
Mean	100	97.34	97.23	95.11	98.82	91.01	87.41
SD	0	10.59	8.16	10.56	7.41	12.96	8.28

Data presented as a percentage of solvent only treated cells

Table 7-4 Catechin and Epicatechin concentrations detected in blood plasma samples from volunteers that had ingested Oligopin 0 or 8 h prior to sample collection.

Volunteer	Time point	Catechin	Epicatechin	Diadzein	C: D	E: D	Catechin (nM)	Epicatechin (nM)
1	0	35851	0	181350	0.19769	0	340.2574021	0
1	8	34002	0	176395	0.192761	0	331.7737793	0
2	0	29015	0	184115	0.157592	0	271.2422075	0
2	8	49652	0	210518	0.235856	0	405.9489093	0
3	0	36876	0	188496	0.195633	0	336.7173814	0
3	8	40982	0	206808	0.198164	0	341.0748383	0
4	0	38125	0	180577	0.211129	0	363.3885896	0
4	8	49463	0	198766	0.24885	0	428.3139535	0
5	0	21049	0	80269	0.262231	0	127.0497815	0
5	8	40162	0	59755	0.672111	0	325.6352328	0
6	0	35146	0	71345	0.49262	0	238.6726579	0
6	8	20806	0	63270	0.328845	0	159.3239506	0
7	0	16260	0	55108	0.295057	0	142.9539627	0
7	8	21402	0	64743	0.330569	0	160.159184	0
8	0	0	0	73834	0	0	0	0
8	8	6349	0	64365	0.098641	0	47.79097167	0
9	0	0	0	53474	0	0	0	0
9	8	5957	0	45256	0.131629	0	63.77371864	0
10	0	72240	0	267471	0.270085	0	103.4413462	0
10	8	131476	0	231031	0.569084	0	217.956263	0
11	0	47174	0	189556	0.248866	0	95.31435103	0
11	8	131398	0	216850	0.60594	0	232.0718459	0
12	0	33485	0	228031	0.146844	0	56.24054747	0
12	8	64410	0	225792	0.285263	0	109.2541335	0
13	0	50190	0	211050	0.237811	0	91.08040799	0
13	8	94017	0	201591	0.466375	0	178.619298	0
14	0	90327	0	169435	0.533107	0	204.1773616	0
14	8	114637	0	182295	0.628854	0	240.8480772	0
15	0	34692	0	134129	0.258647	0	103.7907411	0
15	8	22801	0	93136	0.244814	0	98.2399821	0
16	0	13471	12964	111605	0.120702	0.11616	74.55372297	49.22019927
16	8	12522	0	99215	0.126211	0	77.95599408	0
17	0	10590	0	79532	0.133154	0	82.24456492	0
17	8	13792	0	77380	0.178237	0	110.0909639	0
18	0	6677	0	92418	0.072248	0	44.62497252	0
18	8	11687	0	102981	0.113487	0	70.09694497	0
19	0	15851	0	95008	0.166839	0	103.0503904	0
19	8	7286	0	79043	0.092178	0	56.93494468	0

20	0	33490	0	91069	0.367743	0	147.5694783	0
20	8	56651	0	88750	0.638321	0	256.1481247	0
21	0	35954	0	83501	0.430582	0	172.7855817	0
21	8	49146	0	81910	0.6	0	240.7704655	0
23	0	38812	0	67821	0.572271	0	229.6433088	0
23	8	49170	0	78165	0.629054	0	252.4293437	0
25	0	8565	0	130323	0.065721	0	40.59377589	0
25	8	22391	0	104637	0.213987	0	132.1725782	0
27	0	30700	0	170938	0.179597	0	309.117523	0
27	8	46198	0	180719	0.255634	0	439.9904268	0

7.4.1 Limitations of the study

There were several limitations in this study; these can be addressed in future work on the same preparation for better results. The major limitation of this work, highlighted above, is that different batches of Oligopin® were used across both studies, and this is currently the most probable reason for the lack of an agreement between the studies. Although Oligopin® is partially standardised in that some of the "active" structures – procyanidin multimers – are quantified and amounts controlled. However, many other potential active structures present are not identified or standardised in this extract. Due to the substantial technical difficulties inherent in the analytical challenges of fully standardising a plant extract, (this batch variation is a limitation, which will likely interfere any project using a complex extract. If further work were to be done using plant extracts, we would recommend the use of a single batch of material that has been shown to have biological activity relevant to the clinical study endpoints (e.g. the *in vitro* screening tool used in this study). This approach will not however solve the problems associated with batch variation (multiple batches would inevitably have to be used in further studies and manufacturing), and thus the testing of multiple different batches of an extract, and the demonstration of biological activity using a screening tool would be recommended. A better approach would be to identify chemical structures that have biological activities (using the *in vitro* screening tool) and then testing these structures, or an extract rich in these structures, in clinical studies. This approach, with the key active component(s) known and identified, would eliminate many of the limitations of using complex plant extracts in addition to providing greater understanding of the mechanism of action. The sample size for

this study was calculated as a pilot study rather than a definitive study. Moreover, we have done this study in health volunteers who have an intact endothelial function; the effect of this preparation on patients with diseased endothelium such as diabetes has not been studied.

8 General Discussion

The thesis had three objectives. The first was to discover whether Oligopin® (commercially available polyphenol preparation) when used in a physiologically relevant dose influenced endothelial function, specifically by influencing nitric oxide bioavailability. These effects of Oligopin® were evaluated *in vitro* using healthy HUVEC cells and the findings were further investigated in healthy volunteers *in vivo* compared to a methylcellulose placebo.

The second objective was to develop and validate a standardized, easily reproducible *in vitro*, angiotensin II-induced model for hypertension to study the effects of dietary polyphenols on endothelial function in hypertensive settings.

In the third objective, brachial artery FMD (using ultrasound) and reactive hyperemia peripheral arterial tonometry RH-PAT (using EndoPAT2000 machine) were used to compare endothelial function in patients with PCOS, which is associated with increased risk of future cardiovascular events, with age and weight matched control patients. The goal of this comparison was to determine the ideal technique for characterizing the vasodilatory response to a hyperemic stimulus in order to aid in future diagnosis and prognosis of endothelial dysfunction in those with increased risk of cardiovascular disease.

8.1 Selection of candidate polyphenol mixture

The increasing use of plant extracts as dietary supplements warrants information on their safety, bioavailability and bioactivity. The uptake and potential to modulate vascular function of three polyphenol preparations (Amlamax® (extract from *Embilica officinalis* also called amla), Oligopin® (French maritime pine bark extract) and pollen shell (sporopollenin exine) encapsulated Oligopin®) was assessed in a clinical trial. The aim of the trial was to select a single preparation that has any effects on vascular function assessed as changes in nitrate and nitrite levels as a measure of nitric oxide bioavailability.

The results showed that all the polyphenol preparations were detectable in plasma from the healthy volunteers, however only Oligopin® increased nitrite

concentrations (after 8 hours post ingestion) above baseline. Moreover the pollen shell exines (sporopollenin) encapsulation that improved the bioavailability of eicosapentaenoic acid found in fish oils (199) had no significant effects on Oligopin® bioavailability and prevented Oligopin® from having any effects on nitrate/nitrite levels, which were observed for Oligopin® without encapsulation.

Although data (see **Chapter 3** for details) on the bioavailability of Amlamax® and Oligopin® were generated, with results showing chemical components from all the preparations were detectable in plasma for up to 8 hours in healthy volunteers. However the lack of complete bioavailability data (due to the low number of samples analysed following an equipment failure), made any robust conclusions regarding the pharmacokinetics (PK) difficult. It was therefore necessary to select the appropriate polyphenol (Oligopin) based on nitrate/nitrite measurements alone.

The PK results were however in line with the bioavailability and bioactivity study (19) for a similar preparation, Pycnogenol® (commercially available French maritime pine bark extract), where its metabolites were detectable in blood for up to 14 hours after ingestion of the preparation.

In a placebo-controlled, double-blind, parallel group study (214) supplementation of 58 hypertensive patients with 100 mg Pycnogenol® (similar polyphenol preparation) over a period of 12 weeks resulted an increased NO levels in plasma but the difference between the groups were not significant. Further work is required for a better understanding of the effects of these preparations on NO availability and hence on vascular health using more robust and direct measuring techniques such as FMD and EndoPAT.

8.2 Effects of polyphenols treatment on endothelial (HUVEC) cells in *in vitro* model

There have been reported claims especially in the popular media that polyphenols have health benefits (1, 215-217). However, the scientific and clinical literature on the effects of polyphenols on the state of the endothelium (a valuable marker of cardiovascular disease risk) mostly consist of *in vitro* studies where large

pharmacological doses of polyphenols have been required to elicit a positive response. Attaining such concentrations *in vivo* is not possible due to the complex absorption and metabolic processes polyphenols have to go through before they are available to the endothelium (215, 218, 219). This limitation leaves conclusions regarding the preventive potential of polyphenols unresolved. To overcome this limitation, it was therefore decided to use a dose in the physiological range (0.1-1µmol/L) obtained from literature (220, 221) and thereafter confirmed by the absorption and bioavailability study (**Chapter 3**) in healthy volunteers.

Oligopin® being a mixture of various bioactive chemicals might be expected to exert beneficial effects on the vasculature through various modes of action (222, 223). The precise mechanisms are not known but it is suggested that an increased NO bioavailability is at the core of these health benefits such that a decreased NO bioavailability has been associated with a number of disorders including hypertension, hypercholesterolemia and coronary artery disease (223, 224). NO is believed to play an important role against atherogenic processes and its bioavailability may serve as a marker for cardiovascular risk (55, 56, 73, 224, 225). The present results are in line with these observations and show that Oligopin® significantly increases NO production and thus NO bioavailability to the endothelial cells that could lead to an improved endothelial cell functioning.

Furthermore, the results suggest that this increase in NO after Oligopin® treatment is a cumulative effect of increased eNOS activity and decreased superoxide oxide production seen *in vitro* in the HUVEC cell model used. Related findings have been reported for the commercially available polyphenol preparation Pycnogenol® using different cell system in which rat aortic ring preparations *in vitro* were treated with Pycnogenol® (1 to 10 µg/ml) leading to relaxation of adrenaline, noradrenaline and phenylephrine-contraction (190) via an endothelium dependent increase in NO production following increased eNOS activity. Similarly, in humans oral administration of Pycnogenol® in combination with L-arginine significantly improved sexual function in men with erectile dysfunction in an endothelium dependent increase in NO production (226).

8.3 Development of in vitro model for hypertension for investigating the effects of polyphenols on HUVECs in a hypertensive state

The important role of dietary polyphenols in the modulation of cardiovascular diseases and the suggestion that they are involved in the protection of vascular endothelium from dysfunction is well known now (57, 216, 227). The underlying mechanisms that are involved in these effects are what is not clear till date and the lack of a consistent, easily reproducible and standardised *in vitro* model of hypertension in the literature is behind this limitation. Development of an advanced human umbilical vein endothelial cells (HUVECs) model of angiotensin II (Ang II)-induced hypertension that is stable, easily reproducible and especially designed to investigate the exposure of endothelium to dietary polyphenols would help in understanding the mechanisms involved.

Angiotensin II has an important role in the development and progression of atherosclerotic diseases such as hypertension and diabetes (76, 204, 228, 229). Angiotensin II mediates these effects mostly through angiotensin receptor type 1 (AT_1) as both acute and chronic AT_1 blocking reverses endothelial dysfunction (80, 196). Angiotensin II treatment of HUVECs for 8 hours in the present study showed no effects on AT_1 protein expression. Our results are in agreement with Salisch et al (197) where even a longer (24 or 48 hours) exposure of HUVECs to Angiotensin II showed no significant effects on AT_1.

Activation of AT_1 results in oxidative stress that is a result of an increased production of ROS by an increased NADPH oxidase activity (85, 86, 88). There is a strong association between oxidative stress and atherosclerosis (67, 79). Although there was a tendency for the expression levels of $P47^{phox}$ to increase after angiotensin II treatment but there were no differences in the protein levels for either of the NADPH subunits ($P47^{phox}$ and $gp91^{phox}$) targeted as markers of NADPH activity. In contrast to our results, there are reports of an increase in $P47^{phox}$ (87) and $gp91^{phox}$ (199, 201). More over our results show that increasing confluence of the cells have a decreasing effect on the expression levels of $P47^{phox}$ and thus a decreased NADPH activity. Similar results have been reported previously (198)

where actively proliferating endothelial cells expressed $P47^{phox}$ more than did quiescent ones.

8.4 Non-invasive endothelial function assessment

While endothelial dysfunction is regarded as an important factor in cardiovascular disease development and progression (66, 225, 230), its assessment clinically can prove as a surrogate marker for cardiovascular risk stratification and for disease prognosis (110, 231-233). Of the available methods for assessing endothelial function, FMD and RH-PAT holds promise, as both are relatively simple and are non-invasive (114, 180). Both the techniques have been shown to correlate with coronary endothelial function (111, 234). A good measure of vascular reactivity that is aimed at improving cardiovascular risk assessment, should not merely have a sound physiological basis, but should be easily reproducible, inexpensive and operator independent (235).

A comparison between the two techniques in the same individuals showed no differences between the two in terms of endothelial function assessment in healthy volunteers (205). In this study, where 40 healthy volunteers underwent 5 minutes of forearm suprasystolic cuff-induced ischemia, Dhindsa and co-workers reported that there was a modest but significant association between the two techniques and suggested that both techniques can be used alternatively in this population group.

Although by knowing all this, the selection of the EndoPAT for the assessment of endothelial function in the clinical trial would have been an easy choice as it is advantageous in that it is easy to set-up, is automated and non-user dependent (236, 237). However, one of the limitations in the above-mentioned study was that they studied only healthy individuals and this may have reduced the strength of the correlation by limiting the spread of data.

To overcome this limitation we compared FMD and EndoPAT in patients with polycystic ovary syndrome and in their age and weight matched controls. As PCOS has shown evidence of increased cardiovascular risk in the absence of clinically obvious disease (238), information regarding the method that is more sensitive in

identifying the subclinical cases in this population group would help in disease risk stratification and management. The results from this study showed that in contrast to the above study there was no association between FMD and RHI. However, there were no differences in endothelial function measurements between PCOS and control groups for either FMD or RH-PAT that is measured as the reactive hyperaemic Index (RHI). Both the techniques have been shown to correlate well with coronary endothelial function and can be used equally in PCOS.

8.5 Double blind, randomized, placebo-controlled, crossover study of the effects of a French maritime pine bark extract (Oligopin®) on endothelial function

One of the well-studied potential uses of French maritime pine bark extract is its use for improving vascular health, a result of an improved endothelial function (227, 239). Bioactive components in the pine bark extract stimulates NO production resulting in improved endothelial function (240). The polyphenol preparation Oligopin® administered in this clinical trial showed no effects on blood pressure and other markers of vascular health including endothelial function (measured as RHI using EndoPAT2000 machine) in healthy volunteers.

In a Double blind, randomized, placebo-controlled clinical study, a similar polyphenol preparation Pycnogenol® administration for two weeks improved endothelial function in healthy young men (240). However, comparing the results with that of the present clinical study is not possible due to the different methods (forearm blood flow response to acetylcholine and sodium nitroprusside) used for assessing endothelial function and the differences in polyphenol administration and duration of treatment. The positive effects of pine bark extract on endothelial function have been reported in several other studies (187, 190, 241).

Administration of Enzogenol, 480 mg pine bark extract in combination with 240 mg vitamin C, in a pilot study including 24 healthy subjects with an age range of 55-75 years resulted in a significant decrease of 7 mmHg in the mean systolic blood pressure which was sustained at 12 weeks with continued use (242). While in contrast to these results in a randomised placebo controlled, double-blind, parallel-

group clinical study, 130 individuals with increased cardiovascular risk were given 200 mg of a water-based pine bark extract (Flavagenol®) once a day for 12 weeks did not show any effects on blood pressure and other cardiovascular risk factors (243).

Although the *in vitro* results were suggestive of an improved endothelial function, the lack of any effects of Oligopin® on vascular function in the present study can be a result of various reasons. One of the reasons may be that the bioactive substances in this mixture of polyphenols that had the effect *in vitro* may not be available in blood in the form that are needed for an effect due to an extensive metabolism that occurs *in vivo* (5, 244). The gut microbiota plays an important role in these catabolic processes yielding a diversity of chemicals including phenolic acids and aromatic components that are absorbed into the circulatory system and are rapidly excreted from the body (245-248). Moreover phase II metabolism in the liver occurs as compounds have been found as their conjugates in plasma samples after ingestion of Pycnogenol® (19).

Another limitation in this study the population group. The study population group in this study contained people from 18 to 65 years old and both sexes were included. It is now known that endothelial function decreases with age (249) and therefore the wide age group will have a lot of variation in the measured values for endothelial function which can affect the results. It is true for gender differences, as differences in endothelial function between males and females reported (208, 250) and estrogen has been suggested to have a preventive effect against endothelial dysfunction (251, 252).

Knowing all this necessitates that great care should be taken while designing such studies. A better approach would be selecting an individual bioactive component of the pine bark extract and evaluate its activity in the *in vitro* cell model and then subject it to a clinical trial carefully designed selecting an appropriate population group matched for age and sex.

8.6 Summary and future direction

Endothelial dysfunction (ED), a failure of the endothelium to suppress oxidative stress, inflammatory and thrombotic processes, is at the core of various diseases such as cardiovascular, cancer and Alzheimer's and has been suggested to be an early sign of atherosclerotic disease progression (10, 14, 17, 47, 66, 113). ED has been detected in coronary and resistance vessels as well as in peripheral arteries and is thus regarded as a systemic condition (59, 111). Endothelial dysfunction has been shown to be an independent predictor of future cardiovascular events and thus assessment of endothelial function can identify individuals at risk of developing cardiovascular disease (69, 70). ED is usually assessed as a reduced endothelium dependent vasodilatory response of the peripheral vasculature (57). Although other vasodilators secreted by the endothelium such as endothelium derived hyperpolarizing factors and prostaglandins may have a role, however it is nitric oxide (NO) that is considered mainly responsible for the endothelial dependent vasodilatation (57, 253-255). Measures targeting an improved NO bioavailability can rescue endothelial function (4, 10, 256-258).

The antioxidant properties of pine bark extracts have been voiced for some time, particularly the standardized French maritime pine bark extract (*Pinus pinaster*). They are available commercially under different trade names such as Oligopin® and Pycnogenol® and have been shown to improve endothelial function in cardiovascular, cancer and other degenerative diseases (2, 214, 240, 259-265). The molecular mechanisms underlying the beneficial effects of these polyphenols are not known. One suggested mechanism is that polyphenols modulate endothelial nitric oxide synthase (eNOS) activity and thus NO bioavailability to endothelium (188, 189).

In this research work, Oligopin® improved endothelial function *in vitro* by increasing NO bioavailability in primary human endothelial cells (HUVEC) after 8 hour incubation in a physiologically relevant dose (1 nM to 1000 nM) range. This increase in NO can be a result of either an increased production by eNOS in the L-arginine/NO pathway and or by a decreased scavenging by reactive oxygen species such as superoxide (192-194). The results suggested that Oligopin® significantly increased eNOS activity and decreased superoxide oxide production both of which

may have resulted in the increased NO bioavailability and thus an improved functioning endothelium. Similar results have also been shown in different cell models and using different polyphenol preparations *in vitro* (83, 93, 190, 221, 266-268).

Although the doses used here were in the physiological range obtained from literature and earlier confirmed in bioavailability in healthy volunteers, in a double blind, randomized, placebo-controlled, crossover study in 24 healthy volunteers, Oligopin® showed no beneficial effect on endothelial function using the EndoPAT 2000 as an indirect measure of NO bioavailability. While this may suggest the ineffectiveness of this preparation on NO function at this concentration, subsequent in vitro analysis showed that the preparation that was used in the clinical study appeared in active. Thus, the study does need to be repeated in the future and with a dose response curve using increasing doses both acutely as performed here, as well as long-term studies to investigate if there is a protracted effect that develops slowly. Ideally having shown safety in normal subjects then the studies need to repeated in subjects with compromised endothelial dysfunction, such as that seen in hypertension and type 2 diabetes, as it may well be that the doses used here may show a more overt effect on NO bioavailability.

This research is encouraging that polyphenol preparations may have a direct effect on endothelial function and the overall vasculature that may be of therapeutic value. There is a plethora of polyphenol preparations and the *in vitro* assays that were developed in this PhD may have value in screening those preparations for selective activity for future use and evaluation in future clinical trials.

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10 Appendix

Dietary intervention for the avoidance of polyphenols in the diet

Polyphenols are common constituents of plants and act as part of their defence system against animal, insects and humans. As this trial is looking at the effect of polyphenols on the body, we need to ensure that you do not consume a high intake during the trial period. Many foods and drinks contain polyphenols; the most common foods that you will need to avoid as part of this trial are listed below.

- All products containing soya – milk, tofu, soy flour, miso
- Almonds
- Apple cider and juice
- Apples
- Apricots
- Artichoke
- Aubergine
- Beans
- Black- or red currants
- Blackberries
- Blueberries
- Broccoli
- Cabbage
- Cashews
- Celery
- Cherries
- Chick peas
- Chocolate
- Coffee
- Cola
- Corn
- Cranberries
- Curly kale
- Dates
- Elderberries
- Garlic
- Goose berries
- Grape juice
- Grapes
- Green tea
- Hazelnuts
- Kiwi
- Leeks
- Lemon
- Lentils
- Limes
- Mangoes
- Nectarines
- Olive oil
- Olives
- Onions – red and white
- Oranges
- Parsnips
- Peach
- Peanuts
- Pear
- Peas
- Pecans
- Pistachios
- Plums and prunes (dried plums)
- Pomegranates
- Pumpkin seeds
- Pure Fruit juice (not from concentrate)
- Quinces
- Raisins
- Raspberries
- Raw spinach
- Red cabbage
- Red wine
- Rhubarb
- Rooibos (red bush) tea
- Shallots
- Spring onions
- Strawberries
- Sunflower seeds
- Sweet potatoes
- Tea
- Walnuts
- Whole grain cereals, wholegrain bread

Table 10-1 Nitrite measurement results

	Nitrite (μM)											
	Placebo						Active					
Patient	**0**	**2**	**4**	**8**	**9**	**24**	**0**	**2**	**4**	**8**	**9**	**24**
1	0.899777	0.883852	0.7883	0.801571	0.623739	0.85731	0.740524	0.759104	0.814842	0.812188	0.836076	0.615777
2	0.878543	0.881198	0.950207	0.950207	0.790954	0.777683	0.822805	0.812188	0.833422	0.828113	0.944899	0.974095
3	0.775029	0.820151	0.886506	0.854655	0.947553	0.88916	0.995329	1.045759	0.814842	0.767067	0.921011	0.881198
4	1.195652	1.280193	1.222826	1.274155	0.751965	0.70108	1.170351	1.077062	1.08837	0.932889	0.884831	0.819811
5	1.089976	1.11413	1.126208	1.135266	1.25	1.171498	1.16244	1.186594	1.177536	1.147343	1.096014	1.089976
6	1.083937	1.231884	1.165459	1.183575	1.219807	1.210749	1.038647	1.138285	1.099034	1.174517	1.192633	1.099034
7	1.38587	1.379831	1.343599	1.38285	1.171498	1.228865	1.391908	1.331522	1.343599	1.446256	1.479469	1.407005
8	1.080263	1.104151	1.032488	1.027179	1.096189	1.098843	1.0086	1.053721	1.236862	1.09088	1.120076	1.300563
9	1.098843	1.125385	1.207665	1.252787	1.061684	1.05903	1.114768	1.136002	1.234207	1.199703	1.165198	1.202357
10	1.120076	1.09088	1.122731	1.10946	1.096189	1.15989	1.146619	1.122731	1.088226	1.122731	1.162544	1.15989
11	1.175815	1.289946	1.173161	1.175815	0.771459	0.818751	0.851265	0.960629	0.931071	1.028612	1.069993	1.120241
12	0.801017	0.777371	0.866044	0.92516	0.936983	1.025656	0.996098	1.043391	0.759636	0.762592	0.939939	0.954717
13	0.954717	1.031568	1.043391	1.05817	1.111374	1.120241	1.093639	1.007921	1.090683	1.067037	0.984275	0.830575
14	0.777371	0.765547	0.886734	0.913336	1.043391	0.987231	1.13502	1.120241	0.534537	0.588235	0.610203	0.6639
15	0.690749	0.705394	0.768855	0.798145	0.871369	0.866488	0.63217	0.656578	0.829876	0.671223	0.622407	0.680986
16	0.637052	0.654137	0.778619	0.742006	0.773737	0.915304	0.910422	0.96412	0.558946	0.536978	0.617525	0.768855
17	0.666341	0.66146	0.729802	0.773737	0.656578	0.585794	0.702953	0.685868	0.851843	0.834757	0.607762	0.898218
18	1.067487	1.048079	1.10076	1.150668	1.164532	1.228304	1.208895	1.211667	1.061942	1.059169	1.125714	1.145123
19	1.036988	1.031442	1.097987	1.092442	1.120169	1.122941	1.114623	1.084124	1.106305	1.156214	1.023124	1.03976
20	0.886506	0.918357	0.905085	1.053721	0.709561	0.653022	0.938542	0.893311	0.853734	0.808503	0.763272	0.735003
21	0.850907	0.780234	0.732176	0.732176	0.650195	0.616272	0.66433	0.579522	0.585176	0.559733	0.935715	0.873523
23	0.549421	0.525533	0.796263	0.80688	0.663552	0.713982	0.882004	0.828292	0.768926	0.831119	0.723695	0.695426
25	1.200577	1.208895	1.075805	1.089669	1.10076	1.086896	1.012033		1.10076	1.095214		
27	1.208895	1.178395	1.250485	1.203349	1.1383	1.112488	1.156368	1.16153	1.18218	1.169274	1.220897	1.189923
Mean	0.962992	0.978667	1.002132	1.020291	0.946731	0.958645	0.995431	0.99392	0.956106	0.945431	0.958577	0.962868
SD	0.215106	0.232463	0.184025	0.186723	0.207558	0.206101	0.190642	0.194501	0.225946	0.235196	0.22956	0.220021

Table 10-2 Nitrate measurement results

	Nitrate microM											
	Placebo						Active					
Patient	**0**	**2**	**4**	**8**	**9**	**24**	**0**	**2**	**4**	**8**	**9**	**24**
1	1.274822	1.410682	1.742946	1.767549	1.844383	1.825432	1.607664	1.614334	1.451286	1.554937	1.351148	1.691382
2	0.885754	0.744229	0.735186	0.50163	0.869189	1.0308	1.250797	0.955265	0.911939	0.705784	0.614248	0.708142
3	1.134452	0.918897	0.742076	0.758146	0.539002	0.651049	1.053024	0.696446	1.176699	1.066667	0.63498	0.690574
4	2.757046	3.632913	3.533479	3.273082	0.518396	0.7259	0.604673	0.607471	0.394297	0.675075	0.556071	0.777711
5	4.339268	4.079911	3.610497	3.166969	3.068568	2.996802	3.508931	3.03724	2.873163	3.226759	4.147027	3.875397
6	3.509033	3.279419	4.753788	3.749131	3.066094	3.398555	4.671532	4.209291	4.607879	3.242052	3.116135	3.180334
7	2.873898	2.84727	2.798567	3.023918	3.793875	3.700575	3.129195	3.009913	3.11217	3.094447	3.093901	3.107565
8	0.969331	0.913584	1.102064	0.725064	0.541894	0.948096	1.205599	1.253399	0.88707	0.656053	0.706505	0.839298
9	1.136595	0.948103	0.857858	0.796807	0.881713	1.245436	1.274655	0.948106	0.924238	0.5844	0.672003	1.04901
10	1.022439	1.37819	1.378199	0.794114	0.693224	0.618903	1.123333	0.818012	0.89234	0.892349	0.849881	1.030415
11	1.349009	1.030449	1.006524	0.68528	1.052208	1.553386	1.123231	0.863038	0.923447	0.73678	0.801665	1.04622
12	1.23861	1.094287	1.077601	0.92593	1.023802	0.976263	1.204642	1.078507	1.314271	1.13649	1.079121	1.643665
13	1.845913	1.666225	1.561847	1.231697	1.219628	1.735237	1.333346	1.64188	1.452852	1.349664	1.569543	1.61355
14	2.352343	2.251044	1.800774	1.472513	1.232764	1.316348	1.641615	1.560411	1.974221	1.547599	1.531282	1.596242
15	1.476162	1.450217	1.621245	1.512851	1.65999	1.509487	1.469763	1.668543	1.616729	2.55513	2.434436	1.423772
16	1.803902	1.775516	1.546503	1.501185	1.350797	1.356139	1.784798	2.21703	2.514848	2.028283	1.837555	2.090225
17	1.650305	1.463074	1.496438	1.390349	1.821103	1.843859	1.802979	1.715534	1.535433	1.42256	1.313359	1.308246
18	0.90174	0.952725	0.78522	0.781241	0.655425	0.749535	0.642638	0.674313	0.700602	0.63161	0.639701	0.964763
19	0.828898	0.880373	0.684652	0.971515	0.691176	1.098898	0.886181	0.827692	0.839957	0.752731	0.696361	1.047161
20	0.824137	0.672352	0.594094	0.344461	0.630409	1.066315	0.826041	0.652004	0.555844	0.541907	0.642825	0.932128
21	1.1051	0.949545	0.875787	0.764413	0.992573	1.043898	1.201186	1.011039	1.134162	0.954258	0.790583	0.870177
23	2.001741	1.493122	1.584358	1.469328	1.25069	1.287271	1.254986	1.534927	1.399388	1.344156	1.61516	1.900982
25	1.319803	1.505573	0.701492	0.740309	0.443631	0.743082	0.989852		0.695946	0.609993		
27	0.601675	0.510176	0.404813	0.443631	0.988751	1.125844	0.922991	1.010033	1.202406	1.104032	0.902974	1.611171
Mean	1.633416	1.576995	1.5415	1.366296	1.284554	1.439463	1.521402	1.461062	1.462133	1.350571	1.373759	1.521658
SD	0.930325	0.963066	1.091241	0.967537	0.883779	0.828168	0.952149	0.906049	0.963347	0.86185	0.969131	0.846489

Validation of the deconjugation method used during plasma sample preparation for LC-MS analysis of circulation dietary polyphenolics.

The deconjugation of β-estradiol-glucuronide and β-estradiol-sulphate using glucuronidase and sulfatase enzymes was monitored by the detection of β-estradiol using HPLC-UV. Representative chromatographs are shown below for A) glucuronide removal (Figures 10.1 – 10.3) and B) sulphate removal under optimised conditions (2 h, 37°C) (Figures 10.4 – 10.6). Chromatographs are shown for samples containing enzyme solution only, substrate (β-estradiol-glucuronide or sulphate respectively) only, or the full reaction mixture (enzyme and substrate) respectively.

The following HPLC method was used for the detection of β-estradiol, with solvent A being water and solvent B being methanol. An Agilent 1260 quaternary pump, auto sampler, column oven and variable wavelength detector was used in conjunction with an Agilent Eclipse-XDB-C_{18} column (5 µm pore size, 4.6 x 150 mm) column. The flow rate was 1 ml/min, and the column oven was set to 40 °C. An injection volume of 20 µl was used for these experiments. Solvent B was maintained at 35% for 5 minutes, increased to 85% over 15 minutes, maintained at 85% for 5 minutes, before returning to 35% over 1 minute. The starting conditions were regenerated over 6 minutes.

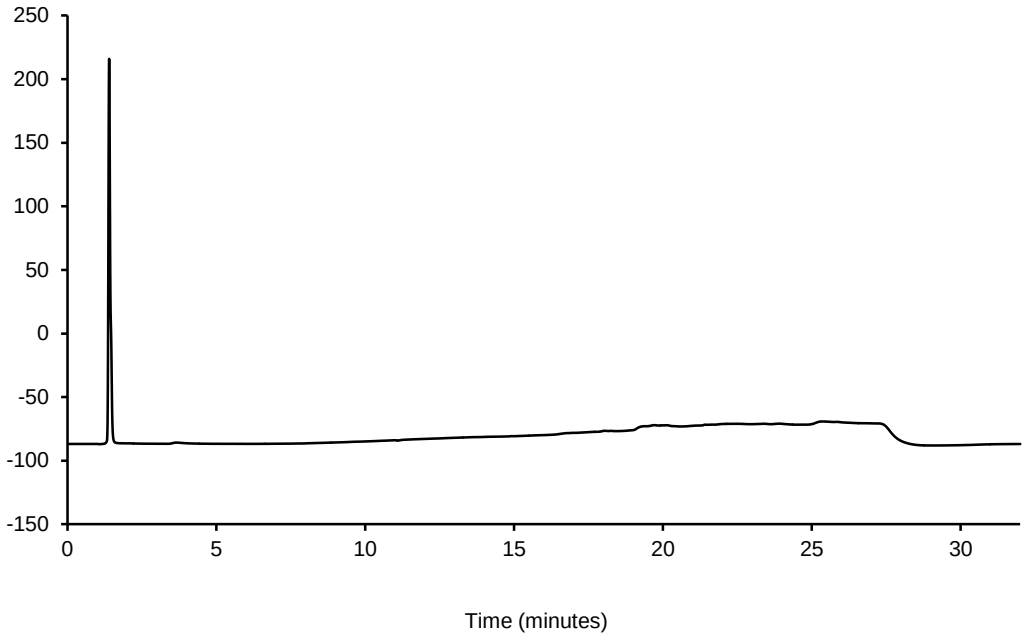

Figure 10-1 Chromatograph showing enzyme only sample (β-glucuronidase). Note the lack of a β-estradiol peak at 16.89 minutes.

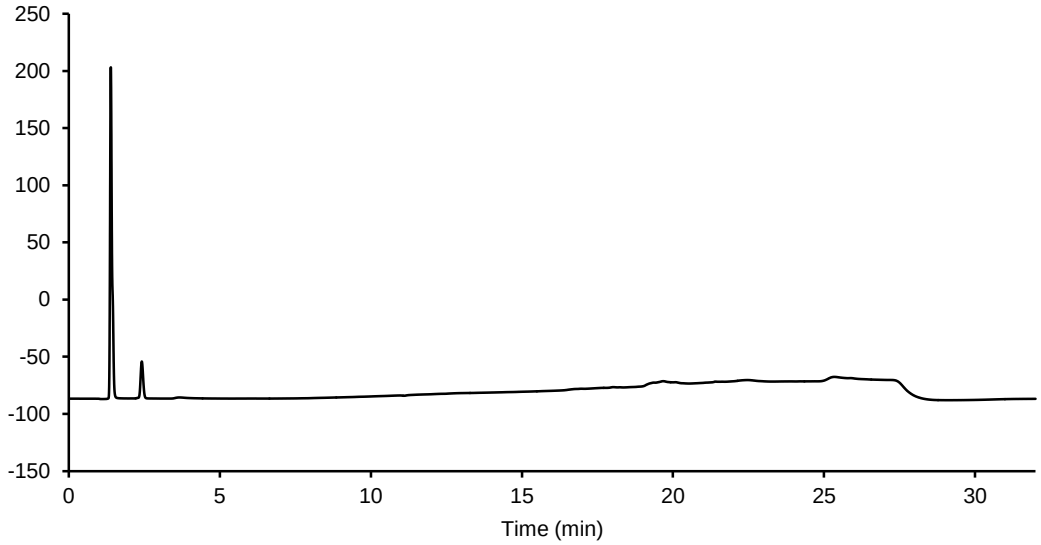

Figure 10-2 Chromatograph showing substrate only sample (β-glucuronidase). Note the lack of a β-estradiol peak at 16.89 minutes. Note the peak at 2.29 minutes that indicates the presence of the substrate.

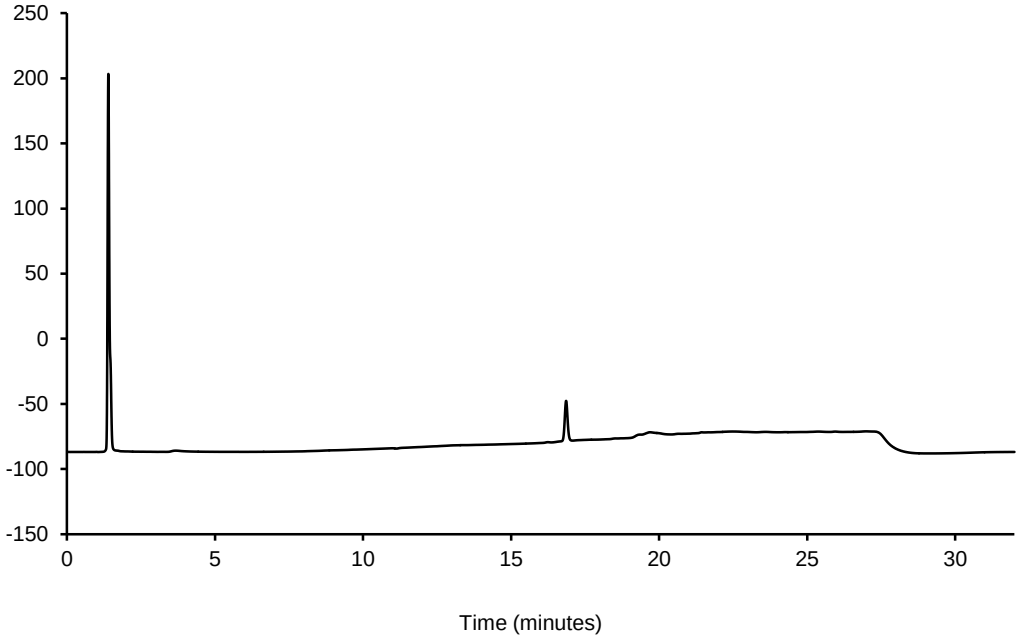

Figure 10-3 Chromatograph showing that when both substrate and enzyme are present the glucuronide is cleaved resulting in the appearance of the β-estradiol peak at 16.89 minutes. Note that the substrate peak at 2.29 minutes has completely disappeared, suggesting that the reaction has run to completion.

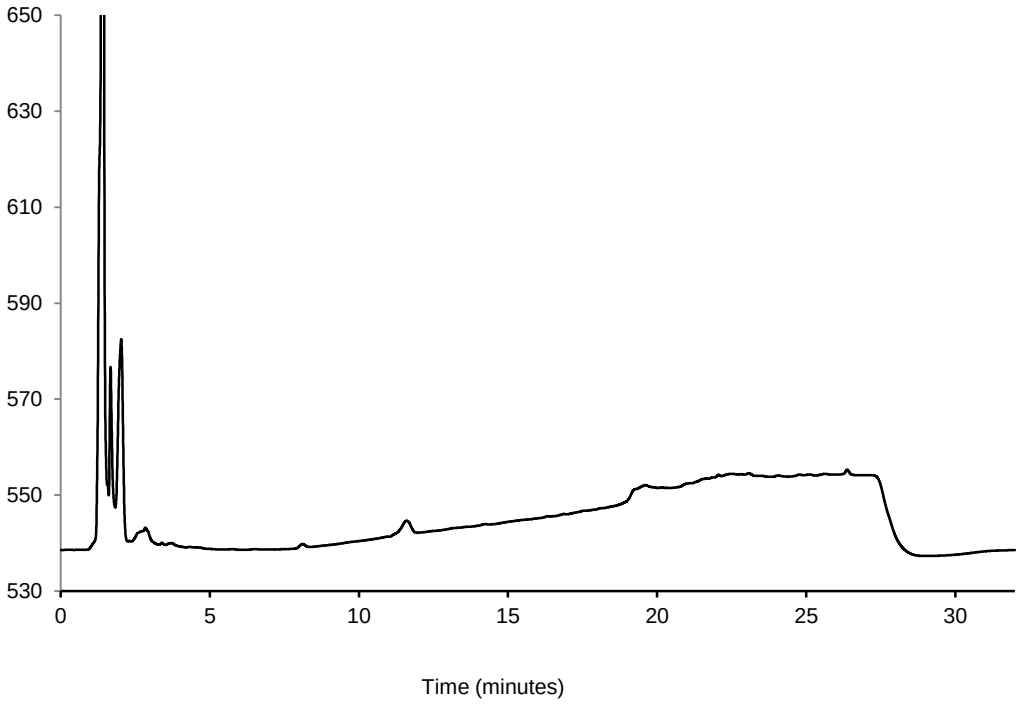

Figure 10-4 Chromatograph showing enzyme only sample (sulfatase). Note the lack of a β-estradiol peak at 16.89 minutes.

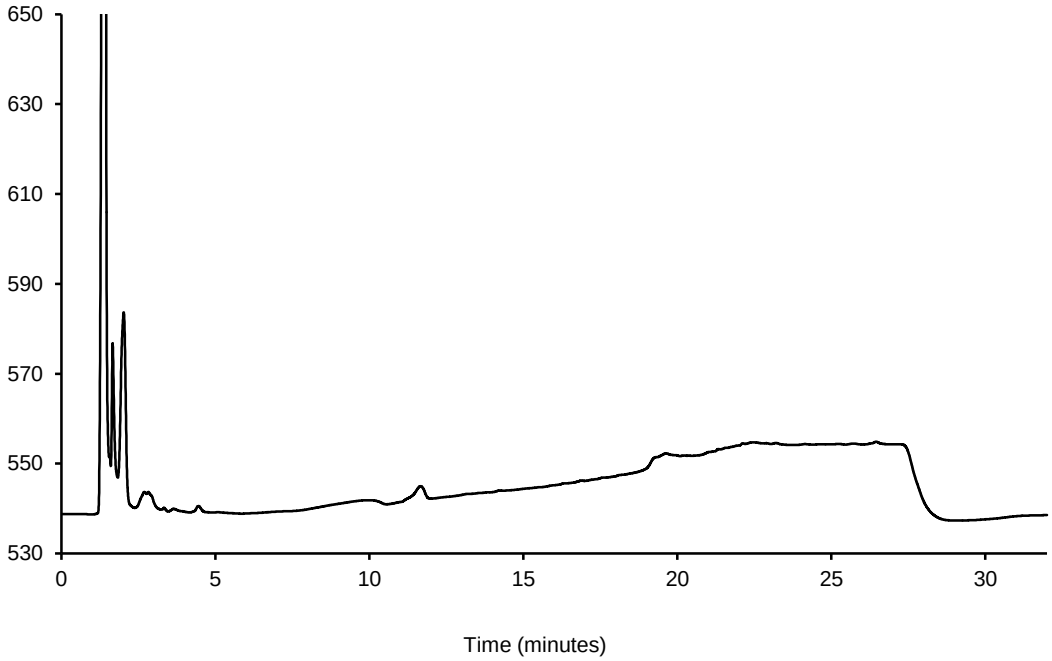

Figure 10-5 Chromatograph showing substrate only sample (β-glucuronidase). Note the lack of a β-estradiol peak at 16.89 minutes.

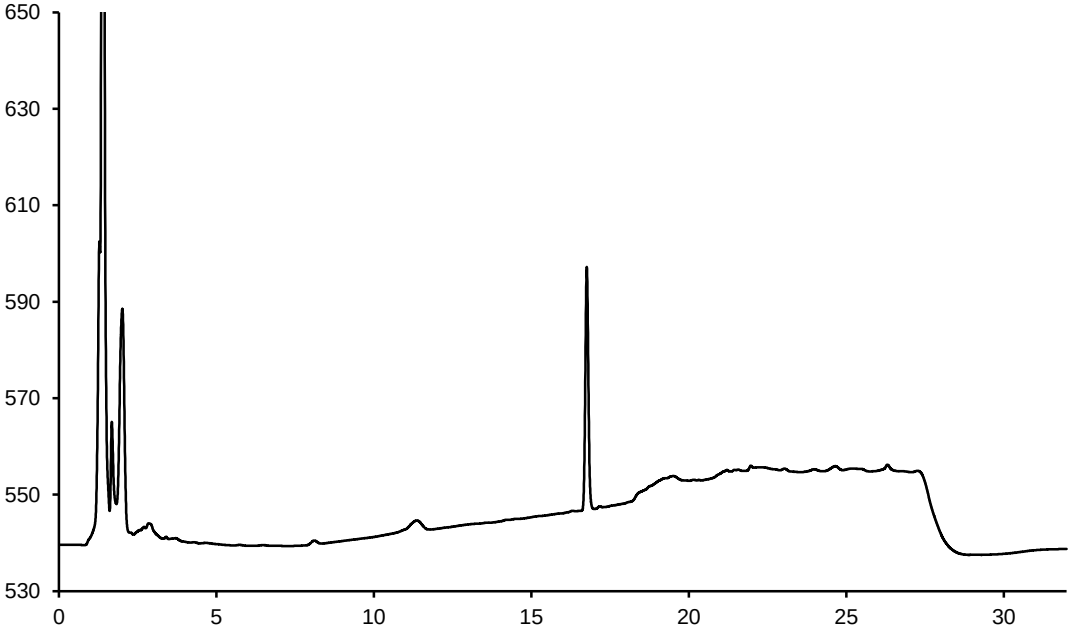

Figure 10-6 Chromatograph showing that when both substrate and enzyme are present the sulphate is cleaved resulting in the appearance of the β-estradiol peak at 16.89 minutes.

Validation of the LC-MS analytical method for the detection of ellagic acid, catechin and epicatechin in blood plasma.

The following LC-MS method was developed to detect Ellagic acid in plasma collected from individuals that had consumed Almamax. Plasma samples (200 µl) were incubated with the deconjugation mix (60 µl sulfatase, 5.1µl β-glucuronidase and 1 µM final concentration of taxifolin or diadzin as an internal standard) for 2 hours at 37 °C. These samples were then deproteinated by addition of 120 µl of acidified DMF (100 µl of DMF plus 20 µl of formic acid) and incubation at room temperature for 10 minutes with regular mixing by vortex. The precipitated protein was removed by centrifugation (16000 x g, 10 minutes) and the supernatant was retained. The supernatant was loaded into a HPLC insert vial and analysed as detailed below.

Chromatography was done using a Shimadzu LC20-AD quaternary pump, SIL-20A HT auto sampler and CTO-10A column oven connected via a FCV-20AH$_2$ switching valve to a LC2020 single quadrupole mass spectrometer. An Agilent Eclipse-XDB-C$_{18}$ column (5 µm pore size, 4.6 x 150 mm) was used for separating the analytes as detailed below, with solvent A comprising of 0.5% v/v formic acid in water, and solvent B comprising 0.5% v/v formic acid in methanol. The column was maintained at 40 °C.

For the measurement of ellagic acid, the method began with a solvent B at 45% over 5 minutes, followed by an increase in solvent B from 45% to 80% over 5 minutes. Solvent B was then maintained at 80% for 5 minutes before returning to 35% over 30 seconds. The initial starting conditions were re-equilibrated over 4.5 minutes. Under these conditions Ellagic acid eluted at RT = 7.1 min and taxifolin at RT = 5.0 minutes. Ellagic acid and taxifolin were detected at a $m\backslash z$ = 301 and 303 respectively.

For the measurement of catechins, the method began with a gradient of solvent B from 35% to 45% over 5 minutes, followed by an increase in solvent B from 45% to 80% over 5 minutes. Solvent B was then maintained at 80% for 5 minutes before returning to 35% over 30 seconds. The initial starting conditions were re-equilibrated over 4.5 minutes. Under these conditions catechin eluted at RT = 3.7 min, epicatechin eluted at RT = 4.7 min, and diadzin at RT = 12.6 minutes. Both catechin and epicatechin were detected at a *m\z* = 289, diadzin at *m\z* = 253.

Standard curves of ellagic acid, catechin and epicatechin were prepared in plasma from fasted individuals, shown not to contain these analytes, for the quantification of the samples collected in this study. A representative chromatogram and standard curve for each analyte is shown below in Figures 10.7 to 10.10.

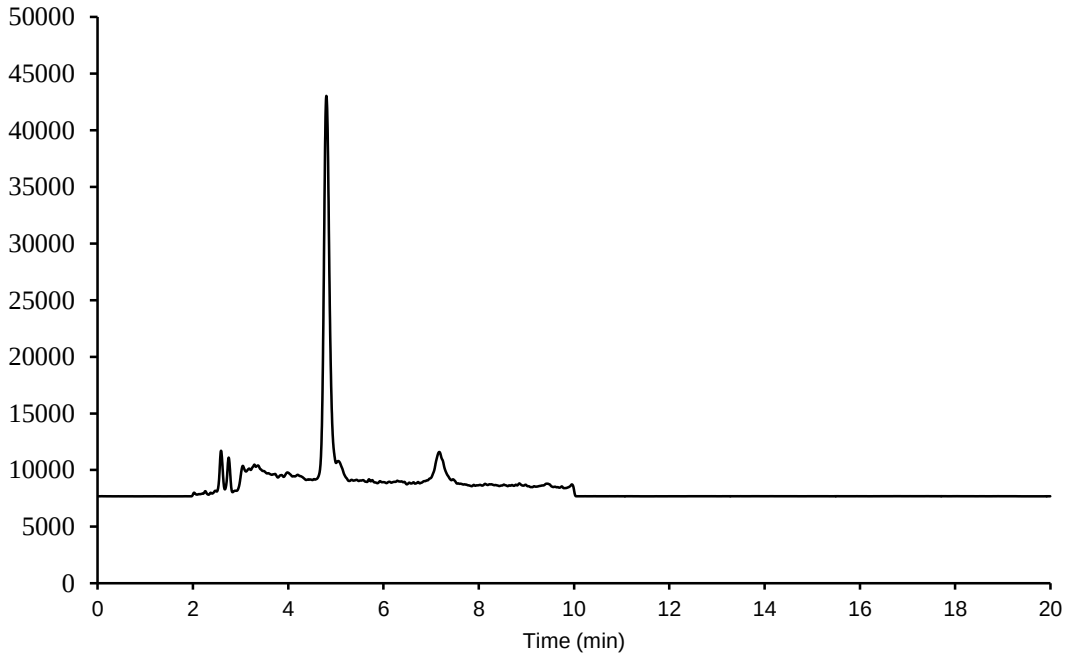

Figure 10-7 Representative chromatogram showing taxifolin (RT = 5 min) and ellagic acid (RT = 7.1 min)

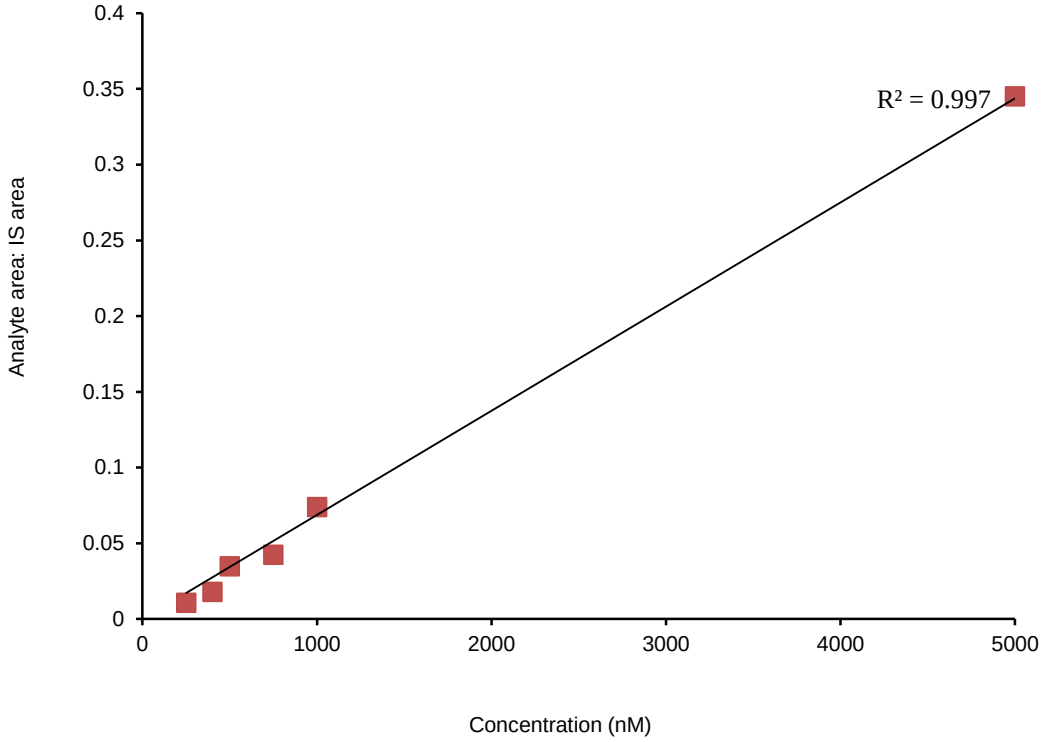

Figure 10-8 A representative standard curve for ellagic acid spiked into blank plasma.

The raw data used to generate this curve is shown below in Table 10.3.

Table 10-3 Raw data used for the Standard curve (figure 10.8) calculation

nM	5000	1000	750	500	400	250
Ellagic acid	518355	108714	57789	34096	13551	5756
IS	1502136	1471698	1361673	979491	764503	536271
Ellagic: IS	0.345079	0.07387	0.04244	0.03481	0.017725	0.010733

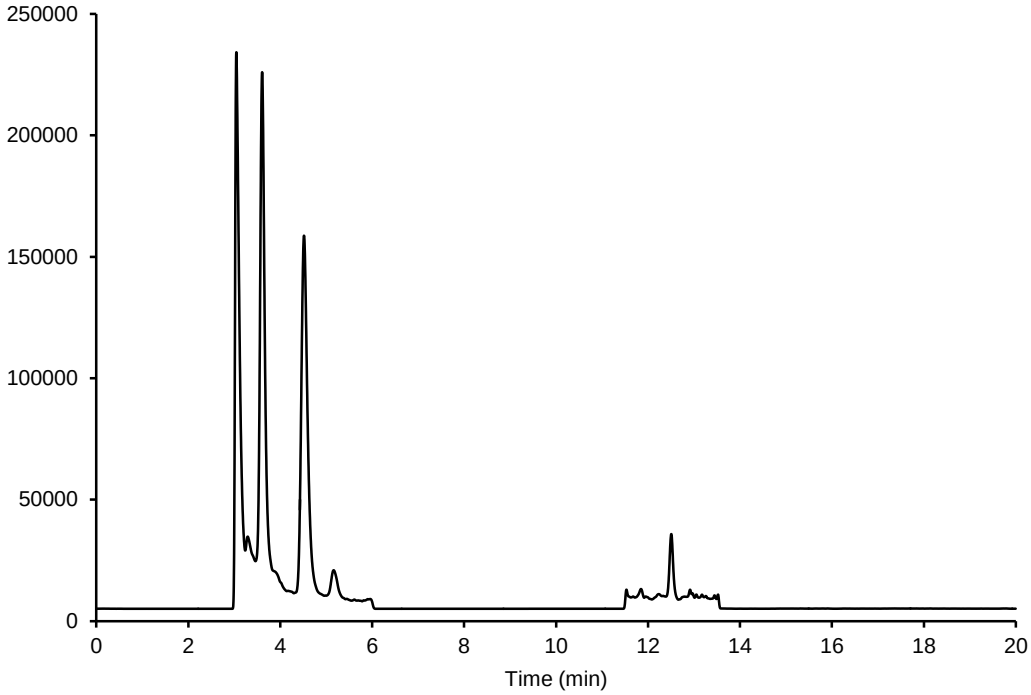

Figure 10-9 Representative chromatogram showing catechin (RT = 3.7 min), epicatechin (RT = 4.7 min) and diadzin (RT = 12.6 min).

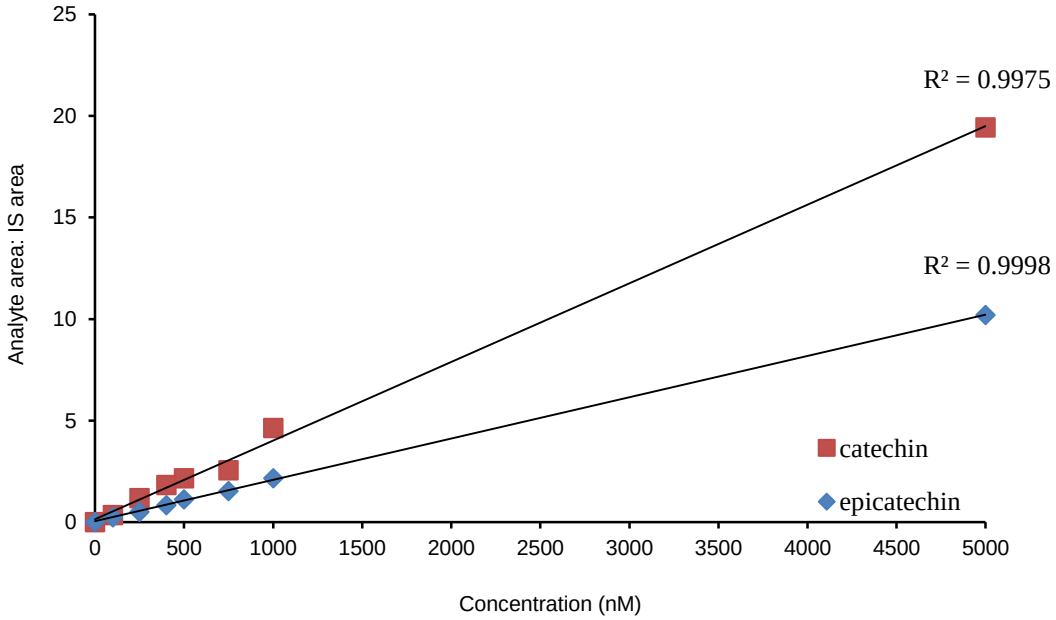

Figure 10-10 Representative standard curves for catechin and epicatechin spiked into blank plasma.

The raw data used to generate these curves is shown below in Table 10.4.

Table 10-4 Raw data for standard curve (figure 10.10)

nM	5000	1000	750	500	400	250	100	0
Catechin	8328012	2054973	1202740	968306	911378	613767	164100	0
Epicatechin	4369364	959673	722212	501532	420105	262366	115272	0
IS	428468	443638	469422	447810	496811	517641	475870	476
Cat: IS	19.43672	4.632094	2.562172	2.162314	1.834456	1.1857	0.344842	0
Epi:IS	10.19764	2.163189	1.538513	1.119966	0.845603	0.506849	0.242234	0