Effects of citrus flavonoids on endothelial function and cardiovascular health

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The candidate confirms that the work submitted is his/her own, except where work which has formed part of jointly-authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

Conference abstracts

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Publications

Li. L , Birch K. M., Bösch C. Effects of blood orange juice on endothelial function in healthy overweight subjects of European origin. *Manuscript in preparation.*

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Abstract

Cardiovascular diseases (CVD) represent one of the leading causes of mortality worldwide. Epidemiological studies have suggested an inverse association between the consumption of citrus fruit and the risk of CVD. However, the molecular mechanisms by which citrus flavonoids contribute to cardiovascular health have not been fully elucidated. In addition, evidence from human studies is limited and contradictory. Therefore, the aims of this thesis were to investigate mechanisms by which citrus flavonoids impact on endothelial cells and their effects on endothelial function and cardiovascular health in an acute manner and following regular consumption.

The potential of selected citrus flavonoids to modulate gene expression relevant to nitric oxide (NO) production and antioxidant status was investigated in EA.hy926 cells; the acute effect of flavanone-rich blond orange juice (total flavanone content=81.7 mg/100 ml) on vascular function (cardiac electrical activity, blood pressure, vascular compliance, peripheral blood flow, pulse transit time and pulse wave velocity) in healthy subjects (n=13) was determined; in a randomized controlled crossover trial, the effect of flavanone-rich blood orange juice (total flavanone content=89.7 mg/100 ml) on endothelial function, blood pressure, lipid profile, inflammation in overweight/obese men and premenopausal women of European origin (n=15) were examined following 2-week consumption (400 ml/d) of blood orange juice and a sugar-matched control drink.

The *in vitro* study demonstrates NOS3 mRNA and protein expression were significantly upregulated by nobiletin and tangeretin; incubation with nobiletin also resulted in a significant reduction in ET-1 mRNA levels and a significant induction of HO-1 and GCLC. Vascular related markers were not acutely altered at 2 h post consumption of a flavanone-rich orange juice in healthy subjects under the investigated conditions. Following 2-week consumption of blood orange juice, endothelial function was significantly improved from 8.15% ± 2.92% to 10.16% ± 3.31%, compared to the control drink (time by treatment interaction: P=0.002). Interestingly, this favourable effect on endothelial function was only observed in overweight subjects but not in obese subjects. Baseline blood pressure, lipid profile, hsCRP and ET-1 were generally within healthy ranges and not altered during juice supplementation. Present data indicate that regular

consumption of blood orange juice exerts beneficial effect on endothelial function in healthy overweight men and premenopausal women of European origin, which may be attributed to elevated NO bioavailability and the induction of cell-protective enzymes as evidenced by the *in vitro* findings.

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List of Abbreviations

AB	Assay buffer
ADME	Absorption, distribution, metabolism and excretion
AUC	Area under curve to peak dilation
BCA	Bicinchoninic acid
BDC	Body correlation factor
BH_4	Tetrahydrobiopterin
BP	Blood pressure
BSA	Bovine serum albumin
CBG	Cytosolic β-glucosidase
CD	Control drink
cDNA	Complementary deoxyribonucleic acid
cGMP	Cyclic guanosine monophosphate
COMT	Catechol-O-methyltransferase
CVD	Cardiovascular disease
DAF-2	4,5-diaminofluorescein
DAN	2,3-diaminonaphthalene
DBP	Diastolic blood pressure
DEPC	Diethy pyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
ECG	Electrocardiography
eNOS	Endothelial nitric oxide synthase
ET-1	Endothelin 1
FAD	Flavin adenine dinucleotide
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FMD	Flow mediated dilation
FMN	Flavin mononucleotide
FROJ	Flavanone-rich orange juice
G	Genistein
GCLC	Gamma glutamyl cysteinyl synthetase
GDP	Gross domestic product
GFJ	Grapefruit juice
GSH	Glutathione
GTP	Guanosine triphosphate
H	Hesperidin
HDL	High-density lipoprotein
HES	Hesperetin
HF	High flavonoid
HFM	High-fat meal

HO-1	Heme oxygenase-1
HR	Heart rate
hsCRP	High-sensitivity C rective protein
HUVEC	Umbilical vein endothelial cell
LCMS	Liquid chromatography–mass spectrometry
LDL	Low-density lipoprotein
LF	Low flavonoid
IL-6	Interleukin 6
L-NAME	N^{ω}-nitro-L-arginine methyl ester hydrochloride
LPH	Lactase phlorizin hydrolase
Lp-PLA 2	Lipoprotein-associated phospholipase A2
mRNA	Messenger RNA
N	Narirutin
NADPH	Nicotinamide adenine dinucleotide phosphate
NAR	Naringenin
NCD	Non-communicable disease
NO	Nitric oxide
NOB	Nobiletin
NOS	Nitric oxide synthase
NQO1	NAD(P)H:quinone oxidoreductase 1
NR	Neutral red
OJ	Orange juice
oxLDL	Oxidized low-density lipoprotein
Nrf2	Nuclear factor-E2-related factor 2
PA	Proanthocyanidin
PCR	Polymerase chain reaction
PGI2	Prostacyclin
PMA	Phorbol-12-myristate-13-acetate
PWV	Pulse wave velocity
QTc	QT interval corrected for heart rate
RCT	Randomised controlled trial
RES	Resveratrol
RH-PAT	Reactive hyperemia-peripheral arterial tonometry
RNA	Ribonucleic acid
RXNO	Nitros(yl)ated species
ROJ	Red orange juice
ROS	Reactive oxygen species
SBP	Systolic blood pressure
SD	Standard deviation
sE-selectin	Soluble E-selectin
sGC	Soluble guanylate cyclase
SGLT-1	Sodium-dependent glucose transporter
sICAM-1	Soluble intercellular adhesion molecule-1

SSA	Serum amyloid A
SULT	Sulfotransferase
sVCAM-1	Soluble vascular cell adhesion molecule-1
TAN	Tangeretin
TC	Total cholesterol
TF	Total flavanones
TG	Triglycerides
TNF-α	Tumour necrosis factor-α
TXA_2	Thromboxane A2
UDP-GT	Uridine-5'-diphosphate glucuronosyltransferase
VEGF	Vascular endothelial growth factor

Chapter 1 Introduction and literature review

1.1 Introduction

Cardiovascular disease (CVD) is a commonly used term to describe disorders of the heart and blood vessels, which are the leading cause of death worldwide (World Health Organization, 2017). Specifically, it is estimated that 17.7 million people die from CVDs each year, accounting for 31% of all deaths globally (World Health Organization, 2017). Worse still, industrialisation and urbanisation contribute to the emergence of CVDs in Africa (Sliwa et al., 2016) and prevalence in China (Wu et al., 2016). In addition, CVDs are the dominant contributors to the global economic burden of non-communicable diseases (NCDs) (Bloom et al., 2012). At a micro-economic level, ample evidence is emerging to suggest CVDs and other NCDs contribute to poverty in low- and middle-income countries (Vorster and Kruger, 2007). Furthermore, deaths from CVDs in low- and middle-income countries account for over 75% of total deaths from CVDs worldwide, due to insufficient medical care and late detection of the disease.

In order to reduce substantial economic loss by CVDs and improve the quality of life at an individual level, several modifiable factors can be controlled to reduce the risk of CVDs. Specifically, reduced risk of CVD has been associated with increased consumption of fruits and vegetables (Aune et al., 2017), reduction of unhealthy diet (high in sugar, salt, fat) (Mozaffarian et al., 2011), regular physical activity (Myers et al., 2015), cessation of tobacco use (Clair et al., 2013) and avoiding excessive alcohol (Holmes et al., 2014).

Notably, the consumption of the Mediterranean diet rich in fruits and vegetables has been associated with reduced rick of CVD (Widmer et al., 2015; Knoops et al., 2004). Its favourable effects on cardiovascular health have been, at least in part, attributed to its rich polyphenol content (Scoditti et al., 2012; Carluccio et al., 2007), which attracts increasing research interest in recent years. Increasing evidence from epidemiological studies has demonstrated an inverse association between consumption of polyphenol-rich foods and the risk of CVD (Kim and Je, 2017; Tresserra-Rimbau et al., 2014; Mennen et al., 2004). Possible mechanisms by which polyphenol impact on endothelial

function and cardiovascular health have been explored via animal models (Anhê et al., 2015; López-Sepúlveda et al., 2008), *in vitro* cell mechanistic studies (Kamiloglu et al., 2017; Martínez-Fernández et al., 2015) and human intervention studies (Kerimi et al., 2017; Kelishadi et al., 2011). The following literature review will be focused on current understanding of progression of CVD, effects of dietary polyphenols on CVD risk factors, identifying gaps in previous research, thereby introducing the rationale of the thesis.

1.2 Endothelial function and cardiovascular health

The endothelium is a monolayer of cells at the internal surface of blood and lymphatic vessels. Up to the mid-sixties, the function of the endothelium was believed to be a barrier between circulating blood in the lumen and the tissues of the vessel wall (Florey, 1966). Its key function in maintaining vascular homeostasis has become evident following the use of endothelial cell culture techniques (Jaffe et al., 1973). Over the last few decades, the endothelium has been recognised to play a pivotal role in regulating vascular tone and structure (Zaborska et al., 2017; Busse et al., 1985). Specifically, in response to various physical and chemical stimuli, healthy endothelial cells produce and release a variety of vasodilators (such as nitric oxide (NO), prostacyclin (PGI2)) and vasoconstrictors (such as thromboxane A2, endothelin and angiotensin II) that not only modulate vascular smooth muscle contractility (Edwards et al., 2010) but also play a role in vascular remodelling (Deanfield et al., 2007). In addition, healthy endothelium plays a crucial role in maintaining cardiovascular homeostasis, exerting anti-inflammatory, anti-coagulant and anti-thrombotic functions (Figure 1.1) (Rubanyi, 1993).

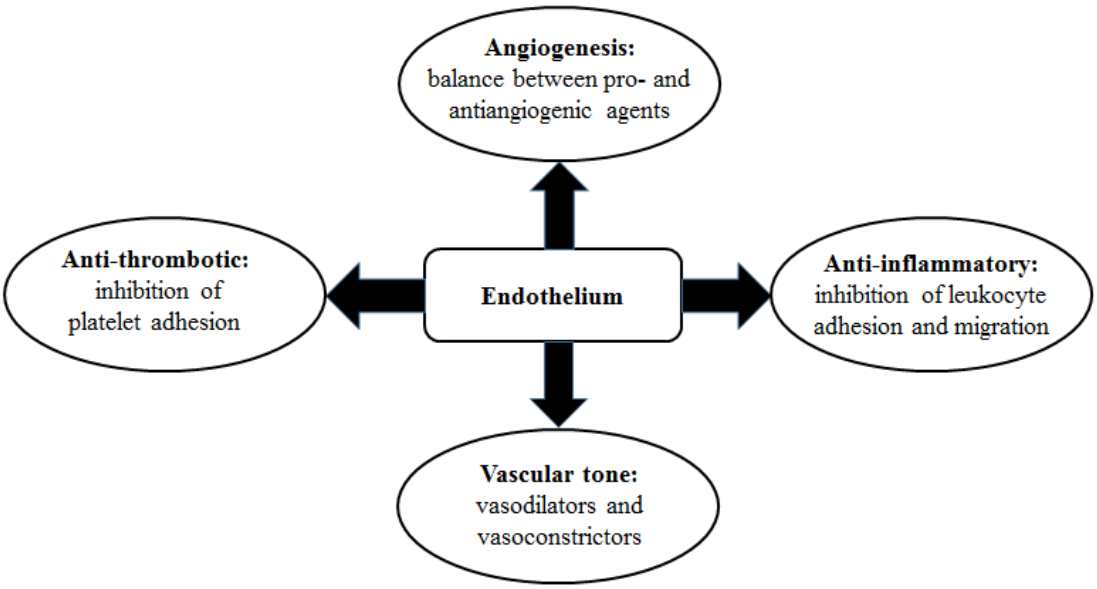

Figure 1.1. Multiple roles of healthy endothelium (Landmesser et al., 2004; Rubanyi, 1993).

Endothelial dysfunction, or endothelial activation, is characterised by a change in signalling from an NO-mediated silencing of cellular processes to reactive oxygen signalling, resulting in activation of the endothelial cells. It should be noted activation of the endothelial cells can occur physiologically as part of normal host defence in the context of immunity, or pathophysiologically as a result of cardiovascular risk factors, which differ in the nature, degree, duration and combination of the proinflammatory stimuli (Deanfield et al., 2007). As a consequence, endothelial dysfunction upregulates cytokines and inflammatory molecules, resulting in increased oxidative stress (Bauersachs et al., 1999). Numerus factors contribute to endothelial dysfunction, including conventional CVD risk factors such as unfavourable lipid profile (low levels of high-density lipoprotein (HDL) cholesterol, elevated low-density lipoprotein (LDL) cholesterol, oxidized LDL and triglycerides), hyperglycaemia, inflammation (systemic inflammatory marker C-reactive protein (CRP); vascular inflammatory marker lipoprotein-associated phospholipase A2 (Lp-PLA 2)), hypertension and smoking (Grover-Páez and Zavalza-Gómez, 2009). Chronic production of reactive oxygen species (ROS) and prolonged endothelial dysfunction then contribute to the development of CVDs, including atherosclerosis, hypertension and diabetes (Vanhoutte et al., 2009). Specifically, endothelial dysfunction occurs in the earliest stages of atherosclerosis,

preceding angiographic or ultrasonic detection of atherosclerotic plaque. This was first evidenced by the study conducted by Ludmer et al. (1986) demonstrating impaired endothelium-dependent vasodilation induced by acetylcholine in subjects with early and late atherosclerosis. Therefore, evaluation of endothelial function has significant clinical relevance in early detection of CVDs and prediction of future CVD incidents.

1.2.1 Evaluation of endothelial function

With growing recognition of the crucial role of the endothelium in cardiovascular health, a variety of methods have been developed to evaluate different aspects of endothelial function. Furchgott and Zawadzki (1980) discovered the functionally normal endothelium was responsible for the relaxation of preconstricted rabbit aorta strips with well-preserved endothelium in response to acetylcholine whereas the aortas did not dilate in response to acetylcholine following removal of the endothelium.

Subsequently, flow mediated dilation (FMD) of the brachial artery was introduced by Celermajer et al. (1992) and has been widely used worldwide to evaluate endothelial function. Flow-mediated dilation is a non-invasive technique that can assess endothelium-derived NO bioavailability and endothelial function when measured appropriately (Green, 2005). The principle of FMD is that the brachial artery dilates when there is an increase in blood flow or, more precisely, sheer stress. During an FMD test, the baseline brachial artery diameter is measured first using Doppler ultrasound, followed by reactive hyperaemia caused by the cuff occlusion in either the upper arm or forearm for a period of time. Reactive hyperaemia created by cuff occlusion can be controlled to result in a primarily NO-dependent dilation of the branchial artery, with the distal occlusion for 5 min. Upon cuff deflation, the hyperaemia increases sheer forces parallel to the long axis of the artery and this change can be sensed by endothelial cells which then convert L-arginine to NO through endothelial NO synthase (eNOS) in the presence of cofactors (Nathan and Xie, 1994). Subsequently, NO diffuses into the tunica media and activates soluble guanylate cyclase (sGC) resulting in the conversion of GTP to cGMP, which leads to the relaxation of the smooth muscle and subsequent vasodilation (Figure 1.2). The result of FMD is the change in diameter post cuff deflation, technically expressed as a percentage of the baseline resting diameter (Harris et al., 2010). Importantly, endothelial dysfunction was detected using FMD (Celermajer et al., 1994) in patients with coronary risk factors, preceding angiographic or ultrasound evidence of structural coronary artery disease. Therefore, evaluation of endothelial function using

FMD provides evidence in the early detection of CVDs, thereby making early treatment of CVDs possible, which may possibly save substantial pain and cost for patients.

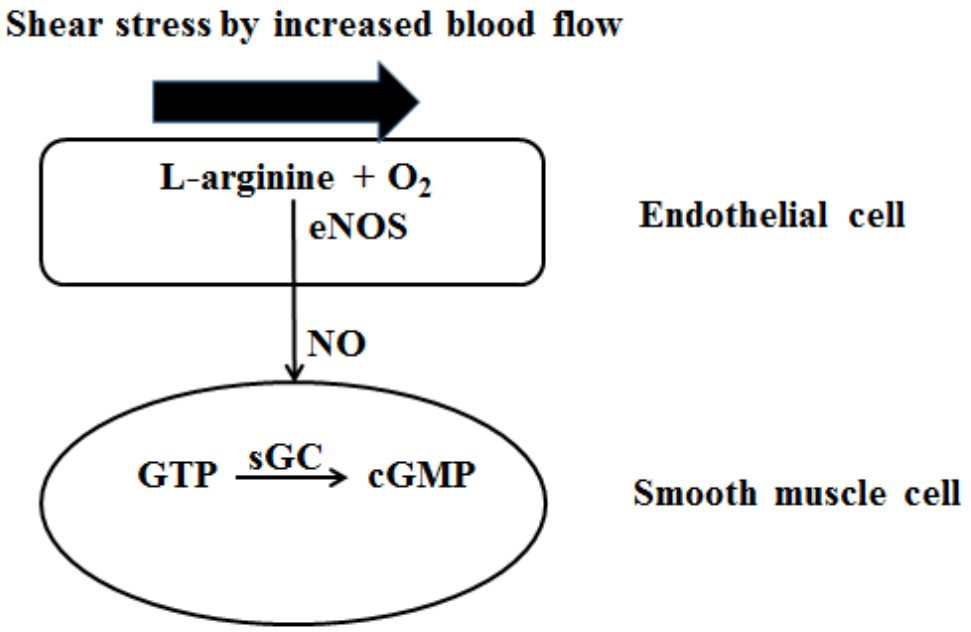

Figure 1.2. Generation of flow-mediated dilation. The increase in blood flow activates endothelial nitric oxide synthase (eNOS), which converts L-arginine to nitric oxide (NO). NO diffuses into smooth muscle cells and activates soluble guanylate cyclase (sGC) resulting in the conversion of GTP to cGMP, which leads to the relaxation of the smooth muscle and subsequent vasodilation.

Although the investigation on the association between brachial FMD and cardiovascular risk has been difficult due to confounding factors and discrepancies in the technical aspects of the FMD measurement across labs, there is ample evidence indicating that the brachial FMD is not only associated with conventional cardiovascular risk factors but also with future cardiovascular events (Matsuzawa et al., 2017; Ras et al., 2013; Green et al., 2011). More specifically, a meta-analysis study by Inaba et al. (2010) demonstrates that a 1% decrease in FMD is associated with a 13% increase in the risk of future cardiovascular events. Although this should be interpreted with caution given significant heterogeneity between studies, the brachial FMD has been recognised as an independent predictor of future cardiovascular incidents and processes independent prognostic value for future CVDs. For example, brachial FMD and brachial artery diameter have been

documented to be significant predictors of CVD events in older adults after adjustment for age, gender and CVD risk factors (Yeboah et al., 2007). Importantly, the association between brachial FMD and cardiovascular risk may differ in individuals according to the baseline cardiovascular risk. More specifically, a large meta-regression analysis of 399 populations (211 selected articles) suggests a strong inverse association between brachial FMD and cardiovascular risk (assessed by the Framingham risk score) in low-risk populations and less strong associations in intermediate-risk and high-risk populations, independent of brachial artery diameter and technical differences in the FMD measurement (Witte et al., 2005). This meta-analysis indicates that the magnitude of the association between brachial FMD and cardiovascular risk decreases as the risk increases. In addition, brachial FMD has been reported to be less reproducible in subjects with CVD, with larger coefficients of variation compared with healthy controls (van Mil et al., 2016; Craiem et al., 2007).

Given its non-invasive nature and better reproducibility and sensitivity than other non-invasive techniques (Donald et al., 2006), FMD has been widely adopted worldwide for assessment of endothelial function. However, there are disadvantages to this technique that are worth noting. Despite its seemingly simple appearance, FMD has been recognised to be technically challenging, which requires at least 100 supervised practice scans to be competent and regular practice (100 scans per year) to maintain competence (Corretti et al., 2002). Given that performing FMD has a significant learning curve, it typically requires several months to attain competence, depending both on the technical skill of the operator and the frequency of practice scans. As a hands-on technique, it also requires repeated practice with an experienced individual who can elucidate how to acquire the optimal image of the brachial artery and lumen interface for detection of the artery diameter and the pulsed-wave Doppler velocity through manual techniques. In addition, the FMD data analysis also requires good understanding of the technique and the principle of the software used for analysis, especially in the selection of the region of interest for data analysis. Inter-individual variations in the FMD data analysis have been observed (Woodman et al., 2001) and therefore caution should be taken in order to obtain accurate data analysis. Taken together, FMD requires considerable amount of training and its reproducibility is highly operator dependent (Vogel et al., 2000).

Hence, other non-invasive techniques such as finger plethysmography and peripheral arterial tonometry, have been adopted as simpler and cheaper alternative methods for assessment of endothelial function, reflecting downstream hyperaemic response induced by occlusion of the brachial artery (Kuvin et al., 2003). An optimal technique for assessment of endothelial function should be inexpensive, non-invasive and reproducible. However, it is worth noting that there is no perfect technique and all the techniques available for evaluation of endothelial function have advantages and disadvantages, as reviewed by Deanfield et al. (2007).

1.2.2 Nitric oxide and its roles in cardiovascular health

Among all the mediators produced by endothelial cells, NO is a key vasodilator (Palmer et al., 1987) and plays multiple roles in cardiovascular protection, including regulation of myocardial contractility, maintaining endothelial integrity, inhibiting inflammation, platelet aggregation and vascular smooth muscle cell proliferation (Loscalzo and Welch, 1995). Given these biological functions, NO is a crucial component in the vascular protection and the endogenous defence against atherosclerosis. However, it is worth noting eNOS plays distinct roles in normal endothelial function and endothelial dysfunction.

Specifically, NO is produced by a variety of cells, including endothelial cells, neurons, platelets and neutrophils (Loscalzo and Welch, 1995). Nitic oxide synthesis by endothelial nitric oxide synthase (eNOS) is illustrated in Figure 1.3. In healthy endothelial cells, in response to various stimuli (such as shear stress, vascular endothelial growth factor (VEGF), bradykinin, insulin, oestrogen), eNOS dimers are rapidly phosphorylated at serine 1177 site, resulting in electrons transferred from nicotinamide adenine dinucleotide phosphate (NADPH) to flavin adenine dinucleotide (FAD) and to flavin mononucleotide (FMN) in the reductase domain of a monomer of eNOS, and then to the oxygenase domain of the other monomer, where electrons interact with tetrahydrobiopterin (BH_4) and the haem iron to catalyse the reaction of oxygen with L-arginine, producing NO and citrulline (Alderton et al., 2001). Importantly, monomers of eNOS are unable to bind BH_4 or L-arginine, and therefore are unable to catalyse NO

production. However, in the presence of CVD risk factors (such as hypertension, hypercholesterolemia, elevated inflammation and diabetes), excessive amounts of superoxide reacts with NO to form peroxynitrite, thereby reducing NO bioavailability and diminishing its vascular protection. Worse still, peroxynitrite leads to eNOS uncoupling and enzyme dysfunction (Förstermann and Münzel, 2006; Landmesser et al., 2003a). Specifically, BH_4 is highly sensitive to oxidation to BH_2 by peroxynitrite. When BH_4 is limited, eNOS becomes uncoupled and electrons derived from NADPH are passed to oxygen rather than L-arginine, thereby producing superoxide instead of NO, which is referred to as eNOS uncoupling (Stuehr et al., 2001). In addition, hydrogen peroxide is produced if the substrate L-arginine is deficient (Förstermann and Münzel, 2006). Thus eNOS is transformed from a protective enzyme producing NO to a detrimental enzyme contributing to oxidative stress in dysfunctional endothelial cells. Taken together, the key roles of eNOS in endothelial function and dysfunction make this enzyme crucial to endothelial homeostasis (Deanfield et al., 2007).

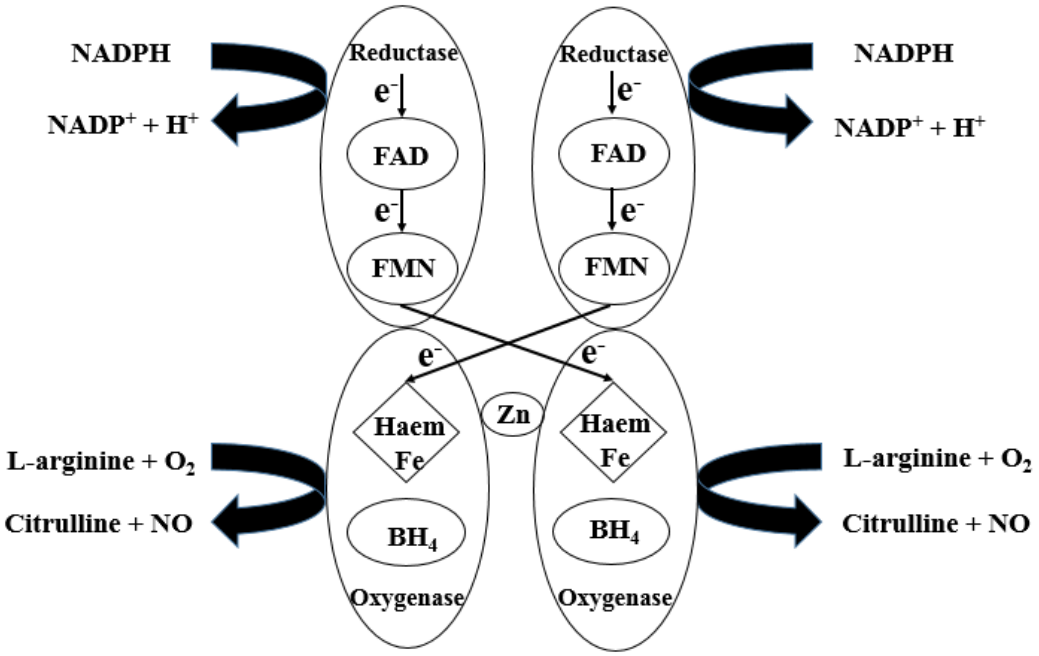

Figure 1.3. Basic structure of eNOS dimer with a zinc-thiolate cluster at the dimer interface and NO synthesis in endothelial cells. Upon activation of eNOS dimers, electrons are transferred from NADPH to FAD and to FMN in the reductase domain of eNOS, and subsequently to BH_4 and the haem iron in the oxygenase domain of eNOS to catalyse the reaction of oxygen with L-arginine, resulting in the

production of NO and citrulline. Uncoupled eNOS is unable to bind BH4 or L-arginine, therefore is unable to catalyse NO synthesis. Modified from (Förstermann and Münzel, 2006).

Accelerated degradation of NO by ROS is believed to be the major mechanism for reducing NO bioavailability in CVDs (Behrendt and Ganz, 2002; Harrison, 1997). Accumulating evidence indicates that reduced NO bioavailability is closely associated with endothelial dysfunction (Sena et al., 2013; Green, 2005), which is not only the primary aetiology of atherosclerosis but also the earliest identifiable event in the development of atherosclerotic cardiovascular disease. Hence the measurement of endothelial function and NO bioavailability is of great importance.

Only low levels (in the pico- to nanomolar range) of NO are generated from eNOS (Moncada et al., 1991) and it is rapidly metabolized to nitrite and nitrate before being excreted in the urine (Moncada and Higgs, 2006; Schechter and Gladwin, 2003). The low cellular production of NO and its short half-life (4 s) make its direct measurement difficult. Therefore, NO-dependent FMD is often used as an alternative, representing *in vivo* NO bioavailability. It is assumed that a small FMD is an indication of low NO bioavailability and increased CVD risk (Inaba et al., 2010). Given the fundamental role of NO in mediating endothelial function, impairment of vasodilation in response to shear stress due to limited endothelium-derived NO is often used as a measure of endothelial dysfunction (Deanfield et al., 2007).

1.3 Flavonoids and cardiovascular health

Given the growing evidence of the potential benefits of flavonoids on cardiovascular health from epidemiological studies (Kim and Je, 2017; Knekt et al., 2002), research interest in flavonoids from dietary sources has been increasing. Flavonoids are polyphenolic compounds that exist prevalently in plants, produced as secondary metabolites with multiple functions in protecting plants from harmful environmental conditions through their antioxidant properties (Agati et al., 2012) and regulating transcription of genes for growth and development (Peer et al., 2004). In addition, many flavonoids are responsible for the colours of flowers, fruits and leaves (Harborne and

Williams, 2000). Polyphenols encompass a wider variety of compounds, divided into flavonoids, phenolic acids, stilbenes and lignans, based on their structural differences (Manach et al., 2004). The focus of this thesis is on flavonoids, especially citrus flavonoids, and their effects on endothelial function and cardiovascular health.

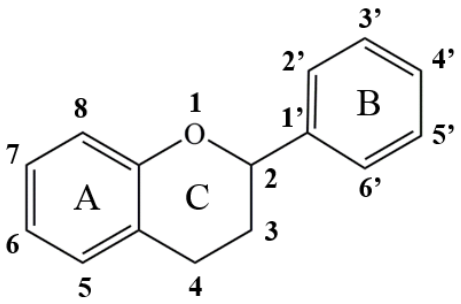

Figure 1.4. The basic structure and numbering pattern of flavonoids (Cook and Samman, 1996).

More than 7000 flavonoids have been identified (Beecher, 2003), sharing a C6–C3–C6 structure with two aromatic rings linked together by a three-carbon unit, as shown in Figure 1.4. Based on the connection of the B ring to the C ring and the degree of oxidation of the C ring, flavonoids are divided into subclasses encompassing flavanones, flavonols, flavanols, flavones, anthocyanidins and isoflavones (Beecher, 2003). In addition, the basic structure of flavonoids allows a multitude of substitution patterns in the benzene rings A and B within each subclass of flavonoids, for example phenolic hydroxyls, O-sugars, methoxy groups, sulphates and glucuronides.

1.3.1 Dietary sources and intake of flavonoids

Fruit and beverages (fruit juice, coffee, tea, red wine) are found to be most abundant in flavonoids, followed by vegetables, dry legumes and cereals (Scalbert and Williamson, 2000). The majority of flavonoids occur in nature as glycosides and other conjugates, with flavanols as an exception which occur in the form of aglycones (Beecher, 2003). Representative flavonoids in different subclasses and their respective major dietary sources are summarised in Table 1.1.

Table 1.1. Flavonoid subclasses with representatives and dietary sources.

Subclass	Representative aglycones	Major dietary sources
Flavanone	Hesperetin; naringenin	Citrus fruits
Flavonol	Quercetin; myricetin; kaempferol	Tea, apples and onions
Flavanol	Catechins; procyanidins	Tea, cocoa and red wine
Flavone	Apigenin; luteolin	Cereals and herbs
Anthocyanidin	Peonidin; cyanidin	Berries
Isoflavone	Genistein; daidzein	Soybeans

Apart from catechins, most flavonoids exist as glycosides in nature.

The main dietary source of flavanones is citrus fruits, which also contain other subclasses of flavonoids such as flavones, flavonols, and anthocyanins (the last exclusively in blood oranges). The genus Citrus encompasses several orange types, including sweet oranges (four subclasses-common, navel, Valencia, and blood), sour oranges, tangerines, lemons, tangors, tangelos and grapefruit. Flavanones usually occur in the form of glycosides, with rutinosides (6-O-α-L-rhamnosyl-D-glucosides) or neohesperidosides (2-O-α-L-rhamnosyl-D-glucosides) attached at position C7. The differences in the sugar moieties of the flavanone glycosides in sweet and sour oranges lead to distinct tastes. Specifically, hesperidin and narirutin with the sugar rutinose have a neutral taste and are relatively high in sweet oranges, tangerines, lemons and limes, whereas neohesperidin and naringin with the sugar neohesperidose possess a bitter taste and are abundant in sour oranges and grapefruits.

Hesperidin is the predominant flavanone in sweet oranges (*C. sinensis*), followed by narirutin, whereas neohesperidin and naringin predominate in sour oranges (*C. aurantium*) (Peterson et al., 2006). Citrus flavonoids in pure blond and blood orange juice are shown in Table 1.2, with data from Phenol-Explorer (Rothwell et al., 2013). Similarly, hesperidin is the predominant flavanone in orange juice with the content of 18-42.8 mg/100 ml in not-from-concentrate orange juice, followed by narirutin ranging from 2.95 to 5.41 mg/100 ml (Vanamala et al., 2006). Although nobiletin and tangeretin are minor in orange juice (Table 1.2), they are more abundant in citrus fruit peels with

the content of 5.89 ± 0.58 mg/g solid extract and 6.41 ± 1.03 mg/g solid extract respectively (Huang and Ho, 2010). Naringin is the primary flavanone in grapefruit juice with the content of 23.5- 37.2 mg/100 ml in not-from-concentrate grapefruit juice, followed by narirutin ranging from 9.1 to 11.7 mg/100 ml (Vanamala et al., 2006). Notably, citrus flavanone content is affected by the growing location, season, the maturity of the fruit (Albach et al., 1981). In terms of the distribution of flavanones in citrus fruits, flavanones are ubiquitous, especially abundant in the peel and seeds. As an example, the albedo and peel of pummelo (*Citrus maxima*) are found to be more abundant in naringin than the flesh, with naringin contents of 3910 μg/g and 220 μg/g, respectively (Yusof et al., 1990). Furthermore, total flavanone content in the whole fruit may be up to 5 times as much as that in a glass of orange juice (Manach et al., 2004). Given that citrus peels and seeds rich in flavanones are not used to produce orange juice, utilisation of these by-products has attracted increasing research interest (Sharma et al., 2017; Ghasemi et al., 2009).

Table 1.2. Citrus flavonoids in blond and blood orange juice (OJ) (pure juice) (mg/100 mL).

	Hesperidin	Narirutin	Didymin	Nobiletin	Tangeretin
Blond OJ	25.85 ± 12.16	5.36 ± 3.05	5.93 ± 0.33	0.07 ± 0.08	0.02 ± 0.01
Blood OJ	43.61 ± 17.98	4.8 ± 1.28	2.43 ± 1.27	0.34 ± 0.02	0.04 ± 0.01

Data from Phenol-Explorer (Rothwell et al., 2013).

As shown in Figure 1.5, hesperetin and naringenin share similar structures with a saturated C ring whereas there is a double bond at 2-3 position at the C ring of nobiletin and tangeretin, with multiple methoxy groups at both A and B rings. The differing structures of these compounds contribute to their differential effects as detailed in Chapter 2.

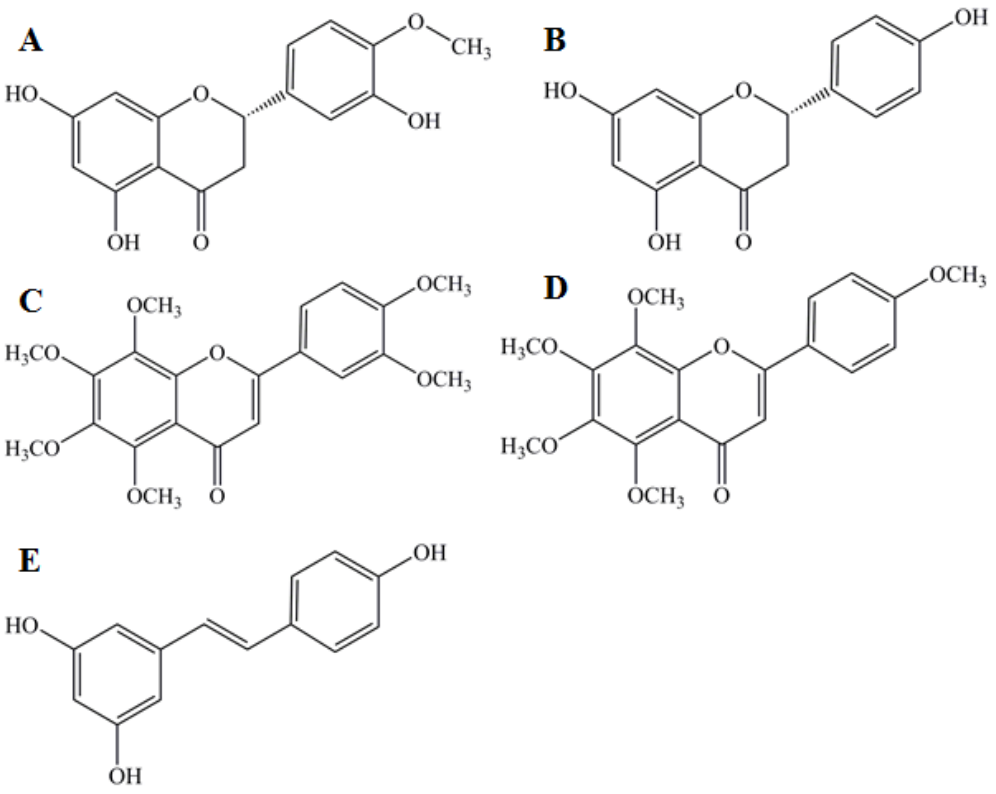

Figure 1.5. Structures of hesperetin (A), naringenin (B), nobiletin (C), tangeretin (D) and resveratrol (E).

With regards to dietary sources of other flavonoids, quercetin, a flavonol, is the most ubiquitous flavonoid in the human diet and has been most intensively researched. It occurs prevalently in plant foods with particular abundance in onions (0.3 mg/g fresh weight) (Hertog et al., 1992) and tea (10–25 mg/L) (Hertog et al., 1993). The main flavanols are catechins. They are abundant in tea and the four major catechins in tea are (−)-epicatechin (EC), (−)-epicatechingallate (ECG), (−)-epigallocatechin (EGC) and (−)-epigallocatechingallate (EGCG) (Balentine et al., 1997). Due to the differences in the manufacturing process, there is a huge difference in the catechin content in different kinds of tea. In green tea, catechins account for 77.1% of the total polyphenols whereas catechins account for 3.3% of the total polyphenols in black tea (Del Rio et al., 2004). During fermentation, the catechins are converted to theaflavins and thearubigins in black tea. Proanthocyanidins (PAs) are oligomeric and polymeric flavanols and exist ubiquitously in plants. According to the study conducted by Gu et al. (2004), the average daily intake of PAs in the U.S. population (>2 y old) was estimated to be 57.7 mg/person, with monomers, dimers, trimers, and those above trimers account for 7.1, 11.2, 7.8, and

73.9% of total PAs, respectively. The main dietary sources of PAs in the American diet are apples (32.0%), chocolate (17.9%) and grapes (17.8%) (Gu et al., 2004). Flavones consist chiefly of glycosides of luteolin and apigenin. The main food sources of flavones are parsley (240–1850 mg/kg fresh weight) and celery (with apigenin contents of 20-140 mg/kg fresh weight) (Manach et al., 2004). The occurrence of isoflavones is mainly restricted to soybean products, with approximately 1 mg of genistein and daidzein per gram of dry bean (Reinli and Block, 1996). There are three types of isoflavones in four chemical forms in soybean: the aglycones daidzein, genistein and glycitein; the acetylglucosides 6”-*O*-acetyldaidzin, 6”-*O*-acetylgenistin and 6”-*O*-acetylglycitin; and the malonylglucosides 6”-*O*-malonyldaidzin, 6”-*O*-malonylgenistin and 6”-*O*-malonylglycitin (Kudou et al., 1991).

It should be noted that the accurate quantification of dietary flavonoid intake has been difficult given the complexity and structural diversity of flavonoids occurring in foods with large variability, their prevalence in a large number of dietary sources and different analytical methods used for quantification (Erdman et al., 2007). In addition, the current methods using food records and food composition tables for the estimation of flavonoid intake fail to take those into account and therefore are not accurate. Despite those challenges and limitations, previous studies have conducted some investigations on dietary intake of flavonoids. The total intake of dietary polyphenols in Western populations who consume several servings of fruits and vegetables per day has been reported to be approximately 1-2 g/day, with flavonoids accounting for two thirds and phenolic acid accounting for one third (Rothwell et al., 2012; Scalbert and Williamson, 2000). It is worth noting that this proportion depends on foods and beverages consumed and may vary substantially from individual to individual, due to their dietary habits and food preferences. For example, heavy coffee consumers are likely to consume more phenolic acids than flavonoids (Clifford, 1999).

Taken together, given that citrus fruits and juices are highly consumed worldwide (Sebastian et al., 2017; Zamora-Ros et al., 2011; Johannot and Somerset, 2006), citrus flavonoids are among the most consumed dietary flavonoids, accounting for a large proportion of the total daily intake of flavonoids (Tomás-Barberán and Clifford, 2000).

Therefore, research on citrus flavonoids and their effects on health is of significant relevance.

1.3.2 Bioavailability of flavonoids

Bioavailability has various definitions depending on the field of study. In nutritional research, the definition of bioavailability is "the rate and extent to which the active ingredient or active moiety is absorbed from a drug product and becomes available at the site of action", according to U.S. Food and Drug Administration (FDA). Accessing bioavailability of flavonoids involves determination of their absorption, distribution, metabolism and excretion (ADME) (Thilakarathna and Rupasinghe, 2013). The understanding of the bioavailability of flavonoid is of great significance to evaluate their biological activities and effects on cardiovascular health. It was initially assumed that the bioavailability of flavonoids was generally low, due to their complex structures, usually conjugated to sugar moieties (Bravo, 1998). However, with increasing understanding of flavonoid absorption and metabolism, recent evidence suggest that flavonoids, especially flavanones and anthocyanins, are more bioavailable than previously reported (Kay et al., 2017; Walle, 2004). Following ingestion, flavonoid aglycones are absorbed by intestinal enterocytes (Scalbert and Williamson, 2000) whereas glycosides undergo phase I deglycosylation and may be hydrolysed by lactase phlorizin hydrolase (LPH) in the brush border of the small intestine (Day et al., 2003), releasing corresponding aglycones, which are less hydrophilic and can be easily absorbed by intestinal enterocytes. Alternatively, glucosides can be transported into the epithelial cells, possibly via the active sodium-dependent glucose transporter (SGLT-1) (Gee et al., 2000). Hydrolysis of glucosides is then mediated by cytosolic β-glucosidase (CBG) within epithelial cells, releasing the respective aglycones. Subsequently, aglycones may diffuse into the hepatic portal vein (Day et al., 2003) or undergo phase II metabolism in the intestinal enterocytes, resulting in sulfates, glucuronides, and/or methylated metabolites, via the action of sulfotransferases (SULT), uridine-5'-diphosphate glucuronosyltransferases (UDP-GT), catechol-O-methyltransferases (COMT), respectively (Scalbert and Williamson, 2000). Thereafter, these metabolites are transported to hepatocytes in the liver, where they may undergo further phase II metabolism (Perez-Vizcaino et al., 2012), before being distributed to the tissues or eliminated by the kidney via urine excretion.

In addition, unabsorbed flavonoids and flavonoid metabolites are carried to the colon, where substantial deconjugation occurs. Specifically, the colonic microbiota break down the conjugation, releasing the aglycones, which are either absorbed or undergo ring fission and fermentation resulting in smaller compounds, such as phenolic acids and phenylacetic acids (Serra et al., 2012). These products may be absorbed by the colon (Déprez et al., 2000), a portion of which circulate to the liver and undergo phase II metabolism, before being excreted in faeces (Monagas et al., 2010).

Large variability has been observed in the bioavailability between different flavonoids (Manach et al., 2005) and between individuals (Fuhr and Kummert, 1995). Numerous factors contribute to this variability, including the chemical structure of flavonoids (Manach et al., 2004), food matrix (Bohn, 2014), endogenous factors such as digestive enzymes and microbiota (Scalbert and Williamson, 2000). With regards to the bioavailability of flavanones, the findings of Pereira-Caro et al. (2014) suggest that hesperidin and narirutin from orange juice are highly bioavailable. Specifically, when their metabolites and catabolites are both taken into account, a recovery over 37% of intake (nearly 100% with hippuric acid taken into calculation) has been estimated.

1.3.3 Cardiovascular protection by flavonoids

Numerus epidemiological studies have demonstrated an inverse association between the consumption of flavonoid-rich foods and the risk of CVD (Steinhaus et al., 2017; Cassidy et al., 2016; Landberg et al., 2011). Citrus flavonoids are of particular interest given that citrus fruits and juices are highly consumed worldwide and flavanones are highly bioavailable, as discussed in section 1.3.1 and 1.3.2. In addition, only a few flavonoid-rich products such as green tea (Babu et al., 2008; Tipoe et al., 2007), cocoa and dark chocolate (Magrone et al., 2017; Basu et al., 2015), have been more extensively researched, with more convincing clinical data suggesting their benefits for cardiovascular health including lowering blood pressure (Negishi et al., 2004) and LDL cholesterol (Maron et al., 2003) as well as improving endothelial function (Loffredo et al., 2017). In contrast, evidence from randomised controlled crossover trials on citrus products (as shown in Table 1.3 and 1.4) suggest the effect of citrus flavonoids on

cardiovascular health is still inconclusive and not fully understood, therefore warrants more investigation.

Following increasing evidence from epidemiological studies indicating that the intake of flavonoids may have possible cardiovascular health benefits, a large number of *in vitro* and *in vivo* studies been conducted to elucidate the mechanisms by which flavonoids potentially improve cardiovascular health. Over decades, flavonoids have simply been assumed as antioxidants (Pietta, 2000) and numerous *in vitro* studies have been performed to investigate their role in scavenging radicals, using supraphysiological concentrations of flavonoids (Rice-Evans et al., 1996), overlooking the complexity of flavonoid metabolism. However, it is unlikely that the antioxidant capacity of flavonoids is the only mechanism for their benefits on cardiovascular health given that complex flavonoid metabolism may decrease the antioxidant capacity of their metabolites, such as sulfate or glucuronide conjugates (Turner et al., 2004; Moon et al., 2001). A previous study by Andriambeloson et al. (1997) has demonstrated that the endothelium-dependent relaxation in the rat aorta, induced by polyphenols from red wine, is not due to their superoxide scavenging activity, but to the induction of NO synthesis by polyphenols. In recent years, with increasing recognition of the pivotal roles of the endothelium and NO, the effect of flavonoids on endothelial function has been under active investigation.

Flavonoids have been reported to exert favourable effects on endothelial function by different mechanisms, some of which are illustrated in Figure 1.6. For example, quercetin upregulates NO production by inducing phosphorylation of eNOS at serine 1179 (Li et al., 2012). (–)-epicatechin scavenges superoxide and its O-methylated metabolites downregulate endothelial NADPH oxidase activity (Steffen et al., 2007). Furthermore, resveratrol upregulates the expression of ROS detoxifying and antioxidant genes such as NAD(P)H:quinone oxidoreductase 1 (NQO1), heme oxygenase-1 (HO-1), gamma glutamyl cysteinyl synthetase (GCLC), through the activation of nuclear factor-E_2-related factor 2 (Nrf2) (Ungvari et al., 2010). Therefore, flavonoids preserve BH_4, a key factor for eNOS coupling by reducing ROS such as peroxynitrite (Papageorgiou et al., 2013). Preventing BH_4 from oxidation is of great significance, given that in presence of oxidative stress, BH_4 is oxidised by peroxynitrite, leading to eNOS uncoupling, which

results in production of ROS insteand of NO, contributing to oxidative stress and endothelial dysfunction (Landmesser et al., 2003b).

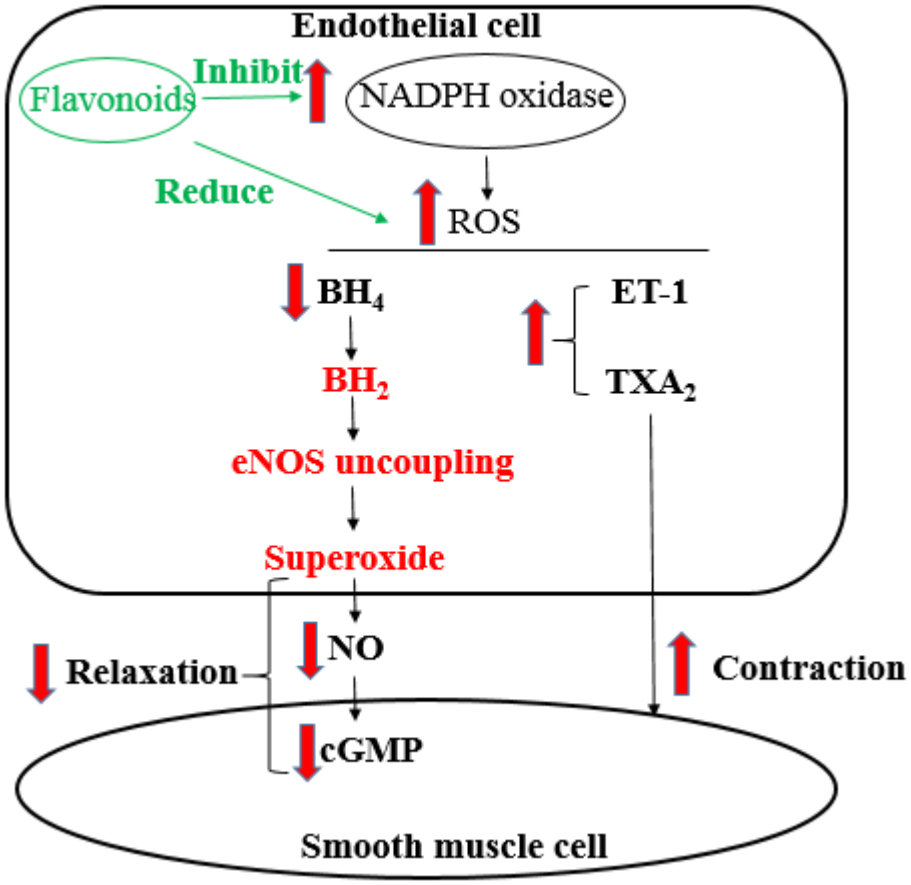

Figure 1.6. Mechanisms involved in pathological artery with increased NADPH expression, resulting in an upregulation of reactive oxygen species (ROS), which leads to a reduction in NO and relaxation as well as an increase in the contraction of the smooth muscle cells. Flavonoids are reported to inhibit NADPH oxidase and reduce ROS, thereby preserving BH_4 and contributing to NO production. cGMP, cyclic guanosine monophosphate; ET-1, endothelin 1; TXA_2, thromboxane A_2.

In addition, intakes of flavanones have been inversely associated with plasma inflammatory biomarkers in healthy subjects (Landberg et al., 2011), the effect of citrus product consumption on inflammatory biomarkers is of great significance and warrants further *in vivo* and *in vitro* investigation, given that inflammation and endothelial dysfunction are considered to be important contributors to the development of atherosclerosis (Deanfield et al., 2007; Friedman et al., 2005).

Specifically, randomised controlled trials (RCT) investigating the effect of citrus products on cardiovascular risk biomarkers following short- or long-term consumption are summarised in Table 1.3. The inclusion criteria are: a crossover study design; with a control drink matched for sugar content of tested juice, or a double-blinded design for a placebo and citrus extracts; outcomes include at least one of the following: endothelial function, blood pressure, lipid profile, inflammatory biomarkers (or antioxidant status).

As shown in Table 1.3, there is conflicting evidence regarding the short- or long-term consumption of citrus products on endothelial function, mostly measured by FMD. Several factors may have contributed to this discrepancy. Firstly, variations in the characteristics of the subjects inevitably lead to mixed results: ethnicity, male or female, premenopausal or post menopausal women, normal-weight or overweight, with or without the presence of CVD risk factors. To our knowledge, ethnicity has not been controlled for in previous studies. However, ethnicity-associated differences have been observed in the enzymes involved in the metabolism of polyphenols, such as LPH (Maskarinec et al., 1998). In addition, given that dietary nitrate also contributes to circulating NO through the nitrate-nitrite-NO pathway (Lundberg et al., 2008), differences in dietary nitrate metabolisms due to different ALDH2 phenotypes in Asian populations and Caucasians (Thomasson et al., 1991; Shibuya and Yoshida, 1988) also give rise to variations in circulating NO and the resultant NO-dependent endothelial function as measured by FMD. Furthermore, previous studies involving premenopausal women have not controlled for the confounding effect of oestrogen, which increases NO production in endothelial cells, mediated partially through oestrogen receptor-α (Muller-Delp et al., 2003), thereby rendering the FMD results questionable. Therefore, further studies controlling for these confounding factors are warranted to minimise confounding effects, thereby investigating effects of citrus products on endothelial function (the primary outcome of this thesis). Secondly, the composition of the juice ingested (high or low contents of flavonoids, fibre and sugar) (Reshef et al., 2005) may have also played a role in the outcomes. Other factors such as juice compliance, food matrix, physical activity may have resulted in variations in endothelial function.

From reviewing previous studies on citrus products, evidence regarding blood orange juice consumption is much less than that on blond orange juice. To our knowledge, only one published crossover study by Buscemi et al. (2012) suggests short-term (1 week) consumption of blood orange juice significantly increases endothelial function as measured by FMD, as shown in Table 1.3. However, there are several limitations to this previous study, such as lack of controlling for confounding factors and another baseline measurement at the beginning of the second intervention arm. In addition, effects regarding blood orange juice consumption on endothelial function and other CVD risk factors for longer than 1 week are still unknown and warrant further investigation.

Table 1.3. Summary of crossover randomised controlled trials investigating the effects of short- or long- term consumption of citrus products on CVD risk biomarkers.

Study	Intervention arms	Duration	Subjects	Results
(Constans et al., 2015)	OJ (600 ml/d) CD (vitamin C controlled)	4 wk	Hypercholesterolemic men (n=25, 50-60 y)	FMD: - sICAM-1, sVCAM-1, sE-selectin: - Lipid profile: - Antioxidant status: ↑ ROS: ↓
(Habauzit et al., 2015)	GFJ (340 ml/d) CD (vitamin C controlled)	6 mo	Healthy postmenopausal women (n=48, 50–65 y)	FMD: - BP: - Inflammation: - PWV: ↓
(Asgary and Keshvari, 2013)	Natural OJ (NOJ) (500 ml/day) Commercial OJ (COJ)	4 wk	Healthy subjects (n=22, 18-59 y)	DBP&SBP: ↓ in COJ
(Buscemi et al., 2012)	ROJ (500 ml/d) CD	1 wk	Subjects with CVD risk factors (n=19, 19–67 y)	FMD: ↑ 2.2% hsCRP, IL-6, TNF-α: ↓
(Morand et al., 2011)	CD+ hesperidin (CDH) (500 ml/d) OJ CD+placebo (CDP)	4 wk	Healthy, overweight men (n=24, 50-65 y)	Microvascular reactivity: - SBP: - ; DBP: ↓ in CDH & OJ Lipid profile:-
(Rizza et al., 2011)	Hesperidin (500 mg/d) Placebo	3 wk	Subjects with metabolic syndrome (n=24, 21-65 y)	FMD: ↑ 2.57% BP: - Lipid profile: TC: ↓;HDL: ↑; LDL&TG:- Inflammation: hsCRP, SAA, sE-selectin all ↓

Study	Intervention arms	Duration	Subjects	Results
(Reshef et al., 2005)	HF citrus juice LF citrus juice	5 wk	12 patients with hypertension (n=12, 52.1±10.1 y)	DBP: ↓

BP=blood pressure, CD=control drink, CVD=cardiovascular disease, DBP= diastolic blood pressure, FMD= flow mediated dilation, GFJ=grapefruit juice, HDL=high-density lipoprotein, HF=high flavonoid, hsCRP= high-sensitivity C-reactive protein, LDL= low-density lipoprotein, IL-6= interleukin 6, LF=low flavonoid, OJ=orange juice, PWV= pulse wave velocity, ROJ=red orange juice, ROS= reactive oxygen species, SAA= serum amyloid A, SBP= systolic blood pressure, sE-selectin=soluble E-selectin, sICAM-1=soluble intercellular adhesion molecule-1, sVCAM-1=soluble vascular cell adhesion molecule-1, TC=total cholesterol, TG= triglycerides, TNF-α= tumour necrosis factor-α, ↑=significant increase, ↓=significant decrease, - =no significant changes.

Regarding acute effects of citrus products on cardiovascular risk biomarkers, the characteristics of some RCTs are summarised in Table 1.4, based on the same inclusion criteria: a crossover study design; with a control drink matched for sugar content of tested juice, or a double-blinded design for a placebo and citrus extracts; outcomes include at least one of the following: endothelial function, blood pressure, lipid profile, inflammatory biomarkers or antioxidant status. Interestingly, a variety of approaches have been used for the evaluation of endothelial function following citrus product consumption in a acute manner, such as FMD (Rendeiro et al., 2016), RH-PAT (Schär et al., 2015), laser Doppler flowmetry and iontophoresis (Morand et al., 2011), resulting in conflicting evidence, which may in part be due to differing sensitivity and reproducibility of the approaches used. Hence, evidence for acute effects of citrus products is still inconclusive and further investigations are needed.

Although some clinical studies suggest that the intake of citrus products may improve endothelial function and contribute to antioxidant status, the molecular mechanisms by which citrus flavonoids exert these effects have not been fully elucidated. However, previous *in vitro* studies on these mechanisms are limited and have incubated cells in supraphysiological concentrations of flavonoids (Appeldoorn et al., 2009; Liu et al., 2008). Hence, more *in vitro* studies investigating effects of different citrus flavonoids at physiological concentrations are needed in order to decipher how they modulate gene expression relevant to NO production and antioxidant status in human endothelial cells.

Table 1.4. Summary of crossover randomised controlled trials investigating the acute effect of citrus product consumption on CVD risk biomarkers.

Study	Intervention arms	Time point	Subjects	Results
(Rendeiro et al., 2016)	OJ (128.88 mg TF)+ HFM FROJ (272.14 mg TF) + HFM Whole orange (452.8 mg TF) + HFM CD + HFM	2, 5, 7 h	Healthy men (n=28, 30–65 y)	FMD: ↑ (7 h); ↓ (CD) Plasma nitrite: ↓ (CD) Plasma nitrate: ↓ RXNO species: -
(Schär et al., 2015)	OJ (767 ml) Hesperidin+Vitamin C CD (match for Vitamin C)	5 h	Men with CVD risk factors (n=16, 51–69 y)	RH-PAT: - BP: - Arterial stiffness: - Cardiac autonomic function: - Platelet activation: - NADPH oxidase gene expression: -
(Morand et al., 2011)	CD+ hesperidin (CDH) (500 ml) OJ CD+placebo (CDP)	6 h	Healthy, overweight men (n=24, 50-65 y)	Microvascular reactivity: ↑ (CDH&OJ)
(Bui et al., 2006)	Bitter orange extract (900 mg) Placebo	every h until 6 h	Young healthy subjects (n=13, 22-29 y)	SBP: ↑ (1-5 h) DBP: ↑ (4,5 h) HR: ↑ (2-5 h)
(Min et al., 2005)	Bitter orange extract (450 mg) Placebo	1, 3, 5, 8 h	Healthy subjects (n=36, >18 y)	QTc interval: - BP: -

BP=blood pressure, CD=control drink, CVD= cardiovascular disease, DBP= diastolic blood pressure, FMD= flow mediated dilation, FROJ=flavanone-rich orange juice, HFM=high-fat meal, HR=heart rate, NADPH= nicotinamide adenine dinucleotide phosphate, OJ=orange juice, PH-PAT= reactive hyperemia-peripheral arterial tonometry, QTc=QT interval corrected for heart rate, RXNO= nitros(yl)ated species, SBP= systolic blood pressure, TF=total flavanones, ↑=significant increase, ↓=significant decrease, - =no significant changes.

1.4 Outline of this thesis

The present literature review describes current understanding on cardiovascular health, evaluation of endothelial function, multiple roles of NO and its synthesis, dietary sources and intake of flavonoids, metabolism and bioavailability of flavonoids and their potential cardiovascular protective effects and possible mechanisms. In addition, based on current *in vitro* and *in vivo* data, remaining limitations to be addressed in future research have been discussed and some of these research gaps were addressed to some extent in the present work.

In summary, CVD is the leading cause of death worldwide, causing great economic loss and diminishing life quality. As one of modifiable factors of CVD, foods or beverages rich in flavonoids have been associated with reduced rick of CVD. Given that citrus fruits and juices are highly consumed worldwide and are highly bioavailable, yet less studied, the effect of citrus flavonoids on cardiovascular health are of great importance.

Indeed, some epidemiological studies have suggested an inverse association between the intake of citrus products and risk of CVD. However, evidence from *in vitro* and *in vivo* human studies is still lacking and conflicting, therefore the possible underlying mechanisms by which flavanones may exert beneficial effects on endothelial function and antioxidant status remain poorly understood.

Therefore, in order to investigate effects of citrus flavonoids on cardiovascular health and address some limitations of previous studies, the objectives of this thesis are as follows:

1. To investigate the effects of selected citrus flavonoids on endothelial NO production at transcription and protein levels as well as gene expression relevant to antioxidant status in human endothelial cells (Chapter 2).
2. To determine and compare flavanone content in commercial orange juices including blond orange juice and blood orange juice (Chapter 3).

3. To investigate the acute effect of flavanone-rich blond orange juice on vascular function, specifically on cardiac electrical activity, blood pressure, vascular compliance, peripheral blood flow, pulse transit time and pulse wave velocity (Chapter 3).
4. To investigate the effects of flavanone-rich blood orange juice on endothelial function (FMD), blood pressure, lipid profile, inflammation, in healthy overweight/obese men and premenopausal women of European origin with consideration of female hormonal cycle (Chapter 4).

Chapter 2 Effects of citrus flavonoids on the basal expression of vascular biomarkers in human endothelial cells

2.1 Abstract

Epidemiological studies have indicated that the consumption of citrus fruit is inversely associated with the risk of cardiovascular disease (CVD). Although clinical studies suggest that the intake of citrus flavonoids might improve endothelial cell function and reduce blood pressure, the molecular mechanisms by which different citrus flavonoids contribute to cardiovascular health have not been fully elucidated. The aim of this study therefore was to investigate the potential of selected citrus flavonoids to modulate gene expression relevant to nitric oxide (NO) production and antioxidant status in endothelial cells.

Confluent human endothelial cells (EA.hy926 hybrid cell line) were incubated with increasing concentrations of hesperetin, naringenin, tangeretin and nobiletin for 18 h. Cell viability was measured by neutral red assay. Expression of endothelial NO synthase (eNOS) and endothelin 1 (ET-1) as well as expression of genes associated with antioxidant and cytoprotective function (gamma glutamyl cysteinyl synthetase, GCLC and heme oxygenase-1, HO-1) were determined by quantitative real time PCR. eNOS protein expression in response to different citrus flavonoids was determined by western blot. NO production from EA.hy926 in response to selected citrus flavonoids was also evaluated.

The results demonstrate that incubation of EA.hy926 cells with nobiletin resulted in significant augmentation of NO production through dose-dependent induction of NOS3 mRNA and protein expression, significant reduction of ET-1 mRNA expression and significant induction of HO-1 and GCLC in a dose-dependent manner. Tangeretin also significantly upregulated NOS3 mRNA and protein levels. Present data indicate that selected citrus flavonoids favourably modulate gene expression in endothelial cells, which could in part contribute to the health effects associated with citrus product consumption.

2.2 Introduction

Epidemiological studies have demonstrated an inverse association between the consumption of citrus fruit and the risk of cardiovascular disease (CVD) (Landberg et al., 2011; Knekt et al., 2002). Some randomised controlled trials (RCT) have indicated that the consumption of citrus juice improves endothelial function acutely (Rendeiro et al., 2016) and chronically (Rizza et al., 2011), as measured by flow mediated dilation (FMD), which is nitric oxide (NO) dependent. However, molecular mechanisms by which citrus flavonoids contribute to cardiovascular health have not been fully elucidated. Previous *in vitro* studies have treated endothelia cells with flavonoids at supraphysiological concentrations, therefore lacking clinical relevance (Kay, 2010; Appeldoorn et al., 2009; Liu et al., 2008). Effect of citrus flavonoids at physiological concentrations on endothelial cells, hence, warrants investigation.

As reviewed in Chapter 1, NO is the major vasodilator produced by endothelial cells and plays a pivotal role in the regulation of vascular homeostasis by inducing vasodilation as well as inhibiting inflammation, platelet aggregation and smooth muscle proliferation (Andriantsitohaina et al., 2012; Deanfield et al., 2007). Accumulated evidence indicates that reduced NO bioavailability is closely associated with endothelial dysfunction, which is not only the primary etiology of atherosclerosis but also the earliest identifiable event in the process of atherosclerotic cardiovascular disease development (Kawashima and Yokoyama, 2004; Oemar et al., 1998). In healthy endothelial cells, NO is produced from L-arginine in the presence of oxygen by endothelial nitric oxide synthase (eNOS) with cofactors such as tetrahydrobiopterin (BH_4). However, when the redox balance between oxidants and antioxidants is disrupted and oxidants outweigh antioxidants, oxidative stress is produced. Consequently, excessive amounts of superoxide reacts with NO to form peroxynitrite, not only reducing NO bioavailability but also leading to uncoupling of eNOS. Specifically, BH_4 is oxidised to BH_2 by peroxynitrite, resulting in eNOS uncoupling (Stuehr et al., 2001). Consequently, electrons derived from NADPH are passed to oxygen rather than L-arginine, thereby producing superoxide instead of NO by eNOS. Prolonged eNOS uncoupling contributes to oxidative stress and leads to endothelial dysfunction (Förstermann and Münzel, 2006; Landmesser et al., 2003a). Given the crucial role of NO in both normal endothelial function and endothelial

dysfunction, the effect of citrus flavonoids on eNOS expression is of particular interest. Only hesperetin and naringenin have been studied for their effect on eNOS expression. Specifically, incubation with hesperetin (12.5-100 μM) for 24 h dose dependently induced NO production (measured as nitrite) in human umbilical vein endothelial cells (HUVECs), whereas no significant changes were observed after treatment with naringenin under the same conditions (Liu et al., 2008). The effect of other citrus flavonoids at lower concentrations has yet to be explored.

Elevated vasoconstrictor endothelin 1 (ET-1) plasma concentrations are associated with hypertension and heart failure (Schiffrin, 2001; Yoshibayashi et al., 1991). ET-1 has been reported to downregulate eNOS promoter activity and protein levels through inducing hydrogen peroxide mediated by endothelin A receptor (Wedgwood and Black, 2005). Moreover, ET-1 upregulated superoxide anions in HUVECs (Duerrschmidt et al., 2000), thereby inducing oxidative stress and reducing NO bioavailability. There is, however, a paucity of data regarding effect of citrus flavonoids on ET-1 expression. Moreover, endothelial function is modulated by the balance between eNOS and ET-1, both influenced by cellular inflammation/oxidative stress.

It has been recognised that oxidative stress and vascular inflammation are closely associated with endothelial dysfunction and vascular damage (Siti et al., 2015). Part of the pathophysiology of CVD, especially hypertension and atherosclerosis, is attributed to mild chronic vascular inflammation (Touyz, 2005; Libby et al., 2002). Furthermore, excessive oxidative stress can turn eNOS from a protective enzyme producing NO to a detrimental enzyme producing superoxide and contributing to oxidative stress. Therefore, redox balance in endothelial cells is of great importance. Several genes contribute to maintaining redox balance. Specifically, the increase in heme oxygenase-1 (HO-1) is associated with a decrease in intracellular pro-oxidants and an increase in bilirubin and carbon monoxide with anti-apoptotic and anti-inflammatory properties in cells (Chen et al., 2010; Szabo et al., 2004). Moreover, HO-1 induction protects eNOS expression from oxidized low-density lipoprotein (oxLDL) and tumour necrosis factor-α (TNF-α) via cellular bilirubin degraded from haem (Kawamura et al., 2005). In addition, the glutathione (GSH) antioxidant system plays a vital role in maintaining

cellular redox balance and protecting against oxidative injury (Hayes and McLellan, 1999). The upregulation of gamma glutamyl cysteinyl synthetase (GCLC) as a subunit of the first rate-limiting enzyme for GSH synthesis, also contributes to cellular antioxidant defence and cell protection against oxidative stress.

The aim of this chapter, therefore, was to investigate the potential of selected citrus flavonoids to modulate gene expression relevant to NO production and antioxidant status in endothelial cells.

2.3 Materials and methods

2.3.1 Chemicals and reagents

Hesperetin, naringenin, phorbol-12-myristate-13-acetate (PMA), N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME) were obtained from Sigma-Aldrich (Dorset, UK). Nobiletin, tangeretin, genistein, resveratrol were purchased from Extrasynthase (Genay, France).

Dulbecco's phosphate-buffered saline (DPBS) without calcium and magnesium, T75 flasks, 6-well plates, PCR single tubes (free of DNA, DNase/RNase and PCR inhibitor) were purchased from Fisher Scientific (Loughborough, UK). Dulbecco's modified Eagle's medium (DMEM), L-glutamine, penicillin-streptomycin, Trypsin-EDTA were obtained from Lonza (Slough, UK). All other chemicals and reagents were obtained from Sigma-Aldrich unless specified otherwise.

2.3.2 Treatment with flavonoids

Stock solutions of all compounds were prepared in 100% dimethyl sulfoxide (DMSO) at 100 mM in aliquots, with the exception of tangeretin (at 25 mM due to its limited solubility in DMSO), and stored at -20°C. Working solutions were diluted from stock solutions to specific concentrations immediately prior to treatment.

Cell viability and compound cytotoxicity were determined by the neutral red (NR) uptake assay. Thereafter, optimisation of NOS3 induction was conducted using positive controls PMA (Li et al., 1998) and resveratrol (Appeldoorn et al., 2009). Subsequent experiments were conducted under optimised conditions for NOS3 induction. Following incubating confluent human endothelial cells (EA.hy926 hybrid cell line) with increasing concentrations of hesperetin, naringenin, tangeretin and nobiletin for 18 h, expression of NOS3, ET-1, HO-1, GCLC were determined using quantitative real time PCR. eNOS protein expression in response to different citrus flavonoids was determined by western blot. NO production from EA.hy926 in response to selected citrus flavonoids was also evaluated using DAN assay and DAF-2 assay.

Specifically, EA.hy926 cells were seeded into 12-well plates at $1x10^5$ cells/ml and left to grow overnight to reach 90% confluence. After media removal, cells were incubated with flavonoids at different concentrations (1, 10, 25, 50, 100 μM) for 18 h, with PMA (10 nM) as a positive control and L-NAME (20 nM) as a negative control for NOS3 induction.

2.3.3 Cell culture

2.3.3.1 Thawing frozen cells

Human endothelial cells EA.hy926 (ATCC® CRL-2922™), derived from the fusion of human umbilical vein endothelial cells and the permanent human cell line A549 (Edgell et al., 1983), were obtained from ATCC (Middlesex, UK) and stored in liquid nitrogen until required. Cells were thawed by placing the vial in a 37 ºC water bath with gentle agitation. Caution was taken to keep the cap and O-ring of the vial out of water to avoid contamination. The vial contents were transferred to a centrifuge tube containing 9 ml of pre-warmed complete media (Dulbecco's modified Eagle's medium with 1000 mg/L glucose, 2 mM L-glutamine, 10% fetal bovine serum and 1% penicillin and streptomycin) under aseptic conditions as soon as the vial contents were thawed, followed by centrifugation at 125 x g for 5 min. The supernatant was removed by an aspirator and the cell pellet was re-suspended with pre-warmed complete media and dispensed into a T75 culture flask. The flask was then incubated in a humidified atmosphere with 5% CO_2 at

37ºC overnight. The cells were checked using an inverted microscope after 24 h and medium was replaced to remove non-adherent cells.

2.3.3.2 Cultivation of EA.hy926 cells

EA.hy926 cells were grown in complete media (Dulbecco's modified Eagle's medium with 2 mM L-glutamine, 1 mM sodium pyruvate, 10% fetal bovine serum and 1% penicillin and streptomycin) in a humidified atmosphere with 5% CO_2 at 37ºC. Medium was changed every two to three days. Cells were subcultured to the next passage when cells were in log-phase growth before confluence was reached. Basically, after removal of the media, cells were gently washed by DPBS to remove any remaining serum, calcium and magnesium which inhibit enzymatic dissociation. Subsequently, 3 ml of pre-warmed Trypsin-EDTA solution was added to a T75 flask, followed by an incubation at 37 ºC for 5 min to facilitate enzymatic dissociation. Dispersal of cells was checked under an inverted microscope before 6 ml of complete medium was added to the T75 flask to deactivate the enzymatic dissociation by Trypsin-EDTA. Cells were transferred to a centrifuge tube by gentle pipetting, followed by centrifugation at 125 x g for 5 min. Supernatant was removed by an aspirator. Cells were then re-suspended in fresh complete media in desired split ratios.

2.3.3.3 Cell counting

Cell counting was conducted using a haemocytometer (Neubauer Improved counting chamber, Assistent**,** Sondheim v. d. Rhön**,** Germany). Cells in suspension were obtained as described in 2.3.3.2. Both the upper and lower chambers were filled with 10 µl of cell suspension. Counting of the cells was conducted in the large central squares containing 25 medium squares in the upper and lower chambers. The average of cell numbers in the upper and lower chambers was calculated and multiplied by 10^4 as an estimation of the number of cells per ml.

2.3.3.4 Freezing cells

Early passages of EA.hy926 cells were frozen and preserved for long-term storage. Specifically, a pellet of viable cells was obtained as described in 2.3.3.2. Subsequently,

cells were re-suspended in recommended freezing media (70% complete media, 20% fetal bovine serum, 10% DMSO) at a concentration of approximately $1x10^6$ cells/ml before being transferred to sterile cryovials. These cryovials were placed in a freezing container which allowed slowly freezing cells by reducing the temperature at the optimal rate for cell preservation (approximately 1 ºC per minute) and left in a -80 ºC freezer overnight, before being stored in liquid nitrogen and a -80 ºC freezer.

2.3.4 Cell viability

Cell viability and compound cytotoxicity were determined by the neutral red (NR) uptake assay (Borenfreund and Puerner, 1985), and the principle is based on the ability of viable cells to accumulate neutral red in lysosomes. NR assay has been recognised to be more cost efficient and more sensitive, with less interference compared with other methods using tetrazolium salts (Repetto et al., 2008).

The NR stock solution was made from 40 mg neutral red dye dissolved in 10 ml DPBS, stored at room temperature and protected from light. NR working solution (40 µg/ml) was prepared one day before treatment, 1:100 diluted from NR stock solution in phenol red-free, serum-free media. For each experiment, each 24-well plate was seeded at $1x10^5$ cells per ml in complete media. After 24 h, cells reached 90% confluence and the medium was removed by an aspirator. Varying concentrations (0, 25, 50, 100 µM) of compounds in phenol red-free and serum-free medium was added to each well in triplicate, followed by an incubation at 37°C for 18 h. Controls included a blank control (medium without flavonoids) and a vehicle control (medium containing 0.4% DMSO which was the highest concentration of DMSO used in this chapter). Subsequently, medium was removed and 1 ml NR-working solution was added to each well, followed by an incubation at 37°C for 2 h. The NR working solution was then removed and the cells were washed gently with DPBS, followed by the addition of 1 ml NR destain solution (50% ethanol, 49% deionized H_20, 1% glacial acid) to each well. The plate was then placed on a plate shaker and shaken rapidly for 15 min. The solution in each well was pipetted up and down to form a homogeneous solution and 200 µl of this solution was added to each well of a 96-well plate in duplicate. The absorbance was measured at 540 nm by a plate reader (Thermo Fisher Scientific, Loughborough, UK), with NR destain

solution without cells as a reference. Viability of treated cells was calculated as percent relative to the blank control (untreated cells).

2.3.5 Optimisation of NOS3 induction

NOS3 was the primary outcome of the present study. Optimisation of NOS3 induction was conducted at the beginning by testing effects of different incubation periods (6 h and 18 h) and different components in the media (glucose and FBS), with resveratrol (Appeldoorn et al., 2009) and PMA (Li et al., 1998) as positive controls. Expressions of two housekeeping genes, GAPDH and ACTB, were compared. The more stably expressed housekeeper gene was selected for all the subsequent experiments.

2.3.6 RNA isolation and real time qPCR

Cell culture medium was removed and cells were washed gently with DPBS. Total RNA was extracted from EA.hy926 cells using a commercially available reagent TRIsure (Bioline, London, UK) according to the manufacturer's instructions. Confluent cells in 12-well plates after treatment were lysed by 600 µl of TRIsure, which protected the integrity of RNA during lysis due to the RNase inhibitory property of TRIsure reagent. Repeated pipetting was performed to facilitate cell lysis and homogenisation, followed by an incubation at room temperature for 5 min. Homogenised samples were then transferred to 1.5 ml tubes and 120 µl of chloroform was added to each tube. Samples were shaken vigorously by vortex for 15 s and then incubated for 3 min at room temperature before they were centrifuged at 12,000 x g for 15 min at 4ºC. The aqueous phase (upper phase) was transferred into new Eppendorf tubes. RNA was precipitated by the addition of 300 µl of ice cold isopropanol per sample. The mixture was incubated for 10 min at room temperature and centrifuged at 12000 x g for 10 min at 4ºC, followed by the removal of the supernatants. The pellet was washed with 800 µl of 75% ethanol. Samples were vortexed and centrifuged at 7500 x g for 5 min at 4ºC. Supernatants were discarded and the pellet was air-dried for 5-10 min. RNA was re-dissolved in 30 µl of DEPC-treated water and stored at -80°C. Total RNA quantity was measured using NanoDrop2000 micro-volume spectrophotometer, with purity assessed by A260/280.

Reverse transcription of RNA to cDNA was conducted using Script ^{TM} cDNA synthesis kit (Bio-Rad, Hertfordshire, UK):1 µg total RNA template was used for cDNA synthesis, with the addition of 4 µl of 5x iScript reaction mix containing oligo (dT) and random hexamer primers, 1 µl of iScript reverse transcriptase pre-blended with RNase inhibitor and nuclease-free water to make a total volume of 20 µl. The mixture was incubated for 5 min at 25ºC, then for 30 min at 42ºC and finally for 10 min at 85ºC. cDNA samples were diluted 1/20 with DEPC-treated water and stored at -20ºC.

Diluted cDNA samples (4 µl) in duplicate were mixed with 16 µl of SYBR™ Green master mix (Bioline, London, UK) containing 5 µl of DEPC-treated water, 10 µl of 2x SensiMix Buffer (Bioline, London, UK), 0.5 µl of forward primer and 0.5 µl of reserve primer. Standard tools were used to design primers (Table 2.1) as previously described (Boesch-Saadatmandi et al., 2012), which were then synthesised by Eurofins Genomics (Ebersberg, Germany). Real time qPCR was conducted using StepOne Real-Time PCR system (ThermoFisher, Warrington, UK). Started with an enzyme activation step for 10 min at 95ºC, reactions underwent 40 cycles of denaturation for 15 s at 95ºC, annealing for 15 s at specific annealing temperature for target genes (usually 57-60ºC), and extension for 15 s at 72ºC. Threshold cycle (Ct) was measured and target gene expression was normalised to ACTB (housekeeping gene), expressed as fold change relative to control (cells without treatment).

Table 2.1. Primers of target genes for RT-qPCR (Sequence (5'-3')).

Gene	Forward primer	Reverse primer
hsACTB	AGAGCTACGAGCTGCCTGAC	AGCACTGTGTTGGCGTACAG
hsGAPDH	CCATCTTCCAGGAGCGAGAT	TGCTGATGATCTTGAGGCTG
hsNOS3	GCAGCCTCACTCCTGTTTTC	GGTCTTCTTCCTGGTGATGC
hsHMOX1	CTTCTTCACCTTCCCCAACA	AGCTCCTGCAACTCCTCAAA
hsGCLC	CAATGGGAAGGAAGGTGTGT	GCGATAAACTCCCTCATCCA
hsEDN1	GATGCCAATGTGCTAGCCAA	GCTGTTTCTCATGGTCTCCG

2.3.7 Protein preparation and quantification

EA.hy926 cells were seeded in large petri dishes at $1.5x10^5$ cells/ml and cultured to 90% confluence. After media removal, cells were incubated for 24 h in fresh complete media with hesperetin, naringenin, nobiletin, tangeretin at 50 µM, and 10 nM PMA and 50 µM

apigenin were used as positive controls. After media removal and DPBS wash, 200 µl of RIPA buffer (150 mM sodium chloride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 50 mM Tris-HCl (pH 8.0), 1/100 proteinase inhibitor cocktail) was added to lyse cells. Cell lysates were transferred to Eppendorf tubes and incubated on ice for 30 min, followed by centrifugation at 12,000 x g at 4ºC for 30 min. Supernatants in aliquots were stored at -80ºC for further analysis.

Protein concentrations of cell lysates were quantified using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Loughborough, UK). The principle of BCA method is based on proteins' capacity to convert Cu^{+2} to Cu^{+1} in an alkaline medium, followed by the highly selective colorimetric detection of the complex formed by Cu^{+1} and bicinchoninic acid. Standards of BSA protein (0-2000 µg/ml) were prepared. Working reagent was freshly made from a mixture of BCA reagent A and B (A: B = 50:1) immediately prior to use.

Measurements were conducted in triplicate: 10 µl of standards and samples were pipetted to each well, followed by the addition of 200 µl of working reagent per well. The mixture was incubated for 30 min at 37°C and the absorbance was measured at 570 nm using a plate reader (Thermo Fisher Scientific, Loughborough, UK). Influence of lysis buffer was tested by diluting the lysis buffer with PBS (1/1000, 1/100, 1/10 and undiluted), followed by protein quantification as mentioned.

2.3.8 Western blot

Protein samples (20 µg) were mixed with 5xLaemmli buffer and denatured at 95°C for 5 min. Subsequently, protein samples were separated on a 10% SDS–polyacrylamide gel (Bio-Rad Laboratories, Hamel Hempstead, UK) in 1xTBS buffer (1/10 diluted from 10x TBS: 60.6 g Tris and 87.6 g sodium chloride in 1 L ultrapure water, pH adjusted by HCl to 7.6) and transferred onto PVDF membranes (Bio-Rad Laboratories, Hamel Hempstead, UK), which were blocked in 5% non-fat milk powder in TBS-Tween-20 buffer (100 ml of 10x TBS and 500 µl of Tween-20 filled with ultrapure water to 1 L). PVDF membranes were then incubated with corresponding primary antibodies (eNOS and β-

actin in 1:1000 dilution) for 2 h, followed by three washes with TBST buffer. Membranes were incubated with corresponding anti-rabbit second antibodies and precision prot streptactin-HRP (both in 1:10000 dilution) for 1 h. Target protein bands were visualised with Pierce enhanced chemiluminescence (ECL) Western Blotting Substrates (Thermo Fisher Scientific, Loughborough, UK) and exposed by ChemiDoc system (Bio-Rad, Hertfordshire, UK). Quantifications were performed with densitometric analysis by Image Lab software. Protein bands were normalised to β-actin band.

2.3.9 Measurement of nitric oxide

Nitric oxide produced by EA.hy926 cells following 18-h incubation with different compounds was measured by the following two assays.

2.3.9.1 2,3-diaminonaphthalene (DAN) assay

Confluent EA.hy926 cells in 12-well plates were incubated for 18 h with different compounds in DMEM (phenol red free and FBS free). 20 µl of medium was taken from each well for measurement of NO production (total nitrate and nitrite) by a nitrate/nitrite fluorometric assay kit from Cayman (Michigan, USA). The principle of this assay is based on the conversion of nitrate to nitrite by nitrate reductase, followed by the quantification of nitrite using a fluorometric assay. Briefly, the reaction between nitrite and 2,3-diaminonaphthalene (DAN) in an acidic solution forms a highly fluorescent product 1(H)-naphthotriazole, the detection of which is enhanced by NaOH.

Stock solution of nitrate (200 µM) was made from 0.1 ml of reconstituted Nitrate Standard and 0.9 ml of Assay Buffer (AB). Nitrate standards were prepared immediately prior to use. The measurement was conducted in duplicate. 60 µl of nitrate standards were added to 20 µl of assay buffer; 20 µl of samples were added to 60 µl assay buffer. The mixture of 20 µl media and 60 µl assay buffer was used as control. 10 µl of the Enzyme Cofactor Mixture and 10 µl of the Nitrate Reductase Mixture were added to each well. The plate was then covered with film and incubated for 1 h at room temperature. Thereafter 10 µl of DAN Reagent was added to each well and the plate was incubated for 10 min followed by the addition of 20 µl of NaOH to each well. Fluorometric

measurement was conducted using an excitation wavelength of 355 nm and an emission wavelength of 410 nm with a plate reader (Tecan SPARK 10M, Männedorf, Switzerland).

2.3.9.2 4,5-diaminofluorescein (DAF-2) assay

The principle of this assay is based on the reaction of low fluorescence 4,5-diaminofluorescein (DAF-2) with NO in the presence of dioxygen to produce highly fluorescent DAF-2T (Kojima et al., 1998). The protocol was modified from previous studies (Elíes et al., 2011; Räthel et al., 2003). EA.hy926 cells were seeded in 96-well plates and grown to confluence, followed by 18-hour incubation with different compounds in DMEM (phenol red free and FBS free). Cells were washed with DPBS, following the removal of cell culture media. Cells were then incubated with L-arginine (100 μM) in DPBS for 10 min. DAF-2 (0.1 μM) was added and cells were incubated in the dark for 5 min thereafter. Then fluorescence was measured at room temperature with excitation wavelength at 485 nm and emission wavelength at 520 nm with a plate reader (Tecan SPARK 10M, Männedorf, Switzerland). Auto-fluorescence of DAF-2 (measured in wells without cells) was subtracted from the total fluorescence in all the wells. Measurements were conducted in duplicate.

2.3.10 Statistical analysis

Results from 3 independent passages were expressed as mean ± standard deviation. Statistical analysis was conducted using Statistical Package for the Social Sciences (SPSS, version 24, IBM Corporation, USA). Treatment effects were analysed by one-way analysis of variance (ANOVA) followed by post-hoc test of Dunnett's (comparing multiple treatments to a single control). $P < 0.05$ was considered as statistically significant.

2.4 Results

2.4.1 Effect on cell viability

EA.hy926 cells were incubated with increasing concentrations of flavonoids (0, 25, 50, 100 μM) or 0.4% of DMSO (the highest concentration used in serial dilutions) for 18 h. Cell viability or compound toxicity is presented in Figure 2.1. Resveratrol was used as a

positive control compound in the optimisation of NOS3 induction and was found to be toxic to cells at 100 µM. Similarly, cell viability was significantly reduced after the incubation with nobiletin and tangeretin at 50 and 100 µM. No significant difference was observed between DMSO treated group (98.78±3.13%) and untreated group (100±2.1%) (p=0.71), therefore DMSO used in experiments at 0.4% or lower, was not considered to have a negative effect on cell viability. Likewise, positive control PMA at 10 nM and negative control L-NAME at 20 nM were not toxic to EA.hy926 cells (87±5.3%, p=0.81 and 91± 2.4%, p=0.68, respectively).

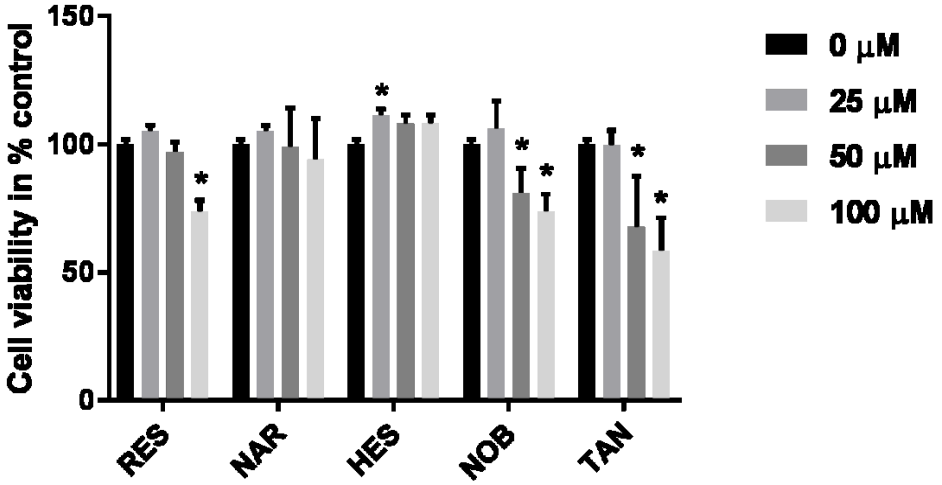

Figure 2.1. Effect of resveratrol, naringenin, hesperetin, nobiletin and tangeretin on cell viability of EA.hy926 cells. Cell viability was determined using neutral red uptake assay after incubation with different flavonoids (0, 25, 50 ,100 µM) for 18 h (RES: resveratrol, NAR: naringenin, HES: hesperetin, NOB: nobiletin, TAN: tangeretin). Data of three independent experiments are presented as mean ± SD. *indicates significant differences between treatment group and untreated control group (p<0.05).

2.4.2 Optimisation of NOS3 induction

Based on the more consistent mRNA levels of ACTB throughout the experiments compared to GAPDH, ACTB was used as the housekeeping gene. Hence, mRNA expression of target genes was normalised to corresponding ACTB mRNA levels in all the following experiments.

PMA was used as a positive control for NOS3 mRNA induction. The effect of different concentrations of PMA (0-1000 nM) on NOS3 mRNA expression was determined (Figure 2.2). NOS3 mRNA levels were augmented by PMA in a concentration-dependent manner from 0 to 10 nM, followed by a decrease at 100 and 1000 nM. Therefore, PMA at 10 nM was used as a positive control in subsequent experiments.

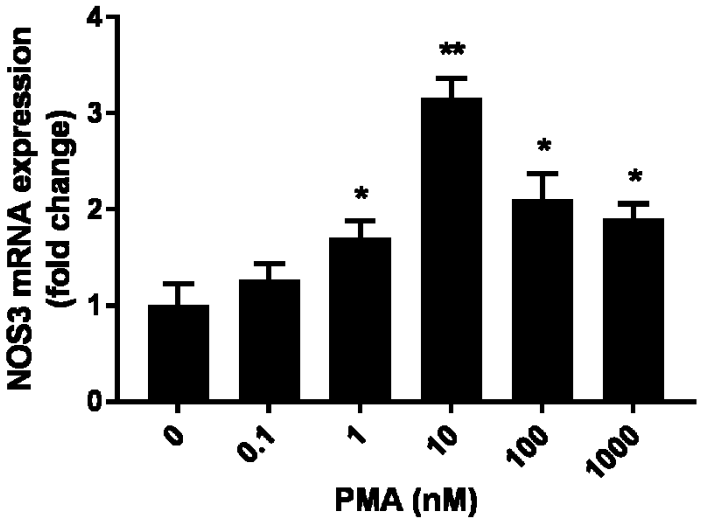

Figure 2.2. Effect of increasing concentrations of PMA on NOS3 mRNA levels. NOS3 mRNA expression after 18 h incubation with PMA at 0-1000 nM, normalised to ACTB mRNA expression. Data of three independent experiments are presented as mean ± SD. *indicates significant differences between treatment group and untreated control group at p<0.05; **indicates significant difference at p<0.01.

Incubation of EA.hy926 cells with PMA (positive control, 10 nM) for 6 and 18 h induced NOS3 mRNA levels similarly (2.8-fold vs. 3.2-fold) (Figure 2.3), however, resveratrol induced NOS3 much stronger following 18 h. Hence, an incubation period of 18 h was conducted for subsequent experiments.

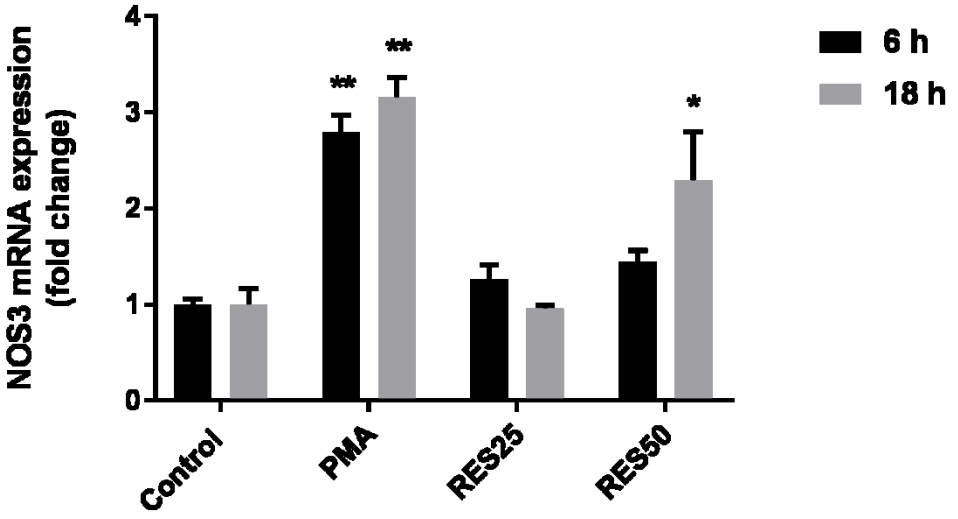

Figure 2.3. Effect of incubation periods on NOS3 mRNA levels. NOS3 mRNA levels after 6 h and 18 h incubation with PMA at 10 nM and resveratrol (RES) at 25, 50 µM, normalised to ACTB mRNA expression. Data of three independent experiments are presented as mean ± SD. *indicates significant differences between treatment group and untreated control group at $p<0.05$; **indicates significant difference at $p<0.01$.

Baseline NOS3 mRNA levels were not affected by the high glucose content in the media whereas fetal bovine serum (FBS) supplementation resulted in a significant upregulation of NOS3 mRNA by around 1.6-fold (Figure 2.4). This increase might be due to oestrogen present in the serum that contributed to NOS3 induction. To minimise confounding effects and optimise NOS3 induction, 18 h incubation with flavonoids in low glucose media with 5% FBS was used for subsequent experiments.

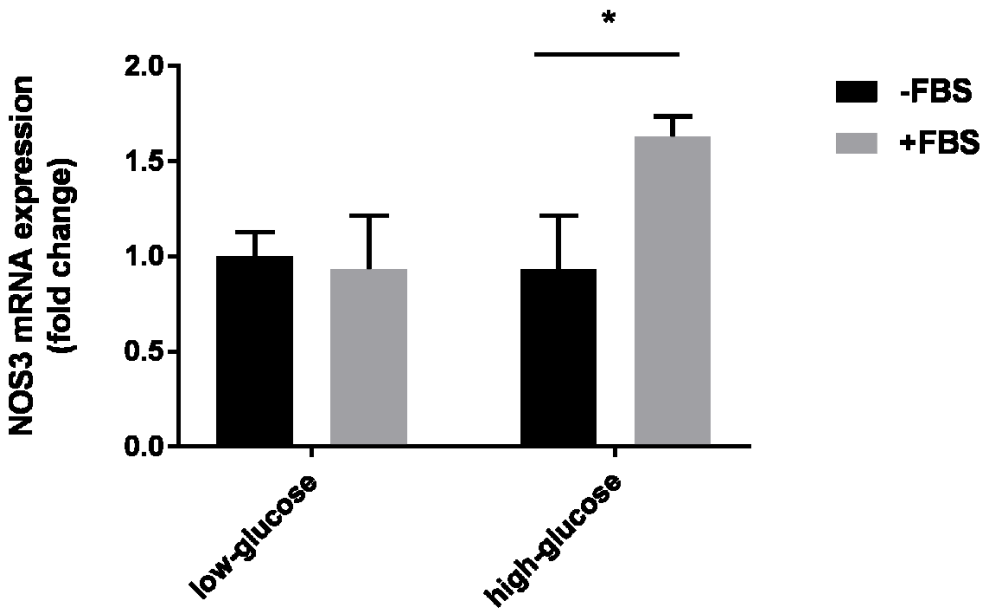

Figure 2.4. Effect of fetal bovine serum (FBS) and glucose on NOS3 mRNA levels. NOS3 mRNA expression following 18 h incubation in low-glucose (5.5 mM) or high-glucose (25 mM) media, with or without addition of fetal bovine serum (FBS 10%), normalised to ACTB mRNA expression. Data of three independent experiments are presented as mean ± SD. *indicates significant differences between high glucose groups (with and without FBS).

2.4.3 Effect on NOS3 mRNA expression

Effect of nobiletin, tangeretin, naringenin and hesperetin on NOS3 mRNA expression was investigated at increasing concentrations following incubation for 18 h. A dose-dependent increase was observed in the upregulation of NOS3 by nobiletin (Figure 2.5A) from 1 µM to 25 µM and followed by a plateau of NOS3 mRNA induction by nobiletin from 25 µM to 50 µM. Nobiletin was found to be more potent in inducing NOS3 mRNA expression at 25 µM, compared to resveratrol which only resulted in a significant induction of NOS3 at 50 µM (Figure 2.3). Similarly, a dose-dependent increase in NOS3 mRNA expression was also induced by tangeretin at lower concentrations (from 1 to 25 µM), with a significant upregulation at 25 µM ($p<0.05$), as shown in Figure 2.5B. A reduction in NOS3 mRNA was observed at 50 µM, probably due to toxicity of tangeretin to cells at this concentration.

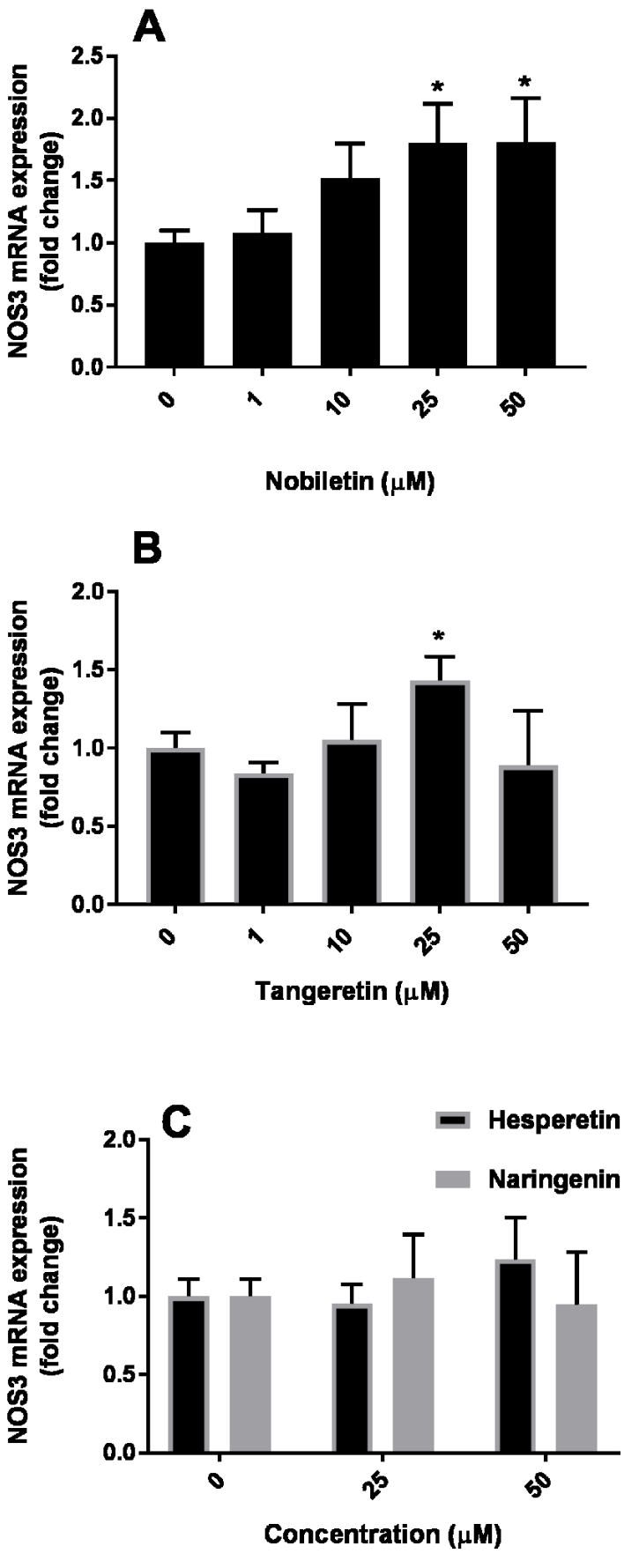
A
NOS3 mRNA expression (fold change)
2.5
2.0
1.5
1.0
0.5
0.0
*
*
0
1
10
25
50
Nobiletin (μM)
B
NOS3 mRNA expression (fold change)
2.0
1.5
1.0
0.5
0.0
*
0
1
10
25
50
Tangeretin (μM)
C
Hesperetin
Naringenin
NOS3 mRNA expression (fold change)
2.0
1.5
1.0
0.5
0.0
0
25
50
Concentration (μM)

Figure 2.5. Effect of nobiletin (A), tangeretin (B), hesperetin (C) and naringenin (C) on NOS3 mRNA levels. NOS3 mRNA levels after incubation with nobiletin, tangeretin, hesperetin and naringenin at 0-50 µM for 18 h, normalised to ACTB mRNA expression. Data of three independent experiments are presented as mean ± SD. *indicates significant differences between treatment group and untreated control group at p<0.05; **indicates significant difference at p<0.01.

As shown in Figure 2.5C, incubation with hesperetin and naringenin at 0-50 µM did not result in significant changes in NOS3 mRNA levels. A 1.24 fold increase in NOS3 mRNA expression was only observed following incubation with hesperetin at 50 µM (p=0.08).

2.4.4 Effect of nobiletin on ET-1 mRNA expression

As shown in section 2.4.3, nobiletin was the most potent compound in NOS3 mRNA upregulation among the flavanones investigated. Hence, the effect of nobiletin on ET-1 mRNA levels was also investigated. As shown in Figure 2.6, nobiletin decreased mRNA expression of ET-1 in a concentration-dependent fashion, with a 35% reduction at 25 µM (p<0.05) and a 45% reduction at 50 µM (p<0.05).

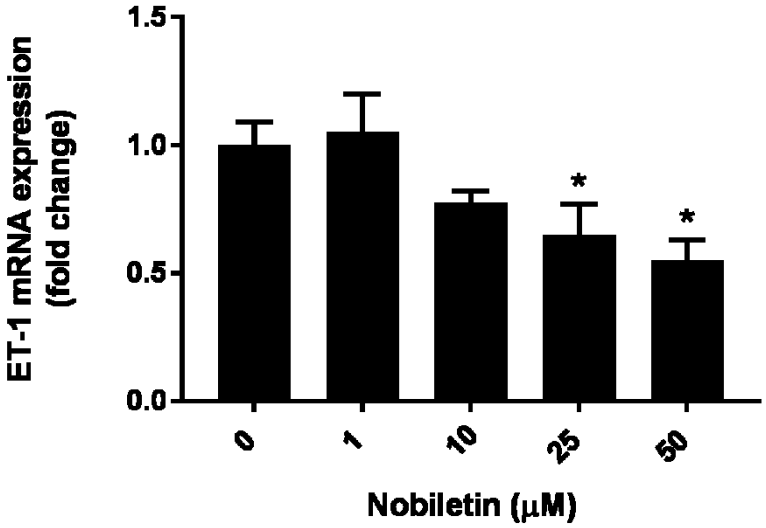

Figure 2.6. Effect of nobiletin on ET-1 mRNA levels. ET-1 mRNA expression after 18 h incubation with nobiletin at 0-50 µM, normalised to ACTB mRNA expression. Data of three independent experiments are presented as mean ± SD. *indicates significant differences between treatment group and untreated control group at p<0.05.

2.4.5 Effect on eNOS protein expression

Nobiletin, tangeretin, naringenin, hesperetin and apigenin at 25 μM were investigated for their effect on eNOS protein following incubation for 24 h, with PMA as a positive control. Housekeeping protein β-actin was used as an internal control. As illustrated in Figure 2.7, eNOS protein was upregulated by nobiletin by 1.23 fold whereas tangeretin significantly upregulated eNOS protein expression by 1.67 fold under the investigated conditions. No significant changes in eNOS protein were observed by other flavonoids.

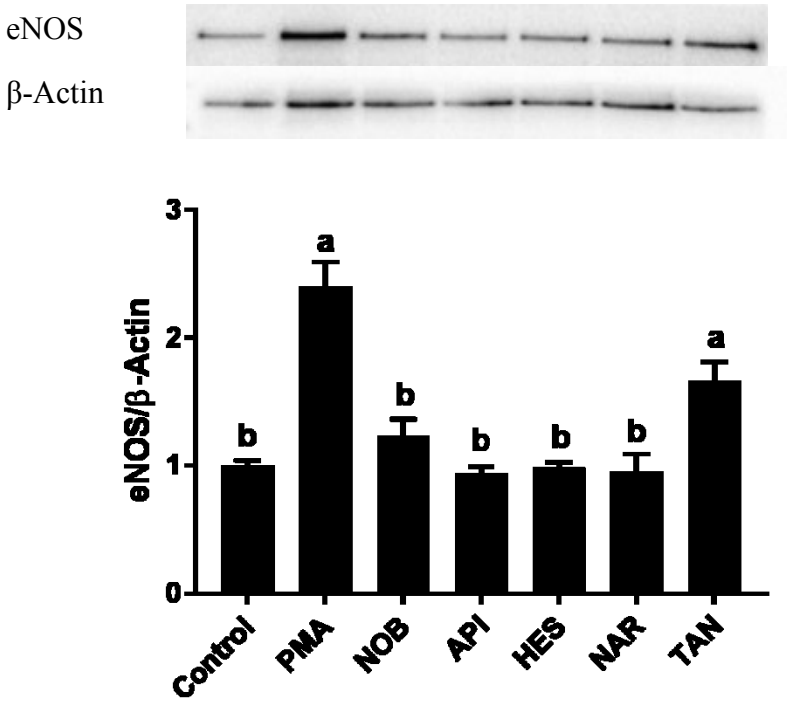

Figure 2.7. Effect of PMA, nobiletin, apigenin, hesperetin, naringenin and tangeretin on eNOS protein levels. eNOS protein expression after 24 h incubation with nobiletin (NOB), tangeretin (TAN), hesperetin (HES) and naringenin (NAR), apigenin (API) at 25 μM, with PMA (10 nM) used as a positive control. Data were normalised to β-actin protein expression. Data of three independent experiments are presented as mean ± SD. Different letters represent significant difference ($p<0.05$).

2.4.6 Effect on nitric oxide production

2.4.6.1 DAN assay

As nobiletin and tangeretin markedly upregulated NOS3 mRNA levels (section 2.4.3) and eNOS protein expression (section 2.4.5), their effect on NO production from EA.hy926 cells was further investigated. NO release from EA.hy926 was measured as the sum of nitrite and nitrate, which is commonly used for NO quantification. Nitrate standard curve for quantification of total nitrate following the conversion of nitrite to nitrate is shown in Figure 2.8.

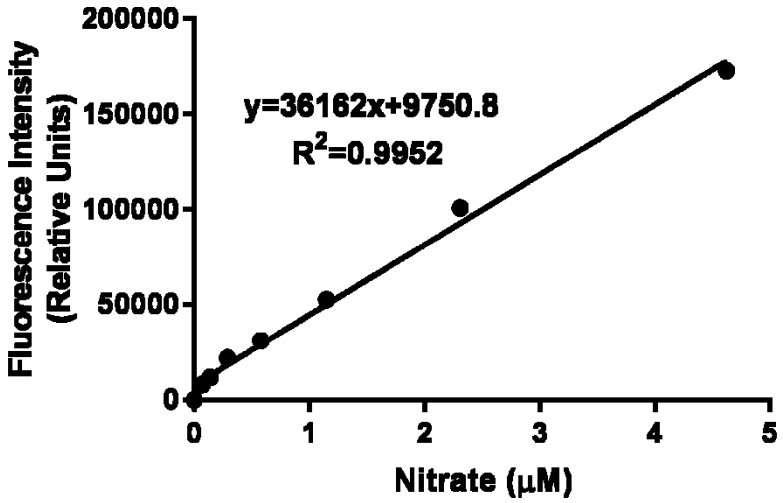

Figure 2.8. Nitrate standard curve. A serial dilutions of nitrate standards were freshly prepared and converted to nitrite utilizing nitrate reductase. Following the addition of DAN and NaOH, samples were measured at an excitation wavelength of 355 nm and an emission wavelength of 410 nm.

PMA was used as a positive control for NO production from EA.hy926 cells. Effect of increasing concentrations of PMA (0-1000 nM) on total nitrate and nitrite was determined (Figure 2.9). In contrast with NOS3 mRNA induction data, PMA at lower concentrations (0.1-100 nM) upregulated NO production similarly (by 1.32-1.46 fold) but only at 1000 nM augmented total nitrate and nitrite significantly.

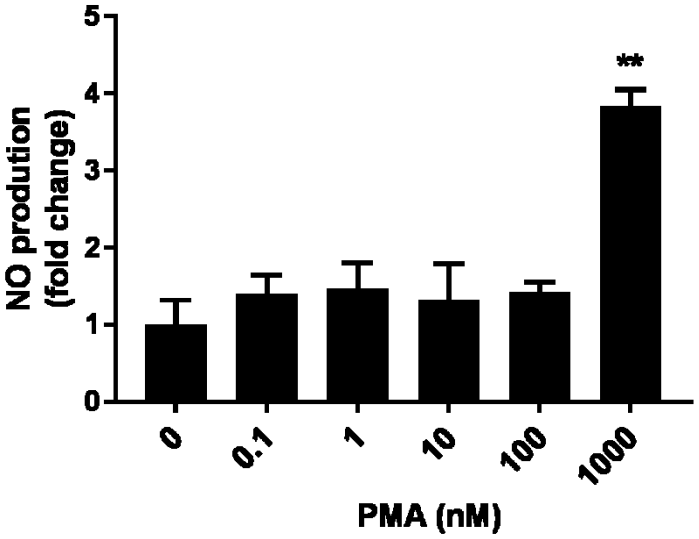

Figure 2.9. Effect of PMA on NO production from EA.hy926 cells. NO was measured as total nitrate and nitrite after 18 h incubation with PMA at increasing concentrations (0-1000 nM). Data were expressed as fold change of control. Measurement was conducted in duplicate and presented as mean ± SD. **indicates significant differences between treatment group and untreated control group at $p<0.01$.

As shown in Figure 2.10, no significant changes were observed in total nitrate and nitrite following incubation with L-NAME and PMA ($p=0.62$ and $p=0.36$, respectively). Similarly, total nitrate and nitrite levels did not change following incubation with nobiletin and tangeretin at 1-25 μM ($P>0.05$).

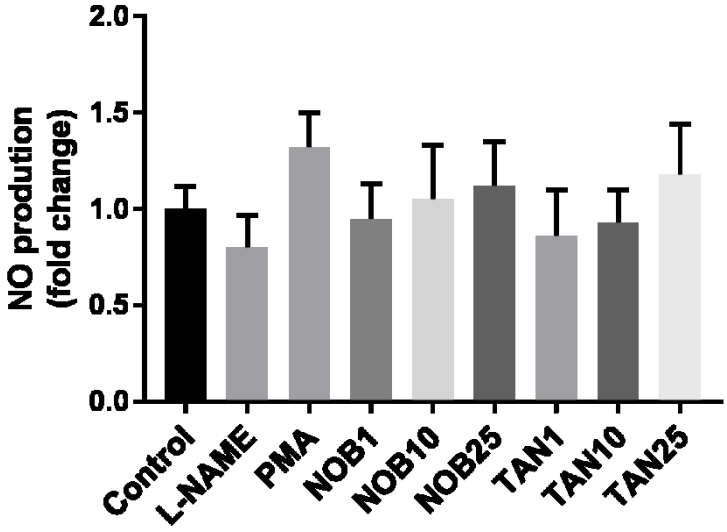

Figure 2.10. Effect of nobiletin and tangeretin on NO production from EA.hy926 cells. NO was measured as total nitrate and nitrite after 18 h incubation with nobiletin (NOB) and tangeretin (TAN) at 1-25 μM, with L-NAME (20 nM) and PMA (10 nM) as negative and positive controls. Data were expressed as fold change of control. Data of three independent experiments are presented as mean ± SD.

2.4.6.2 DAF-2 assay

As shown in Figure 2.11, NO produced by EA.hy926 cells detected by DAF-2 was significantly augmented by nobiletin in a concentration-dependent fashion, from 1.6 fold increase at 10 μM to 7 fold increase at 50 μM. Resveratrol was used as a positive control for NO induction with a 1.6 fold upregulation at 25 μM whereas nobiletin at 25 μM resulted in a 4.98 fold increase in NO release. Therefore, nobiletin appeared to be more potent in inducting NO than resveratrol (both at 25 μM).

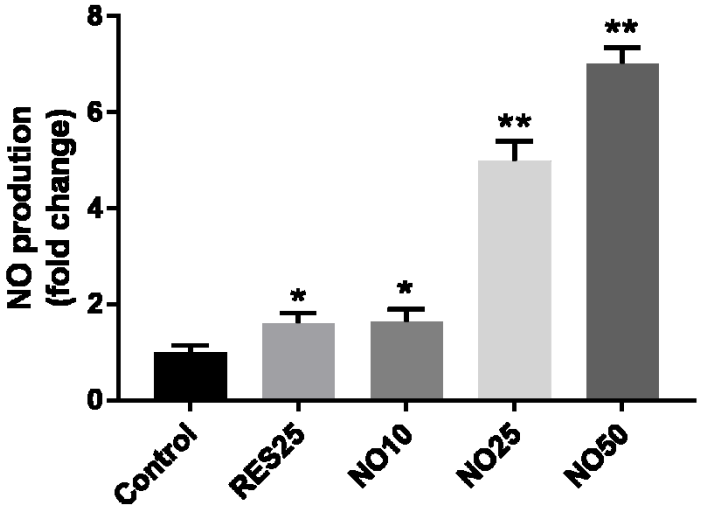

Figure 2.11. DAF-2 detected NO release from EA.hy926 cells after 18 h incubation with nobiletin (NOB) at 10-50 µM, with resveratrol (RES) (25 µM) as a positive control. Data were expressed as fold change of control. Data of three independent experiments are presented as mean ± SD.

2.4.7 Effect on antioxidant status related genes

Citrus flavonoids were also investigated for their effect on HMOX1 and GCLC mRNA expression. In contrast to tangeretin, hesperetin and naringenin, nobiletin significantly increased HMOX1 and GCLC mRNA expression at 25 and 50 µM (Figure 2.12 and 2.13).

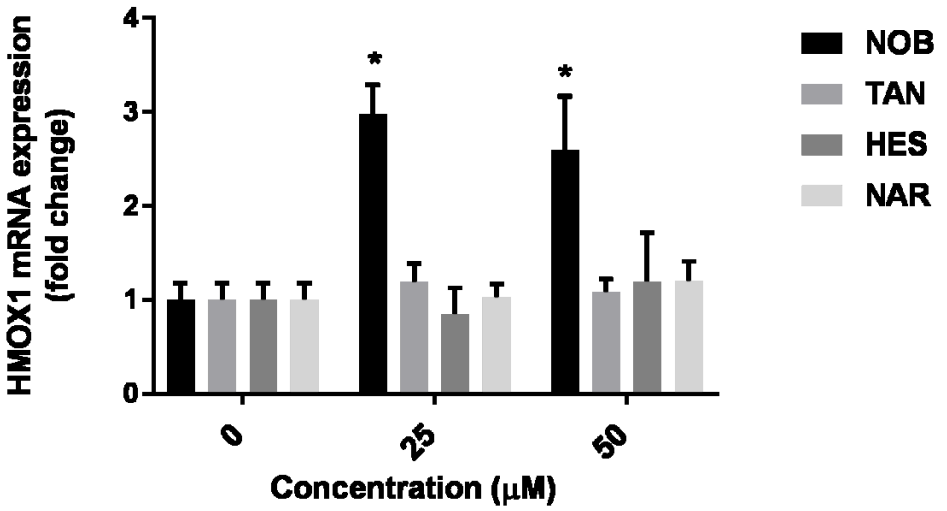

Figure 2.12. Effect of nobiletin, tangeretin, hesperetin, naringenin on HOMX1 mRNA levels after 18 h incubation. HMOX1 mRNA expression after 18 h incubation with nobiletin (NOB), tangeretin (TAN), hesperetin (HES) and naringenin (NAR) at 0-50 μM, normalised to ACTB mRNA expression. Data of three independent experiments are presented as mean ± SD. *indicates significant differences between treatment group and untreated control group at p<0.05.

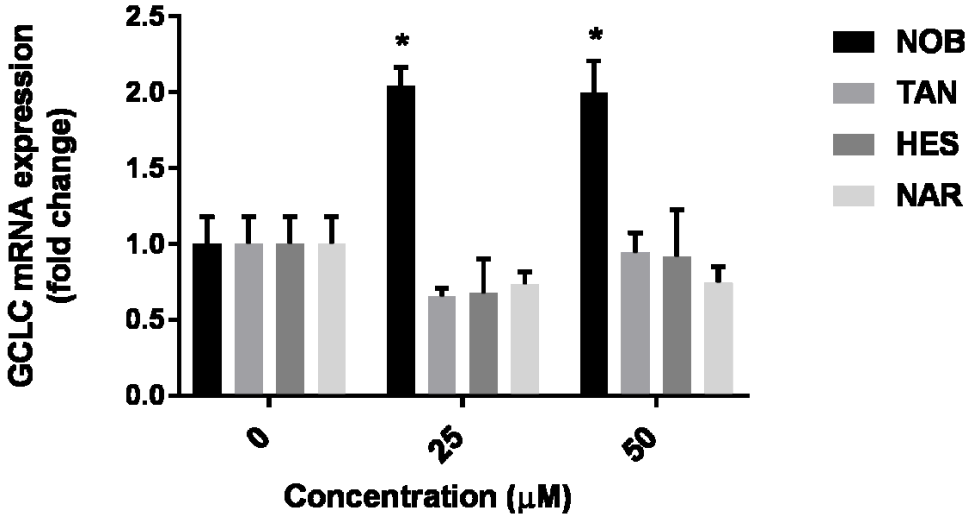

Figure 2.13. Effect of nobiletin, tangeretin, hesperetin, naringenin on GCLC mRNA levels after 18 h incubation. GCLC mRNA expression after 18 h incubation with nobiletin (NOB), tangeretin (TAN), hesperetin (HES) and naringenin (NAR) at 0-50 μM, normalised to ACTB mRNA expression. Data of three independent experiments are presented as mean ± SD. *indicates significant differences between treatment group and untreated control group at p<0.05.

2.5 Discussion

Higher habitual intake of flavanones has been associated with a lower risk of ischemic stroke (Cassidy et al., 2016) and coronary heart disease (Mink et al., 2007) evidenced by epidemiological studies. Some RCTs have demonstrated that the consumption of citrus juice or extracts leads to improved endothelial function (Rendeiro et al., 2016; Buscemi et al., 2012; Morand et al., 2011), reduced blood pressure (Morand et al., 2011; Reshef et al., 2005), elevated antioxidant status (Constans et al., 2015) and reduction in vascular and systemic inflammatory markers (Buscemi et al., 2012; Rizza et al., 2011). In contrast, other RCTs have indicated the consumption of citrus products do not alter endothelial function (Constans et al., 2015; Habauzit et al., 2015), blood pressure (Habauzit et al., 2015; Rizza et al., 2011), and inflammatory markers (Constans et al., 2015; Habauzit et al., 2015). Hence, how citrus flavonoids modulate these markers is not conclusive and the underlying mechanisms have yet to be elucidated.

Given the key role of NO in maintaining normal endothelial function, inhibiting inflammation and platelet aggregation, how citrus flavonoids regulate its synthesis therefore is crucial to cardiovascular health. However, only hesperetin and naringenin have been tested for their effect on NO production at supraphysiological concentrations. In the present work, the potential of selected citrus flavonoids on eNOS expression were explored at physiological concentrations. In addition, redox status plays a key role in cell metabolism, apoptosis and proliferation, contributing to the pathophysiology of a number of chronic diseases, including CVD and cancer (Matés et al., 2008). Furthermore, redox status is also crucial to eNOS coupling (Landmesser et al., 2004), thereby determining whether NO or superoxide is produced by eNOS. Therefore, in the present work the effect of selected citrus flavonoids on the expression of antioxidant status related genes was investigated in EA.hy926 cells, which is one of the most widely used and characterised endothelial cell lines, with many endothelium-specific phenotypes (Unger et al., 2002). In the present study, effects of citrus flavonoids on transcription of genes related to vasomotor, antioxidant and cytoprotective function were investigated. Induction of eNOS protein expression by citrus flavonoids and NO production by nobiletin and tangeretin were also evaluated.

2.5.1 Modulation of endothelial cell function

The effect of naringenin and hesperetin on eNOS expression has been more investigated than other citrus flavonoids. Treatment with hesperetin (12.5-100 μM) for 24 h increased NO production (measured as nitrite) in human umbilical vein endothelial cells (HUVECs) in a concentration-dependent manner, whereas no significant changes were observed after treatment with naringenin under the same conditions (Liu et al., 2008). Similarly, the present study also demonstrated that hesperetin appeared to be more potent in increasing NOS3 mRNA levels in EA.hy926 cells than naringenin (at 50 μM for 18 h). In the previous study by Liu et al. (2008), treatment with hesperetin (50 μM, 25 h) also upregulated eNOS protein expression in HUVECs whereas naringenin showed no effect. In contrast, incubation with hesperetin and naringenin at 25 μM for 18 h on eNOS protein expression resulted in no significant changes in eNOS protein expression in EA.hy926 cells in the present study, suggesting cell type- and/or time point-associated differences. Hesperetin and its plasma metabolite 7-O-β-D-glucuronide exert vasoprotective function through the inhibition of superoxide anion production via NADPH oxidase activity in HUVECs (Takumi et al., 2012).

Nobiletin and tangeretin, polymethoxyflavones (PMFs) from citrus fruit peels, have been reported to exert anti-inflammatory and anti-tumour effects (Ho and Kuo, 2014; Li et al., 2009; Yoshimizu et al., 2004). Their effect on eNOS transcription and protein expression in endothelial cells has yet to be elucidated. In the present study, nobiletin promoted NO production (detected using DAF-2) in EA.hy926 cells via augmentation of eNOS expression at mRNA level and protein level. Similarly, eNOS transcription and protein expression were also upregulated by tangeretin. Nobiletin and tangeretin were found to be more potent in inducing eNOS mRNA expression than hesperetin and naringenin, probably due to the presence of double bond at 2-3 postion at the C ring, which has been associated with an increase in eNOS mRNA expression (Martínez-Fernández et al., 2015). Arguably, this might be due to the reduction potential of double bond.

It should be noted that changes in eNOS mRNA and protein levels might not necessarily lead to changes in NO. Specifically, phosphorylation and activation of eNOS, Ca^{2+} concentrations, redox status in endothelial cells, eNOS coupling as dimers are crucial to

NO production (Fleming and Busse, 2003). Cellular NO is produced at low levels in endothelial cells (picomoles to nanomoles) (Moncada, 1992). The short half-life of NO makes its measurement very difficult. In the present study, NO production was measured using two different assays, DAN and DAF-2. NO production was measured as total nitrate and nitrite using DAN assay. However, total nitrate and nitrite may not be a good reflection of NO produced by the endothelial cells. There is a growing body of evidence that plasma nitrite reflects NO-dependent endothelial function/dysfunction (Rendeiro et al., 2016; Kleinbongard et al., 2006). In contrast, the detection of NO was specific using DAF-2 assay, given that NO reacted with DAF-2 in the presence of dioxygen, producing highly fluorescent DAF-2T. In addition, it appeared that DAF-2 assay was more sensitive whereas the interference from the media in NO measurement was large in DAN assay: only PMA at 1000 nM induced a significant increase in NO detected by DAN. Taken together, DAF-2 assay may be more suitable for detection of low levels of NO released from EA.hy926 cells and can reflect changes in NO production more accurately. Resveratrol (0.1 -100 μM) is documented to be potent in upregulation of NOS3 mRNA expression in HUVECs (Nicholson et al., 2008) and in EA.hy926 cells (Appeldoorn et al., 2009). In the present study, nobiletin was found to be more potent in augmenting NOS3 mRNA expression than resveratrol at 25 μM in EA.hy926 cells.

In the present study, EA.hy926 cells were cultured in low-glucose media. Exposure to high glucose (22.2 mM) for 5 days resulted in increased levels of NOS3 gene expression, protein expression, and NO production (measured as nitrite) in human aortic endothelial cells (Cosentino et al., 1997). It should be noted that in this previous study O_2^- production was also markedly increased (by more than threefold) after prolonged exposure to high glucose, whereas the increase in nitrite was only 40%. Despite the increase in nitrite, this imbalance between nitrite and O_2^- is associated with endothelial dysfunction.

Pretreatment with hesperidin (1-100 μM) for 30 min inhibited ET-1 release from strain-stimulated HUVEC (Chiou et al., 2008). In a study by Martínez-Fernández et al. (2015), hesperetin and naringenin at 1 μM significantly inhibited ET-1 mRNA expression in H_2O_2-treated HUVECs whereas no effect was observed on NOS3 mRNA expression. The present study demonstrated that nobiletin (1-50 μM) concentration-dependently

upregulated NOS3 expression and also suppressed ET-1 mRNA expression, which may contribute to the improvement of endothelial function.

2.5.2 Modulation of cytoprotection

Hesperidin, the β-7-rutinoside of hesperetin, concentration-dependently (20-80 μM) augmented HO-1 mRNA expression, protecting Hepatic L02 Cells from hydrogen peroxide-stimulated cell damage (Chen et al., 2010). The induction of HO-1 was also enhanced by hesperetin from 25 μM to 50 μM (by 19.5%) in the present study. Naringenin was found to upregulate HO-1 mRNA and protein levels in rat vascular smooth muscle cells (VSMCs) in a concentration-dependent fashion (25-100 μM), thereby inhibiting VSMCs proliferation and migration (Chen et al., 2012). In agreement with previous studies, the induction of HO-1 was also upregulated by naringenin from 25 μM to 50 μM (by 20%) in the present study.

Induction of HO-1 in endothelial cells by nobiletin and tangeretin has not been previously investigated. In the present study, HO-1 transcription was increased similarly (by 18.7%) by tangeretin at 25 μM followed by a decrease in HO-1 to the baseline level treated with tangeretin at 50 μM. In contrast, HO-1 was significantly upregulated by nobiletin at 25 μM (by 2.98 fold) and at 50 μM (by 2.6 fold), making nobiletin the most potent citrus flavonoid in HO-1 induction among the citrus flavonoids investigated in this study.

Expression of GCLC contributes to antioxidant and detoxification function (Dickinson and Forman, 2002). There is, however, a notable paucity of studies investigating effect of citrus flavonoids on GCLC expression. The present study indicates the effect of nobiletin is most pronounced on GCLC induction. The structures of nobiletin and tangeretin are identical except for the presence of a methoxy group at 5' position at B ring in nobiletin, which in part contributed to its induction of HO-1 and GCLC mRNA levels. Other citrus flavonoids which did not show a significant effect in this study might improve cardiovascular health by different mechanisms such as inhibition of ROS-forming enzymes (such as NADPH oxidase, xanthine oxidase and lipoxygenases),

platelet aggregation (Beretz et al., 1982) and leukocyte adhesion, which have been observed by some flavonoids.

2.5.3 Conclusions

Taken together, these findings indicate citrus flavonoids, especially nobiletin, exert vasoprotective effects, at least in part through upregulation of NO evidenced by induction of eNOS transcription and protein levels. Augmentation of mRNA expression of genes associated with antioxidant and cytoprotective function also contributes to these vasoprotective effects by citrus flavonoids. Under the conditions investigated, nobiletin seems to be the most potent compound to induce NOS3 and therefore potentially increase NO levels as well as enhance cell protective enzymes. The mechanisms by which other citrus flavonoids might improve endothelial function warrant futhter investigation.

2.5.4 Limitations and future work

The present study has provided novel insight into how citrus flavonoids modulate vascular function at molecular, transcriptional and protein levels. However, there were some limitations to this work. Flavonoids go through complex metabolism producing sulfate and glucuronide metabolites and gut-derived phenolic compounds (Pereira-Caro et al., 2014; Roowi et al., 2009). Future studies should investigate effects of these compounds at physiological concentrations on the expression of vascular biomarkers in human endothelial cells on basal expression as well as under stressor stimulated conditions.

Acknowledgements:

Miss Yue Wu, a previously master students at University of Leeds, collected the raw data for Figure 2.7.

Chapter 3 Acute effects of orange juice on vascular function

3.1 Abstract

Cardiovascular diseases (CVD) represent one of the leading causes of mortality worldwide. Epidemiological studies have indicated that the consumption of citrus products is inversely associated with the risk of CVD. However, clinical evidence regarding effects of orange juice on vascular function are limited and conflicting. The aims of this chapter were to compare the citrus flavonoid contents in a range of commercially available blond and blood orange juices and to investigate in a preliminary study the acute effects of orange juice consumption on vascular function using plethysmography.

The major citrus flavanones in commercially available blond and blood orange juices were identified and quantified by LCMS. Healthy adult subjects (n=13) were recruited for this study. Measurements were conducted prior to and following flavanone-rich blond orange juice consumption. All subjects were instrumented to record cardiac electrical activity via a 3-lead ECG, peripheral pulse amplitude via infra-red plethysmography and the finger pulse signal recorded via a pulse transducer. Data were recorded during baseline for 10 min, occlusion of forearm blood flow for 5 min and recovery post cuff deflation for 10 min. Cardiac electrical activity, vascular compliance, peripheral blood flow, pulse transit time and pulse wave velocity were calculated from recorded signals.

The present results demonstrate substantial variations in the flavanone contents of commercial orange juices. An increase in vascular compliance was observed during reactive hyperaemia post orange juice consumption, indicating improved vascular function, however this increase was not significant. Other vascular related markers were not acutely altered 2 h following consumption of orange juice under the investigated conditions.

3.1 Introduction

Given that cardiovascular diseases (CVD) are the leading cause of death in both men and women worldwide (World Health Organization, 2017), there is an increasing interest in research on modifiable lifestyle factors like physical activity (Francois and Little, 2017; Harris et al., 2014) and diet (Shivappa et al., 2017; Ros et al., 2014). Growing evidence from epidemiological studies has demonstrated an inverse association between polyphenol-rich foods and the risk of CVD (Steinhaus et al., 2017; Tresserra-Rimbau et al., 2016; Tresserra-Rimbau et al., 2014). In support of this, numerous animal studies have suggested favourable effects of polyphenol-rich foods on vascular function and inhibition of insulin resistance and inflammation (Anhê et al., 2015; Boesch-Saadatmandi et al., 2012). Likewise, *in vitro* cell mechanistic studies have also indicated promising positive effects of polyphenols on genes associated with endothelial function and antioxidant status as detailed in Chapter 2 and also in the literature (Kamiloglu et al., 2017; Boesch-Saadatmandi et al., 2009). In addition, randomised controlled trials (RCT) have been conducted to investigate the impact of polyphenol-rich foods on cardiovascular health (Lockyer et al., 2017; Salden et al., 2016; Egert et al., 2010), however, with conflicting results.

Compared with some flavonoids (for example, chocolate and cocoa, green tea) that have been more extensively studied with convincing favourable effects observed, the effects of citrus products on cardiovascular health are still inconclusive, however, are of significant relevance as citrus products, especially orange juice, are highly consumed worldwide. Furthermore, recent studies have suggested that flavanones, exclusively from citrus products, demonstrate the highest bioavailability among flavonoids (Pereira-Caro et al., 2014; Manach et al., 2005).

Although numerous epidemiological studies have suggested that higher habitual intakes of citrus fruits are associated with a lower risk of CVD in women (Landberg et al., 2011) and men (Cassidy et al., 2016), clinical data regarding the acute effect of orange juice consumption on vascular function have been inconclusive. Specifically, a recent study by Rendeiro et al. (2016) suggests the efficacy of orange juices with flavanone content ranging from 128.88 to 452.8 mg/240 mL in attenuating postprandial impaired

endothelial function, induced by high-fat double meal. In contrast, no acute vascular effects were observed after orange juice consumption despite the presence of circulating flavanone and phenolic metabolites (Schär et al., 2015). Acute effects of orange juice therefore still warrant more investigation.

Vascular function can be evaluated by a variety of approaches such as flow mediated dilation (FMD), reactive hyperemia-peripheral arterial tonometry (RH-PAT), laser Doppler flowmetry, acetylcholine iontophoresis and finger plethysmography. The term plethysmograph is derived from two Greek words "plethysmos" meaning "to increase" and "graph" meaning "write" (Alnaeb et al., 2007). Finger plethysmography provides non-invasive, continuous measurement of pressure via infrared light. As the arterial pulse expands and contracts the microvasculature, changes in pulsatile blood flow from the finger are recorded via an infrared photoelectric sensor. The waveform acquired by this technique represents pulsatile peripheral blood flow and indicates both peripheral and central hemodynamics (Dennis, 2013). The digital volume pulse (DVP) acquired by this technique is a waveform that estimates pulse wave velocity (PWV), which is an indicator for arterial stiffness, predicting CVD risk (Theilade et al., 2013). In addition, vascular compliance calculated from this technique has also been used as an indicator of cardiovascular health (Alsop and Hauton, 2016). As shown to be reliable and reproducible (Gunarathne et al., 2008; Thijssen et al., 2005), finger plethysmography is used in this study to evaluate the effect of orange juice on vascular function.

Furthermore, information on flavanone contents in commercial orange juices, especially the comparison of blond orange juice and blood orange juice, is limited. In addition, Chapter 2 demonstrates favourable effects of flavanones on gene expression relevant to nitric oxide (NO) production and antioxidant status in endothelial cells. The aims of this study therefore were to determine and compare total flavanone contents in selected commercial citrus products, including blond orange juice and blood orange juice, and to select an orange juice for a preliminary study to investigate its acute effect on vascular function (cardiac electrical activity, blood pressure, vascular compliance, peripheral blood flow, pulse transit time and pulse wave velocity).

3.2 Materials and methods

3.2.1 Materials

Flavonoid standards (hesperidin and narirutin) and internal standard genistein were purchased from Extrasynthase (Genay, France). Methanol (LCMS grade), acetonitrile (LCMS grade) and formic acid were purchased from Sigma Aldrich (Poole, UK). Water was purified using a Millipore, Millipore UK Ltd (Merck, UK). Tested orange juices were purchased from Morrisons, Tesco, Waitrose, Leeds, UK. Information on citrus products is shown in Table 3.1. Buxton still mineral water (Buxton, UK), low in nitrate (<0.1 mg/L) was purchased from Tesco, Leeds, UK.

Table 3.1. Analysed commercially available citrus products.

Code	Product name	Product type	Product labelled contents
1	Tropicana Orange Juice With Extra Juicy Bits	Blond orange juice	100% orange juice with juicy bits
2	Tropicana Orange Juice Smooth	Blond orange juice	100% smooth orange juice
3	Morrisons Orange Juice with Bits	Blond orange juice	100% orange juice from concentrate
4	Morrisons Orange Juice Smooth	Blond orange juice	100% orange juice from concentrate
5	Tesco 100% Pure Squeezed Orange Juice With Bits	Blond orange juice	100% orange juice with juicy bits
6	Tesco 100% Pure Orange Juice Smooth	Blond orange juice	100% smooth orange juice
7	Innocent Sicilian Blood Orange Juice	Blood orange juice	100% blood orange juice from Sicilian oranges
8	Waitrose 1 Sicilian blood orange juice	Blood orange juice	100% blood orange juice from Sicilian oranges
9	Tropicana Sanguinello Blood Orange Juice	Blood orange juice	100% blood orange juice

3.2.2 Extraction and quantification of citrus flavonoids

3.2.2.1 Extraction of citrus flavonoids from orange juice

Nine commercially available orange juices (Table 3.2) were analysed to determine citrus flavonoid content. According to the previous work by Chebrolu et al. (2011), optimization of extraction conditions (solvent, juice to solvent ratio, centrifuge speed and temperature, extraction cycles) of flavanones was conducted. The polarity of the solvent has a major effect on extraction: dimethyl sulfoxide (DMSO) > acetonitrile (ACN) > methanol (MeOH) >ethanol (EtOH). However, samples for LCMS analysis cannot be prepared in DMSO. ACN and MeOH were used as extraction solvents. After centrifugation (5 min at 5000 x g, 20°C), there were two phases in the supernatant in the samples extracted by ACN and this was consistent with that in the previous work by Chebrolu et al (2011). Therefore MeOH was selected as the solvent for extraction.

Orange juice (1 ml) in triplicate was mixed with 100% MeOH (2 ml). The mixture was vortexed for 2 min and then centrifuged (5 min at 5000 x g, 20°C). The supernatant was removed into a new tube and 2 ml of MeOH was added to the remaining pellet. The extraction was conducted for three times. Supernatants from three times were combined and vortexed. Internal standard genistein (final concentration of 5 μg/ml) was added to all the samples and standards and then the mixture was filtered (0.2 μM PTFE filter) prior to LCMS analysis.

Table 3.2. Composition of tested orange juice according to the label (/100 ml).

Product code	Energy (kcal)	Carbohydrates (g)	Sugars (g)	Vitamin C (mg)	Fibre (g)
1	42	8.6	8.6	34	0.8
2	43	8.9	8.9	33	0.6
3	42	9.2	9.2	20	0.1
4	42	9.2	9.2	20	0.1
5	44	10.1	8.1	30	0.1
6	45	10	10	30	0.1
7	47	11	9.5	42	N/A
8	47	10.9	9.1	24	0.2
9	52	10.4	10.4	47	0.8

3.2.2.2 LCMS analysis

The method was adapted from a previous method (Sweidan, 2015). LCMS 2020 from Shimadzu was used for detection. Separation was carried out using a C18 Zorbax column (4.6 x 50 mm; internal diameter 1.8 µm, Agilent, Berkshire, UK). A gradient of water-formic acid (solvent A; 100:0.1, v: v) and acetonitrile-formic acid (solvent B; 100:0.1, v: v) was applied with a total flow rate and column temperature of 0.5 ml/min and 30ºC, respectively. The gradient is shown in Table 3.3. Injection volume was 10 µL. The DAD scanned from 190 to 800 nm. In SIM and negative mode, specific detection was carried out at 269, 579, 609 (m/z), for detection of genistein, narirutin and hesperidin, respectively. Blank water samples were run in regular intervals, at the beginning, after the standards, between samples and at the end of the sequence. Standards of narirutin and hesperidin were also analysed for quantification of narirutin and hesperidin, respectively.

Table 3.3. LCMS gradient.

Time (min)	% of mobile phase B
0	5
5	10
20	40
25	95
28	95
29	5
33	5

3.2.3 Subjects of human study

Given that some made-from-concentrate orange juices contain added sugars whereas not-made-from-concentrate orange juice contains naturally occurring sugars in citrus fruits, following determination of flavanone contents in nine orange juices, the blond not-from-concentrate orange juice with highest flavanone content was then selected for a preliminary study to ascertain its acute effects on vascular function. The effects of blood orange juice on vascular function were investigated in Chapter 4.

The study was reviewed by the ethical committee of the University of Leeds Faculty of Mathematics and Physical Sciences and Engineering (Ethics Reference Number: MEEC 14-028), in accordance with ethical principles of the Declaration of Helsinki of 1975 as revised in 2013. Subjects were recruited from the University of Leeds through word of mouth. Interested subjects were informed of all aspects of the study and what it involved. The eligibility was confirmed verbally. Potential subjects were given at least 48 h to consider the participation and to ask any questions.

Female and male adults aged between 20 and 50 years who were generally fit and healthy, free from diabetes, liver, cardiac or kidney disease were recruited for the study. Smokers and anyone taking dietary supplement were excluded. Thirteen subjects provided written informed consent prior to commencement of the study.

3.2.4 Experimental protocol

Given that exercise has been reported to improve vasodilation during reactive hyperaemia in healthy adults (Clarkson et al., 1999), subjects were asked to stay fasted and restrain from exercise for 12 h before measurements in the morning. On the study day, participants arrived at the human study room around 9.00 am. Anthropometric measurements including body mass and height were performed upon arrival.

As 15% of dietary nitrate is from drinking water and also exerts vascular effects (Lidder and Webb, 2013), participants were then asked to drink low-nitrate water (Table 3.4) (4 ml/kg body mass). Meanwhile, the information of the study and the procedures of their involvement were explained to participants. Compliance with overnight fast and length of sleep the night before were ascertained before measurements, given that sleep has a significant influence on blood pressure, heart rate and sympathetic nervous system activity (Gangwisch et al., 2006).

Table 3.4. Characteristics of Buxton still natural mineral water.

	Typical analysis (mg/L)
Calcium	55
Magnesium	19
Potassium	1
Sodium	24
Bicarbonate	248
Chloride	37
Sulphate	13
Nitrate	<0.1
Dry Residue at 180°C	280
pH at Source	7.4

After participants rested for 15 min, systolic blood pressure, diastolic blood pressure and heart rate were measured in triplicate by a blood pressure monitor (Omron BP760, Omron Healthcare UK Ltd., Milton Keynes, UK). Specifically, systolic and diastolic blood pressures were measured in overnight fasted subjects in a quiet, temperature-controlled room (22°C ± 1°C), the subject was in a seated position for at least 15 min to reach a cardiovascular steady state prior to the measurement. The cuff was placed on the left upper-arm at the same level as the heart, with the blue stripe aligned with the middle finger. The subject was instructed not to speak during the measurement, which was conducted three times with 2-minute intervals. The averages of three recordings were calculated.

The set-up of the measurement of peripheral vascular function is illustrated in Figure 3.1. Participants were seated in a comfortable position and instrumented to record cardiac electrical activity via a 3-lead ECG module (Bio-Amp, ADInstruments, Oxford, UK) with adhesive electrodes attached to the skin of the right shoulder, the left forearm and one ankle. A sphygmomanometer cuff was put on the right forearm of the participant. Changes in tissue blood volume, caused by the arterial pulse expanding and contracting the microvasculature, were detected by an infrared (at the wavelength of 950 nm) photoelectric sensor (MLT-1020PPG, ADInstruments, Oxford, UK) attached to the right thumb by a velcro strap. Caution was taken to make the strap firm on the finger without restricting blood flow. The amplitude of the infrared photoelectric sensor is in proportion to blood volume in the capillary bed detected by the sensor. The finger pulse signal was

detected by a pulse transducer (TN1012/ST) using a piezo-electric element that develops a voltage in response to an applied force on the active surface of the transducer. Powerlab (4/35, ADInstruments, Oxford, UK) was used to record ECG and peripheral pulse amplitude. Optimal settings for the channel of ECG recording were as follows: range 2 mV, low pass 1000 Hz (to reduce high frequency noise), mains filter on, sampling rate 1000/s. Optimal settings of peripheral pulse amplitude via infra-red plethysmography were as follows: range 50 mV, AC coupling on, sampling rate 1000/s. Optimal settings of pulse signal recorded at the index finger were as follows: range 500 mV, low pass 1000 Hz (to reduce high frequency noise), sampling rate 1000/s. Cardiac electrical activity and peripheral pulse amplitude were recorded for 10 min followed by occlusion of the blood flow for 5 min via inflating a sphygmomanometer cuff to 50 mm Hg above systolic blood pressure of the participant to occlude arterial inflow. Cardiac electrical activity and peripheral pulse amplitude were continuously recorded during this period until 10 min after the release of the blood pressure cuff. During the experiment, participants were spontaneously breathing.

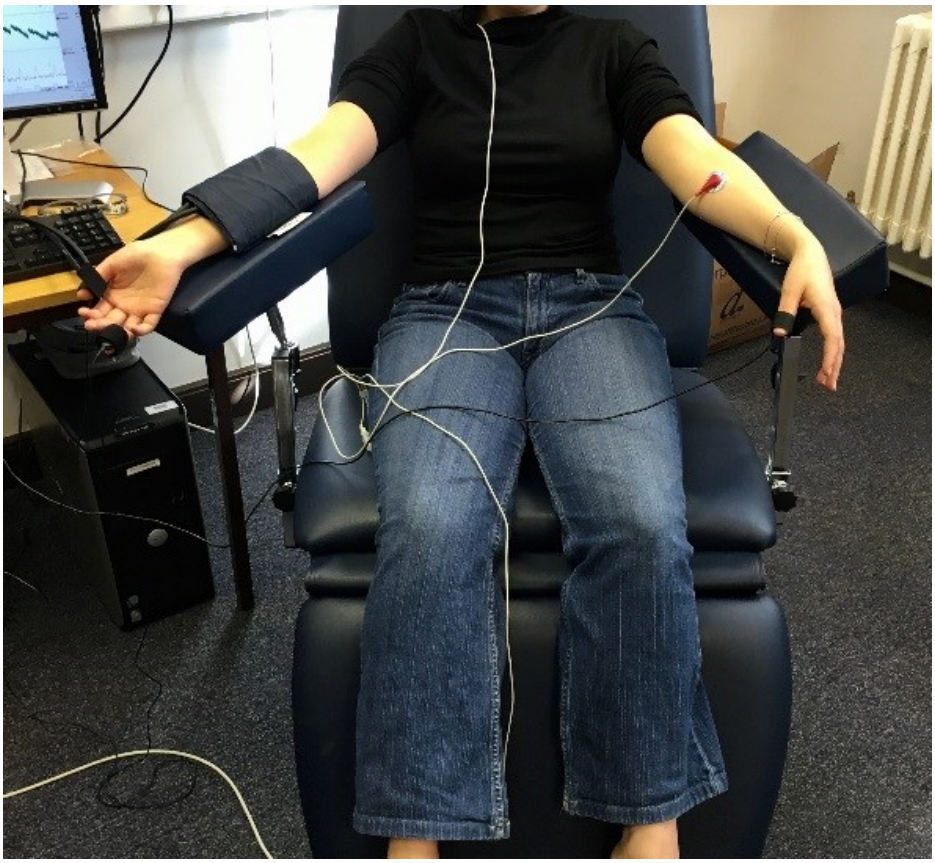

Figure 3.1. Set-up for the measurement of peripheral vascular function: cardiac electrical activity via a 3-lead ECG, peripheral pulse amplitude via infra-red plethysmography attached to the right thumb and the finger pulse signal recorded via a pulse transducer attached to the right fourth finger (the ring finger).

After the initial measurements, participants were asked to drink orange juice (Table 3.5) (4 ml/kg body mass) and refrain from any food for 2 h and then come back to repeat the measurements.

Table 3.5. Characteristics of orange juice used for the preliminary human study.

Typical Values	100ml contains
Energy	186kJ (44kcal)
Fat	<0.1g
Saturates	<0.1g
Carbohydrate	10.1g
Sugars	8.1g
Fibre	<0.1g
Protein	0.8g
Salt	<0.01g
Vitamin C	30.0mg (38% of NRV)

Information from the label of Tesco 100% pure squeezed orange juice with bits.

3.2.5 Data analysis

LabChart software (LabChart 8.0, ADInstruments, Oxford, UK) was used for data recording and analysis. Figure 3.2 is a screenshot of cardiac electrical activity (channel 1), peripheral pulse amplitude (channel 2), the finger pulse signal (channel 3) and transformed heart rate data (channel 4). It should be noted that plethysmography signals (channel 2) are inverted, with a higher signal representing lower light transmission due to increased tissue blood flow during systole.

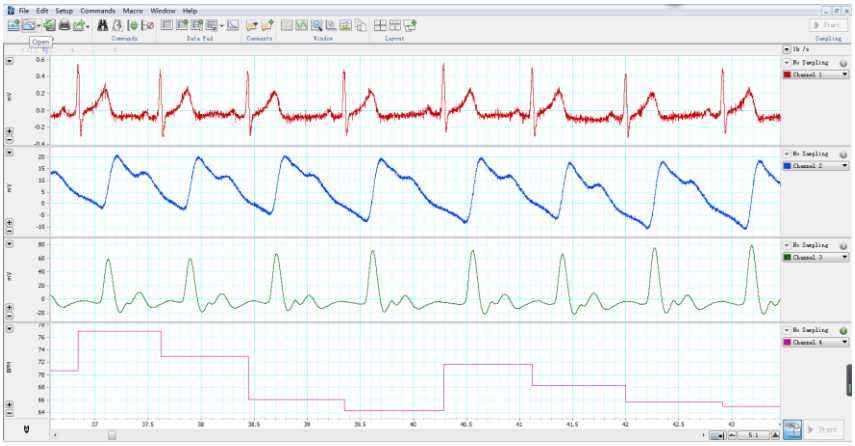

Figure 3.2. LabChart software (LabChart 8.0, ADInstruments) for recording and analysing cardiac electrical activity via a 3-lead ECG (channel 1), peripheral pulse amplitude detected via infra-red plethysmography (channel 2) and the finger pulse signal detected via a pulse transducer (channel 3). Channel 4 was transformed from signals in channel 1 for calculation of heart rate. Signals were shown at a scale of 5:1.

Relative flow (as A in the Figure 3.3) was calculated as the differences in the voltages between the foot and the peak of the pulse pressure waveform measured by an infrared photoelectric sensor on the thumb. As illustrated in Figure 3.3, vascular compliance was calculated as the ratio of the magnitude of the dicrotic notch (B) to total amplitude (A) (A/B *100%) from pulse pressure waveform as an indicator of vascular function (Chowienczyk et al., 1999). Compliance and blood flow was calculated in triplicate every 2 min during baseline, every 10 s during the first minute after cuff deflation, every 15 s during the second minute and every 2 min until 10 min after cuff deflation.

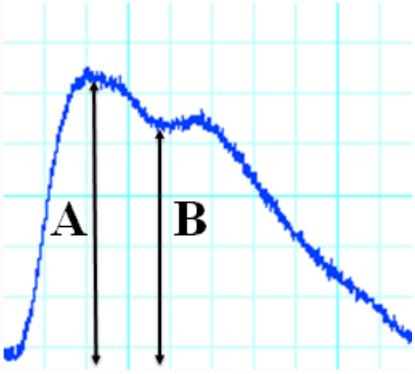

Figure 3.3. Pulse amplitude recorded via infra-red plethysmography attached to the thumb. The total amplitude (A) and the dicrotic notch (B) were calculated and the pulse amplitude ratio of B to A *100 was calculated as vascular compliance. Mean compliance was made from 3 consecutive pulse waveforms.

As shown in Figure 3.4, pulse transit time (PTT) was calculated as the time difference between ECG R-wave peak and the peak of pulse pressure waveform measured by a pulse transducer at the fingertip. Pulse wave velocity (PWV) was calculated according to the following formula (Davies and Struthers, 2003), with BDC as body correlation factor being 0.5 when calculating peripheral pulse wave at the finger for adults (Gesche et al., 2012):

$$\text{PWV(cm/ms)} = \frac{\text{BDC} \times \text{height (cm)}}{\text{PTT (ms)}}$$

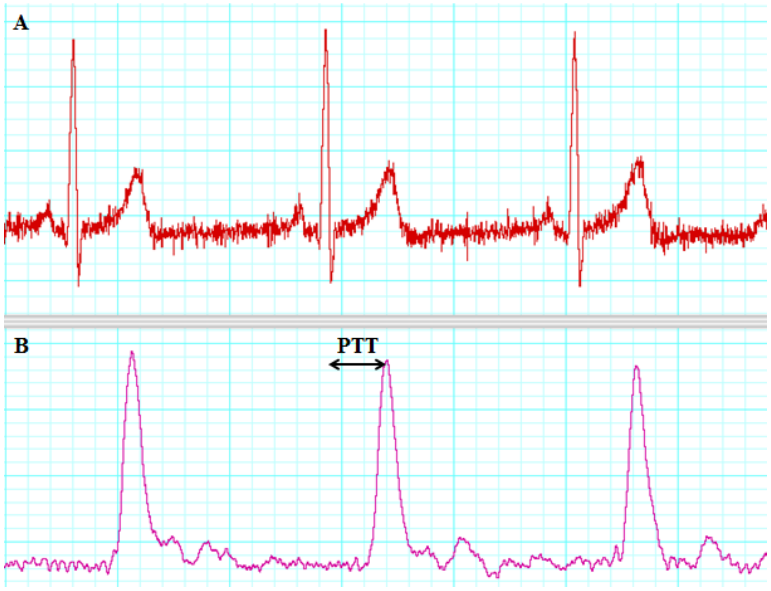

Figure 3.4. Calculation of pulse transit time (PTT). A: cardiac electrical activity via a 3-lead ECG. B: the finger pulse signal detected via a pulse transducer. Pulse transit time was calculated as the time difference between the R-wave peak of ECG and the peak of finger pulse waveform. Mean PTT was made from 3 consecutive calculations. Signals were shown at a scale of 5:1.

As illustrated in Figure 3.5, peripheral pulse amplitude and the finger pulse signal were continuously recorded during baseline for 10 min, and then an occlusion of forearm blood flow (the experiment arm) for 5 min and recovery for 10 min. As shown in the Figure 3.5., peripheral pulse amplitude and the finger pulse signal were negligible during occlusion, followed by an increase in the amplitude upon cuff deflation. In addition, peripheral pulse amplitude was also recorded on the contralateral arm at the beginning of the study, used as a control for the concurrent endothelium-independent changes in vascular tone.

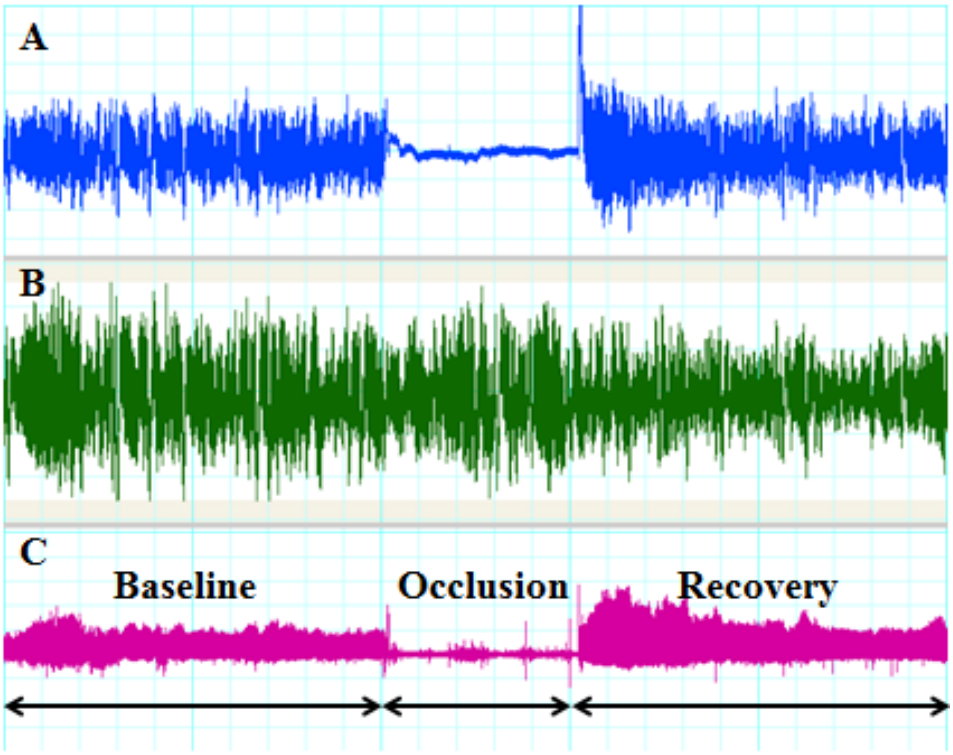

Figure 3.5. Peripheral pulse amplitude and the finger pulse signal continuously recorded during the experiment. A: Peripheral pulse amplitude detected via infra-red plethysmography on the experiment hand during baseline (10 min), occlusion (5 min) and recovery (10 min). B: Peripheral pulse amplitude simultaneously detected on the contralateral arm, used as a control for the concurrence of endothelium-independent changes in vascular tone. C: The finger pulse signal via a pulse transducer simultaneously detected on the experiment hand. Signals were shown at a scale of 2000:1.

3.2.6 Statistical analysis

Based on previous data, at least 12 subjects were required to investigate the effect of orange juice on peripheral vascular function measured by plethysmography (Alsop and Hauton, 2016).

Statistical analysis was conducted using Statistical Package for the Social Sciences (SPSS, version 24, IBM Corporation, USA). Data were tested for normality using Shapiro-Wilk test. Markers before and after orange juice consumption were analysed by analysis of variance (ANOVA), with treatment and time as within-subject factors or two-tailed t-tests (for blood pressure). The flavanone contents in different juices were analysed using one-way ANOVA, with post hoc Tukey's test. $P < 0.05$ was considered as statistically significant.

3.3 Results

3.3.1 Citrus flavonoid content in different citrus products

Flavanones are the predominant flavonoinds in orange varieties. Two flavanones, hesperidin and narirutin, were identified and quantified in the citrus products analysed. As shown in Figures 3.6, standard curves were constructed of pure citrus flavonoid compounds, for quantification of citrus flavonoids in unknown samples. Identification of citrus flavonoids were based on the corresponding retention times and the MS spectra of pure standards and spiking of selected samples with authentic standards. Genistein was used as an internal standard and the results indicated there was no retention time shift from sample to sample. Specifically, narirutin, hesperidin and genistein eluted at 12.5, 13.3, 18.5 min, respectively (Figure 3.7).

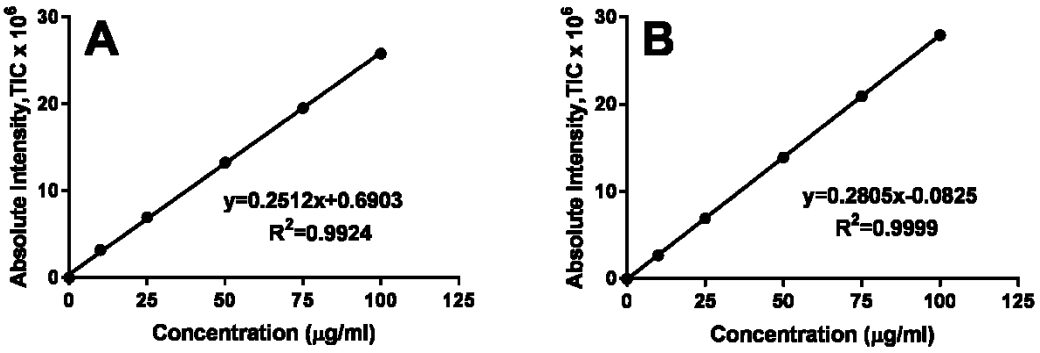

Figure 3.6. Standard curve of hesperidin (A) and narirutin (B). A: specific detection was carried out at 609 (m/z) at 13.3 min using LCMS for hesperidin. B: specific detection was carried out at 579 (m/z) at 12.5 min using LCMS for narirutin.

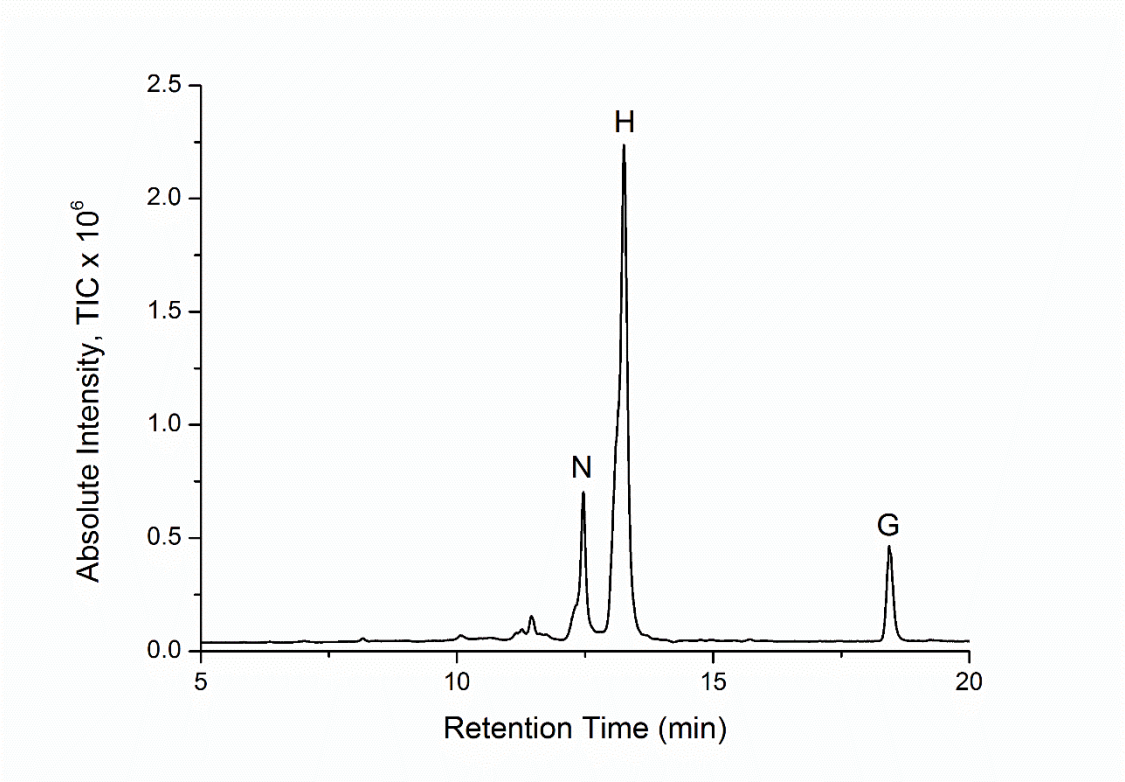

Figure 3.7. A typical LCMS chromatogram of citrus flavonoids in orange juice after extraction by methanol. N is narirutin, H is hesperidin and G is genistein; specific detection of these compounds was conducted at 12.5, 13.3, 18.5 min, at 579, 609, 269 m/z, respectively.

Nine brands of orange juice, including blond and blood orange juice, were analysed for their flavanone contents. After extraction by methanol, hesperidin was found to be the most abundant flavanone in all juice samples ranging from 60.7 ± 0.5 to 82.1 ± 2.4 mg/100 ml in blond orange juice and ranging from 58.6 ± 3.0 to 80.2 ± 2.7 mg/100 ml in blood orange juice, followed by narirutin ranging from 13.0 ± 1.5 to 20.4 ± 0.7 mg/100 ml in blond orange juice and ranging from 5.1 ± 1.2 to 9.5 ± 0.1 mg/100 ml in blood orange juice. As shown in Table 3.6, the concentrations of hesperidin, narirutin and total flavanones in the orange juice from Morrisons (both with bits and smooth from concentrate) were found to be the highest among all the juices tested, followed by those in the blood orange juice from Innocent. In contrast, hesperidin, narirutin and total flavanones were found to be least abundant in the blood orange juice from Waitrose. Regarding the impact of orange juice manufacturing process on flavanone content, smooth orange juices and the corresponding orange juices with bits were found to be comparable in hesperidin and narirutin levels. In addition, the narirutin-to-hesperidin

ratio for blond orange juice and blood orange juice ranged from 0.17 to 0.25 and from 0.08 to 0.12, respectively. In addition, hesperidin contents in blond orange juice and blood orange juice were comparable (71.7 ± 9.8 mg/100 ml and 69.8 ± 9.6 mg/100 ml, respectively). In contrast, narirutin was found to be more abundant in blond orange juice than blood orange juice (15.5 ± 4.5 mg/100 ml and 6.8 ± 2.2 mg/100 ml, respectively). Furthermore, the flavanone contents of orange juices with bits and smooth were comparable.

Table 3.6. Flavanone contents in nine commercially available brands of orange juice (mg/100 ml).

Code	Product	Hesperidin	Narirutin	Total flavanones
1	Tropicana (with bits)	66.3 ± 3.1^{c}	10.6 ± 0.7^{bc}	76.9 ± 3.8^{cd}
2	Tropicana (smooth)	60.7 ± 0.5^{c}	13.0 ± 1.5^{b}	73.7 ± 1.3^{cd}
3	Morrisons (with bits)	80.6 ± 1.4^{ab}	20.2 ± 1.4^{a}	100.8 ± 2.0^{a}
4	Morrisons (smooth)	82.1 ± 2.4^{ab}	20.4 ± 0.7^{a}	102.5 ± 3.1^{a}
5	Tesco (with bits)	69.1 ± 2.6^{abc}	12.6 ± 0.5^{b}	81.7 ± 2.9^{bc}
6	Tesco (smooth)	68.4 ± 3.2^{abc}	12.2 ± 0.9^{b}	80.6 ± 3.4^{bc}
7	Innocent (blood orange juice)	80.2 ± 2.7^{ab}	9.5 ± 0.1^{bc}	89.7 ± 2.6^{abc}
8	Waitrose (blood orange juice)	58.6 ± 3.0^{c}	5.1 ± 1.2^{c}	63.7 ± 2.6^{d}
9	Tropicana (blood orange juice)	70.7 ± 1.7^{bc}	5.8 ± 1.5^{c}	76.5 ± 0.4^{cd}

Means within the column without the same letter (a, b, c, d) are significantly different ($P < 0.05$; one-way ANOVA with post hoc Tukey's test).

3.3.2 Baseline subject characteristics

The baseline characteristics of subjects in the present study are presented in Table 3.7. Thirteen healthy subjects (body mass index 25.7 ± 3.3 kg/m^2, aged 22-43 y) were enrolled in the study,including seven premenopausal women and six men. Although male subjects were relatively younger than female subjects (28.3 ± 4.8 and 32.3 ± 5.4 y, respectively), the BMIs of both genders were similar (26.1 ± 3.1 and 25.4 ± 3.8 kg/m^2, respectively). Moreover, the subjects were normotensive with average blood pressures of 111.9 mmHg (systolic) and 71.7 mmHg (diastolic). The average heart rate was 76.9 ± 12.7 bpm at baseline.

Table 3.7. Baseline characterisation of subjects.

	Mean ± SD	Range
Age (y)	30.5 ± 5.4	22-43
Subjects (n)	13	M=6 / F=7
BMI (kg/m^2)	25.7 ± 3.3	20.2-31.4
Systolic blood pressure (mm Hg)	111.9 ± 8.3	99.5-124.5
Diastolic blood pressure (mm Hg)	71.7 ± 9.2	58.0-90.0
Heart rate (bpm)	76.9 ± 12.7	57.7-111.0

3.3.3 Effect on cardiac electrical activity and blood pressure

Heart rate was calculated as 30 s averages every 2 min for 10 min during baseline, every 1 min for 5 min during cuff occlusion and every 2 min for 10 min post cuff release. As illustrated in Figure 3.8, heart rate during occlusion significantly decreased under both conditions ($p<0.05$). No significant changes in heart rate were observed prior to and 2 h following orange juice consumption ($p=0.25$), indicating no significant alterations in the sympathetic nervous system tone.

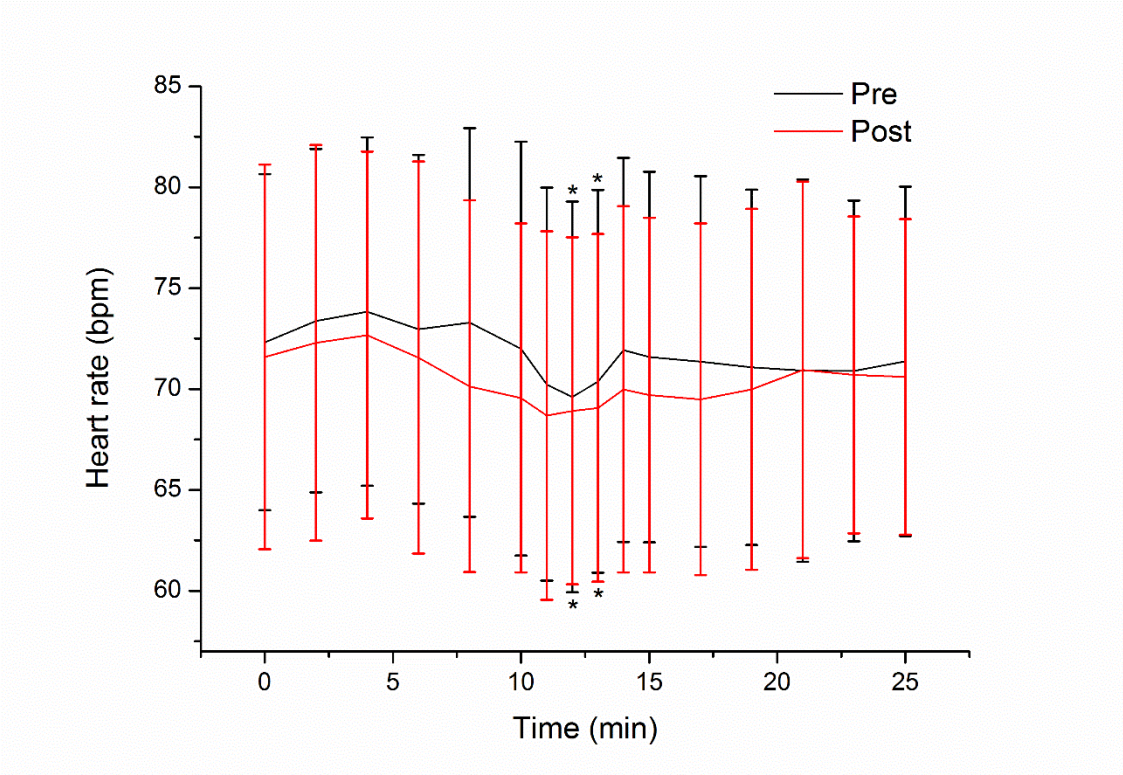

Figure 3.8. Cardiac electrical activity expressed as heart rate during baseline, cuff occlusion and recovery. Data represent mean ± SD (n=13). Heart rate during occlusion (t=12, 13 min) significantly decreased compared with baseline and recovery levels, in both pre and post orange juice consumption conditions ($p<0.05$).

As shown in Table 3.8, no changes were observed in diastolic blood pressure prior to and at 2 h following the consumption of orange juice (p=0.5) while a 3% reduction in systolic blood pressure was observed (p= 0.08).

Table 3.8. Blood pressure prior to and at 2 h following consumption of orange juice.

	Pre	Post
Systolic blood pressure (mm Hg)	111.9 ± 8.3	108.6 ± 9.1
Diastolic blood pressure (mm Hg)	71.7 ± 9.2	71.4 ± 8.5

Data are mean ± SD (n=13).

3.3.4 Effect on peripheral blood flow

As shown in Figure 3.9 relative flow during baseline slightly decreased at 2 h post orange juice consumption. Relative flow was negligible in the fingertip during cuff occlusion, followed by an increase post cuff deflation and recovery to baseline thereafter (Figure 3.10). Specifically, relative flow significantly augmented at 40 s post cuff deflation compared with baseline relative flow prior to orange juice consumption.

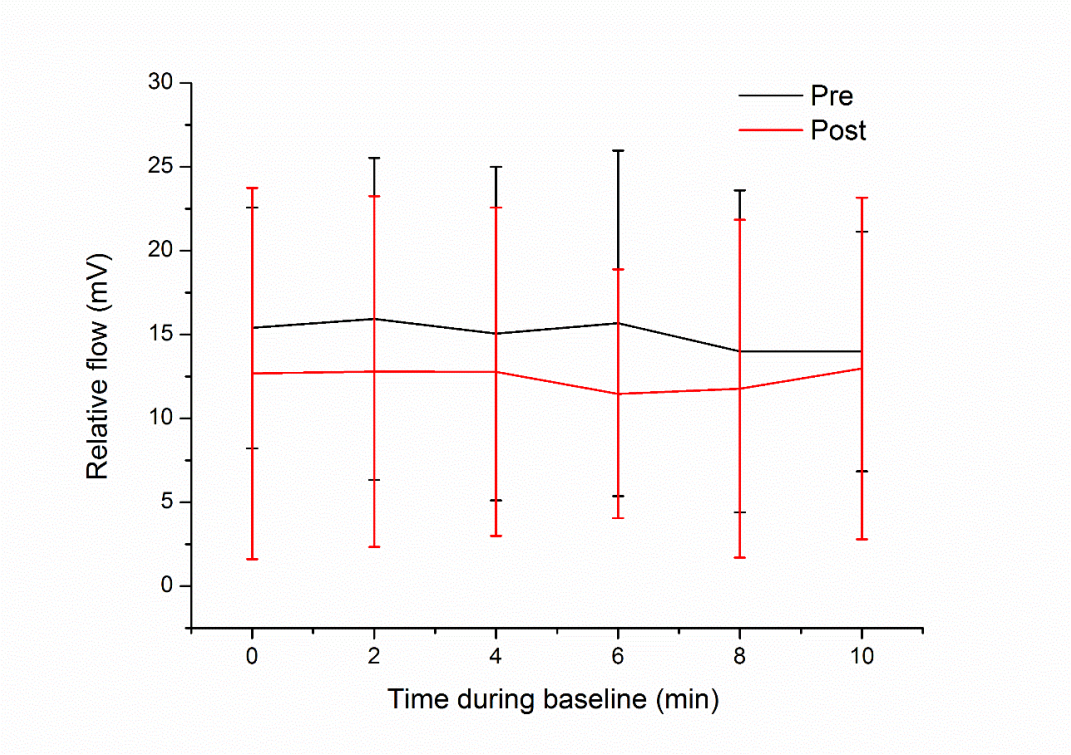

Figure 3.9. Relative flow data during baseline, calculated from peripheral pulse amplitude in triplicate pre and post orange juice consumption. Data represent mean ± SD (n=13). No significant differences were observed.

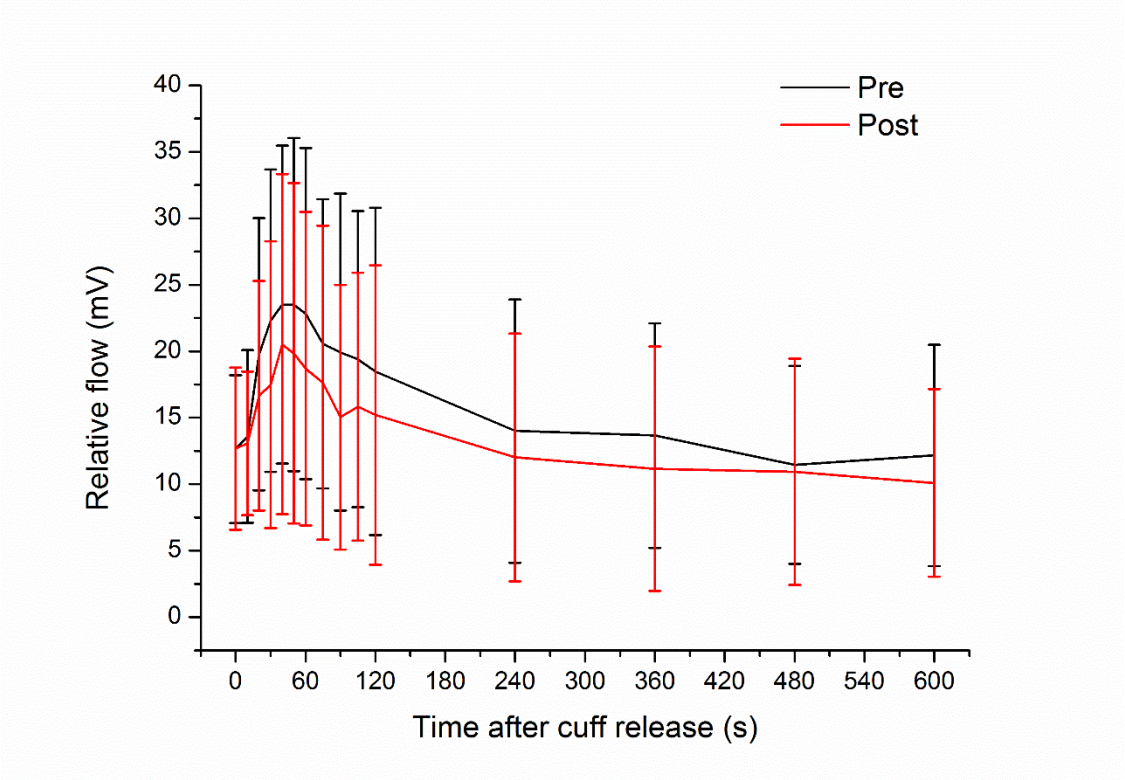

Figure 3.10. Relative flow data post cuff deflation, calculated from peripheral pulse amplitude in triplicate pre and post orange juice consumption. Data represent mean ± SD (n=13).

3.3.5 Effect on vascular compliance

Vascular compliance was measured as pulse amplitude ratio. As shown in Figure 3.11, no changes were observed in pulse amplitude ratios during baseline prior to and following orange juice. Pulse amplitude ratio was 0 during cuff occlusion and increased to 100% upon cuff release, followed by a gradual decrease to a plateau. Specifically, during the first 20 s following cuff deflation pulse amplitude ratio significantly increased compared with baseline level prior to orange juice consumption (Figure 3.12). Similarly, pulse amplitude ratio was significantly augmented during the first 20 s post cuff deflation compared with baseline level following orange juice consumption. In contrast, pulse amplitude ratio was elevated post cuff deflation following orange juice consumption compared with that prior to orange juice consumption.

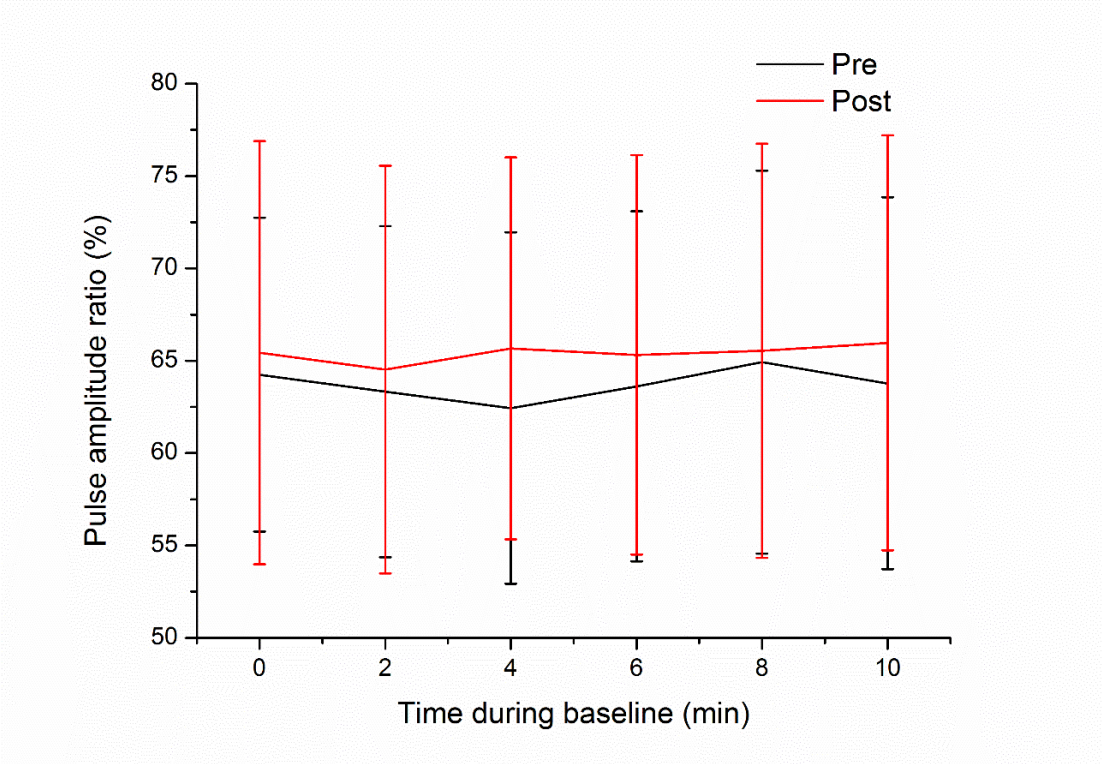

Figure 3.11. Vascular compliance at baseline, expressed as mean of three consecutive values of pulse amplitude ratio (%) pre and post orange juice consumption. Data represent mean ± SD (n=13).

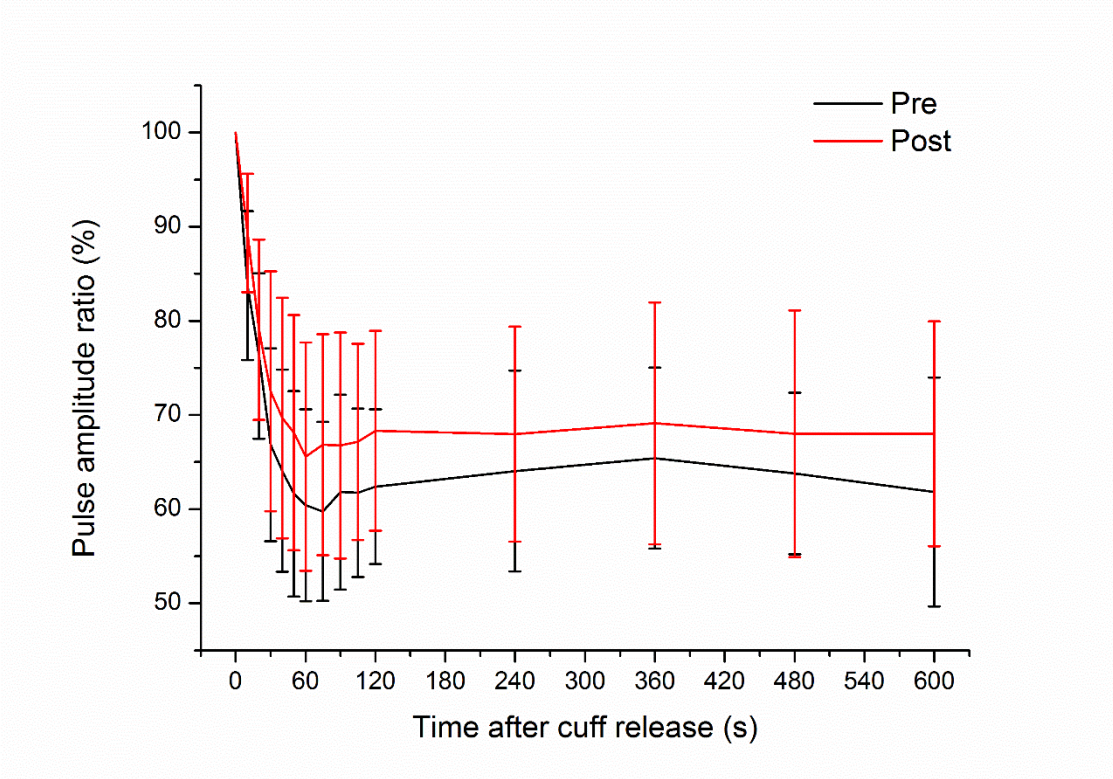

Figure 3.12. Vascular compliance post cuff deflation, expressed as mean of three consecutive values of pulse amplitude ratio (%), pre and post orange juice consumption. Data represent mean ± SD (n=13).

Delta of vascular compliance during baseline and post cuff release was calculated, as shown in Figure 3.13 and 3.14. Although there was an increase in the mean of vascular compliance following orange juice consumption during baseline and post cuff release, no significant changes were observed ($p>0.05$).

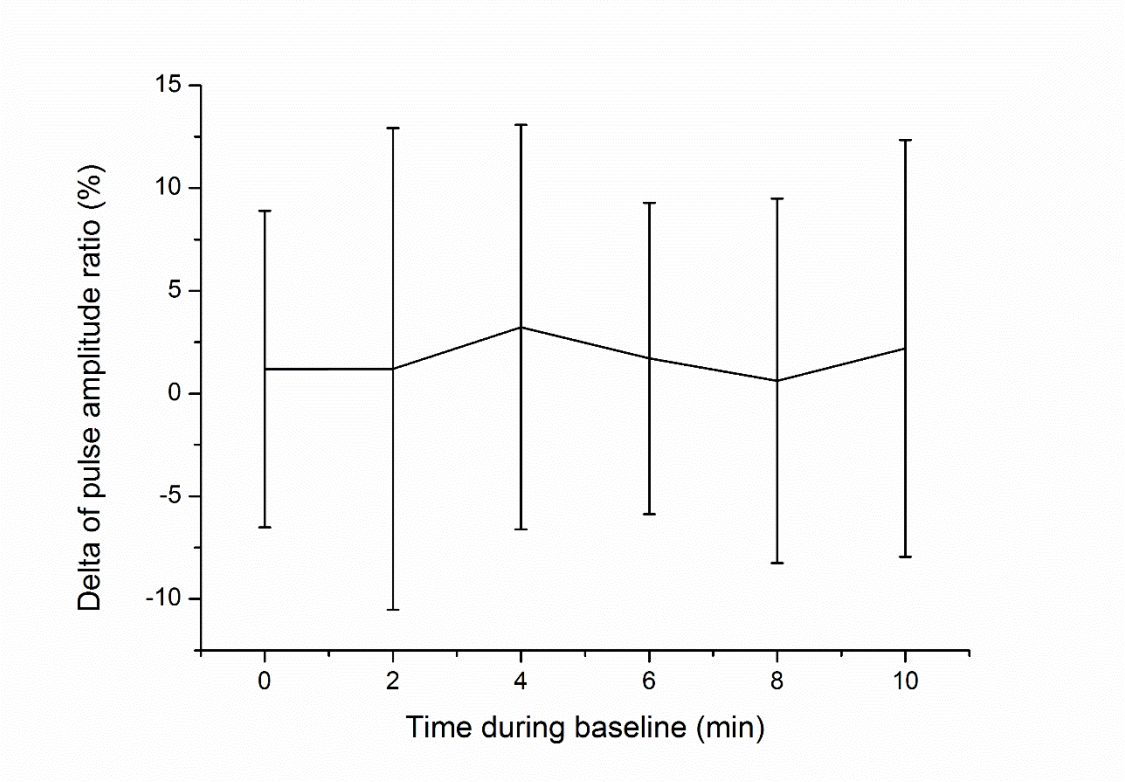

Figure 3.13. Delta of vascular compliance following orange juice consumption during baseline data recording. Data represent mean ± SD (n=13).

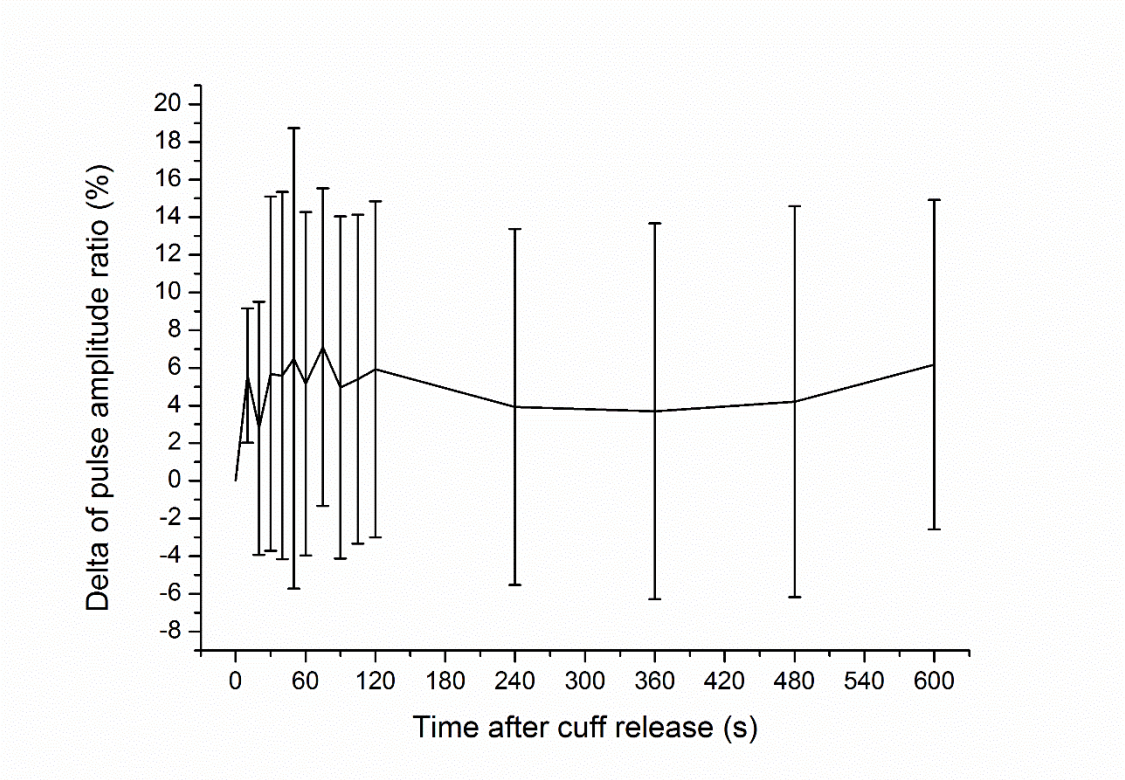

Figure 3.14. Delta of vascular compliance following orange juice consumption post cuff release. Data represent mean ± SD (n=13).

3.3.6 Effects on pulse transit time and pulse wave velocity

As illustrated in Figure 3.15, no significant differences were observed in pulse transit time at baseline pre and post orange juice consumption, with averages of pulse transit time as 256.1 ± 2.4 and 255.7 ± 2.7 ms, respectively. Given that pulse amplitude was negligible during cuff occlusion, pulse transit time could not be calculated. Upon cuff deflation, pulse transit time slightly elevated then gradually returned to baseline value (Figure 3.16). No significant differences were observed in pulse transit time following cuff deflation.

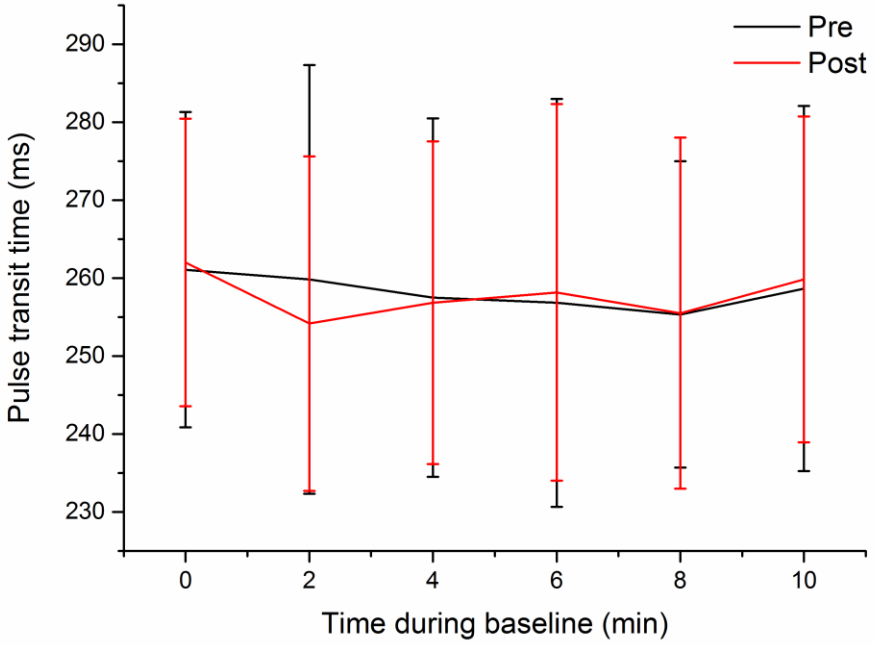

Figure 3.15. Pulse transit time during baseline, calculated by subtracting the ECG R-wave peak time from the peak time of pulse pressure waveform. Data represent mean ± SD (n=13).

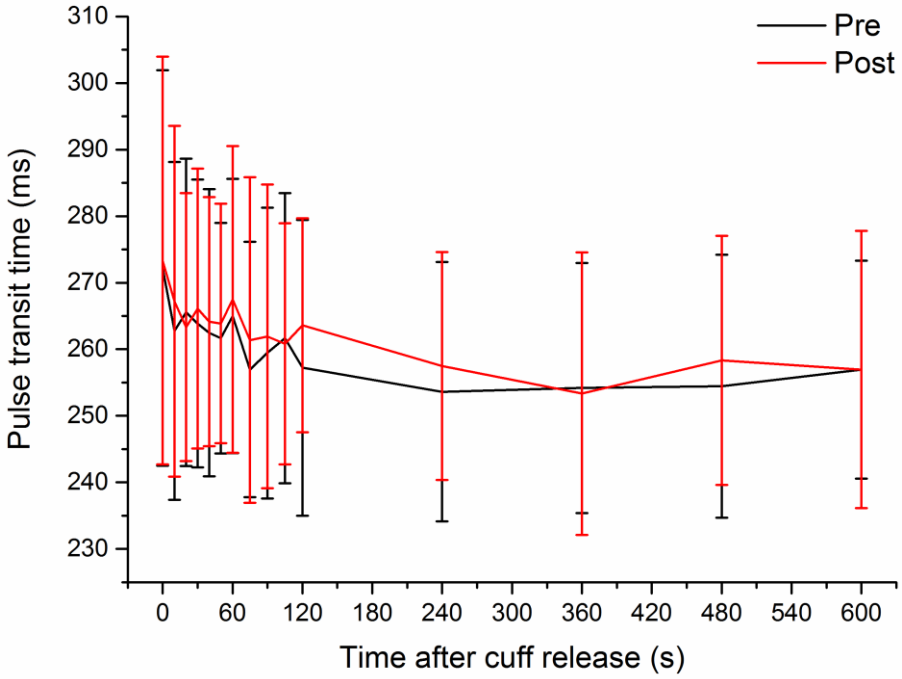

Figure 3.16. Pulse transit time post cuff deflation, calculated by subtracting the ECG R-wave peak time from the peak time of pulse pressure waveform. Data represent mean ± SD (n=13).

In addition, negative correlations were found between systolic blood pressure and pulse transit time prior to and following orange juice ingestion (r=-0.185 and r=-0.088, respectively). Moreover, no significant changes were observed in pulse wave velocity during baseline (Figure 3.17) and post cuff deflation (Figure 3.18) prior to and following orange juice consumption.

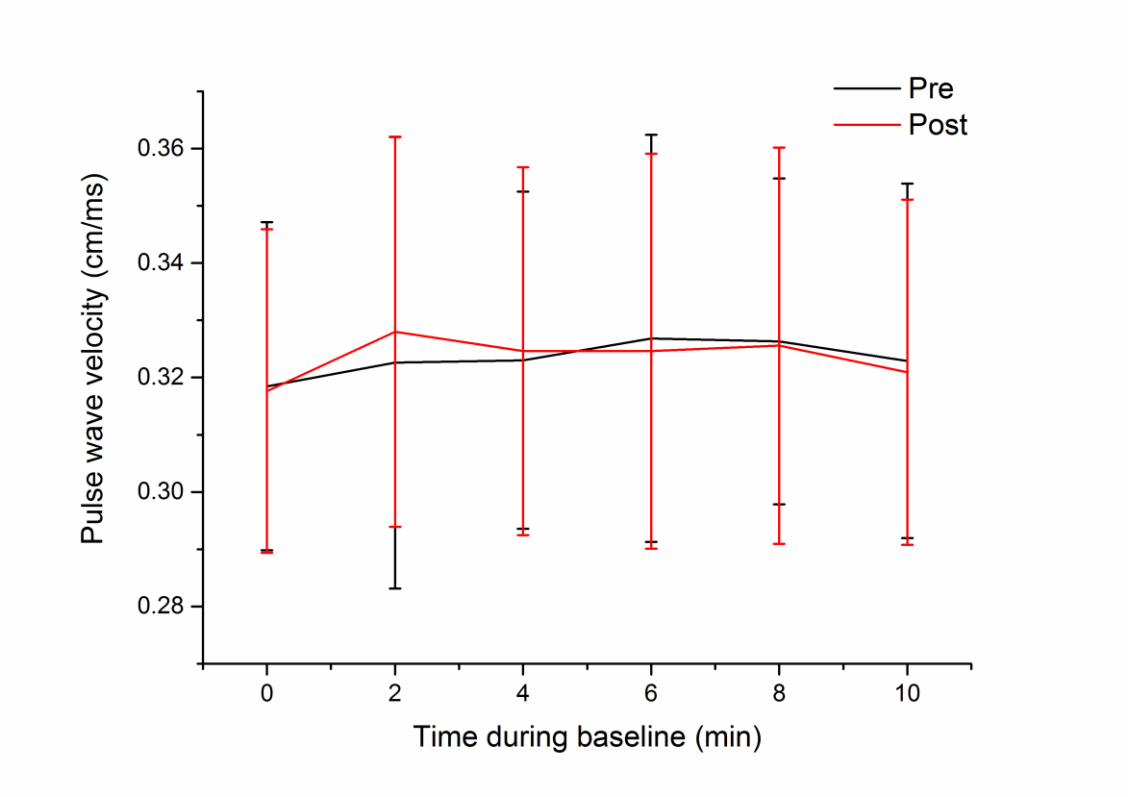

Figure 3.17. Pulse wave velocity during baseline, calculated from height and pulse transit time. Data represent mean ± SD (n=13).

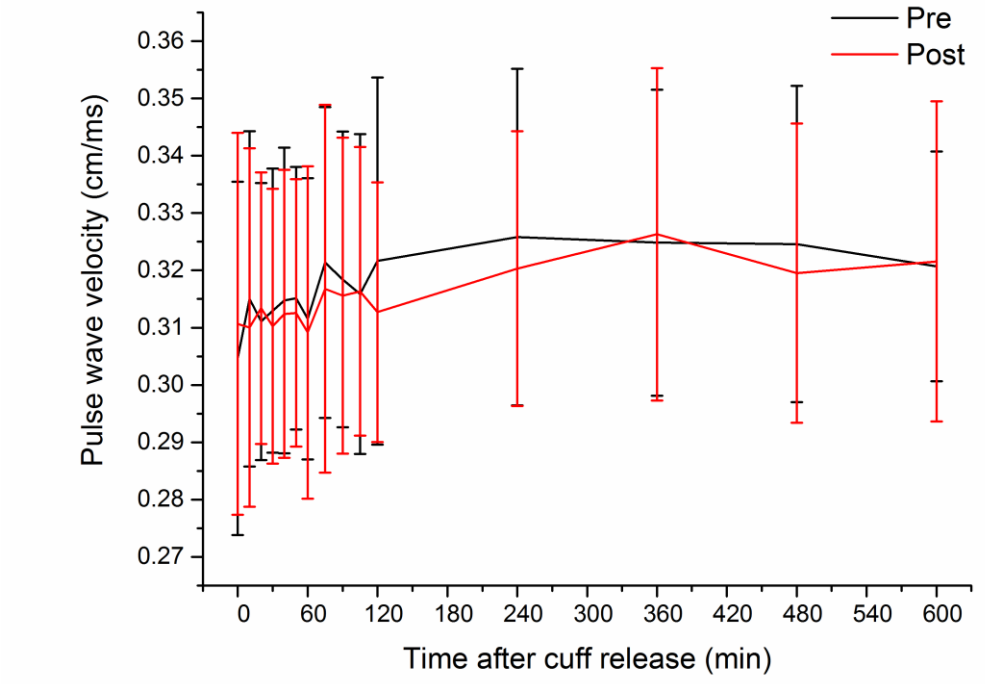

Figure 3.18. Pulse wave velocity post cuff deflation, calculated from height and pulse transit time. Data represent mean ± SD (n=13).

3.4 Discussion

In the present study, flavanone contents of nine commercial orange juices were determined. The blond not-from-concentrate orange juice with the highest flavanone content was then selected for a preliminary study to investigate the acute effects of flavanone-rich orange juice in healthy subjects on cardiac electric activity, blood pressure, peripheral blood flow, vascular compliance and pulse wave velocity prior to and at 2 h following consumption. The present study demonstrated substantial variations in flavanone content of commercially available orange juice, which in part might have contributed to the conflicting results in previous studies regarding changes in health outcomes following orange juice intake in an acute or chronic manner. Although elevation of plasma flavanone metabolites have been observed at 2-7 h following orange juice consumption in healthy subjects (Rendeiro et al., 2016), the present study suggested that flavanone-rich blond orange juice did not significantly improve cardiac electrical activity, blood pressure, vascular compliance, peripheral blood flow, pulse transit time and pulse wave velocity in an acute manner, 2 h post ingestion of juice.

3.4.1 Comparison of flavanone content in orange juices

Over 60 flavonoids have been identified in citrus fruits, including 4 types of flavonoids (flavanones, flavonols, flavones and anthocyanins) (Benavente-García et al., 1997; Horowitz and Gentili, 1977). The flavonoids in citrus are usually in the form of glycosides, with the predominated flavonoids being flavanones (Lee et al., 1990). In keeping with previous studies (Aturki et al., 2004; Kawaii et al., 1999), hesperidin was found to be the most abundant flavonoid in orange juices analysed in the present study, followed by narirutin. The narirutin-to-hesperidin ratio has been proposed for quality control of commercial orange juice (Rouseff, 1988). The narirutin-to-hesperidin ratios of the studied blond and blood orange juices were in accordance with those reported in previous studies (Aturki et al., 2004; Rouseff, 1988). The levels of hesperidin in orange juice reported in the literature vary substantially, ranging from 35 to 70 mg/100 ml (Mears and Shenton, 1973). In the present study, flavanones were found to be slightly higher than those reported by Vanamala et al. (2006) ranging from 18.0 to 54.8 mg/100 ml for hesperidin and ranging from 2.9 to 8.0 mg/100 ml for narirutin. Similarly, the concentrations of hesperidin and narirutin in blood orange juice were also found to be slightly higher than those reported by Kelebek et al. (2008) with hesperidin ranging from 11.3 to 14.3 mg/100 ml and narirutin ranging from 2.9 to 3.3 mg/100 ml. This inconsistency might be due to variations in the flavanone content of the fruits, different techniques used to manufacture orange juice (squeezing, mild or standard pasteurisation, concentration and freezing), storage conditions and differences in the extraction and analytical methods of flavanones. Specifically, substantial variations in flavanone content of citrus fruits might be attributed to numerous factors: varietal variations, environmental and growing conditions, harvest seasons and degree of fruit maturity (Mears and Shenton, 1973). Differences in extraction solvent, solvent to juice ratio and extraction cycles are reported to result in considerable variations in flavanone extracted (Chebrolu et al., 2011). Specifically, the flavanone levels in the commercial orange juices in the present study were found to be generally higher than those reported by Sweidan (2015) on the same juice products including Tropicana orange juice and Morrisons orange juice. It should be noted there were several differences in the extraction methods: in the present study 100% methanol was used as the extraction solvent with a solvent-to-juice ratio as 2:1 whereas 80% methanol was used with a solvent-to-juice ratio as 1:1 in

that previous study, suggesting higher concentrated methanol with a higher solvent-to-juice was more effective in the extraction of flavanones from orange juice samples.

Moreover, total flavanone content was found to be higher in made-from-concentrate orange juice in comparison to not-from-concentrate orange juice, in agreement with the foundlings of Vanamala et al. (2006). In support of this, total flavanones are reported to increase following concentration due to the precipitation of flavanones from the soluble faction (Gil-Izquierdo et al., 2002).

3.4.2 Acute effect of orange juice on vascular function

The measurement of peripheral vascular function provides important diagnostic and prognostic information on cardiovascular health and the risk of CVD (Brevetti et al., 2003). In the present study, during each experiment arm, the increase in compliance upon cuff release validated the experiment, together with increased relative flow and unchanged heart rate, indicating the dilation of the blood vessel. Post juice consumption, heart rate, systolic blood pressure and relative flow reduced, whereas vascular compliance increased meanwhile pulse transit time and pulse wave velocity remained unchanged. The decrease in relative flow post juice consumption was consistent with a minor reduction in systolic blood pressure. Specifically, baseline compliance after drinking orange juice increased but not significantly. This increase might be due to the increase in insulin after drinking orange juice. Insulin has a vasodilatory role through induction of endothelial NO and therefore can alter vascular compliance (Cohen and Townsend, 2002). Notably, vascular compliance increased during reactive hyperaemia following orange juice consumption, with a concomitant reduction in systolic blood pressure, indicating protective effects in healthy subjects (Bahra et al., 2012). Although the increase in vascular compliance at 2 h post juice consumption was not statistically significant, it might still be clinically meaningful given that reduced vascular compliance has been associated with hypertension, atherosclerosis and diabetes mellitus (Izzo Jr and Shykoff, 2001; Glasser et al., 1997). It remains possible that a more pronounced increase in vascular compliance might be observed at a different time point or following prolonged consumption of orange juice.

Absolute pulse amplitude was increased after participants consumed chocolate and sandwich compared to that at the fasted state as control during baseline and recovery (Langer, 2012). In addition, it should be noted that there are several factors affecting the pulse wave magnitude. Vasodilation due to pharmacological factors (for example, nitroprusside), physiologic conditions (warming, sedation), or anaesthetic factors (regional sympathetic blocks) can lead to increase in waveform amplitude (Shelley, 2007). Vasoconstriction resulting from pharmacological factors (phenylephrine, ephedrine) or physiologic factors (cold, surgical stress) can decrease waveform amplitude (Shelley, 2007). Therefore, the present study was conducted in a quiet, temperature-controlled room to minimise fluctuations in vascular tones.

The findings regarding acute effect of orange juice on vascular function in the literature have been conflicting. Specifically, in the previous study by Schär et al. (2015), endothelial function in the microvasculature of the finger as measured by reactive hyperaemia-peripheral arterial tonometry (RH-PAT) (a similar technique as the present study), pulse wave velocity and central augmentation index as measures of central arterial stiffness, cardiac baroreflex sensitivity, platelet reactivity and NADPH oxidase gene expression were unchanged at 5 h post consumption of orange juice, compared with a control drink matched for vitamin C and sugars, despite elevated plasma concentrations of flavanone and phenolic metabolites in old men with increased cardiovascular disease risk. However, acute favourable effects of orange juice on vascular function in the postprandial state have been observed in studies using different techniques. Specifically, flavanone-rich juices were found to attenuate transit decline in endothelial function measured by flow-mediated dilation (FMD) induced by double meal high in fat, concomitant with increased plasma hesperetin and naringenin metabolites as well as plasma nitrite levels at 2 to 7 h post juice consumption in middle-aged healthy men, compared to a sugar-matched control drink (without vitamin C) (Rendeiro et al., 2016). In addition, a previous study by Morand et al. (2011) suggests that both orange juice and a sugar-matched control drink plus hesperidin significantly augmented postprandial microvascular endothelial reactivity measured by combined laser Doppler flowmetry (LDF) and iontophoresis, in relation to a sugar-matched control drink plus placebo,

assessed at baseline and 6 h post consumption in healthy overweight old men. In agreement with these positive effects by flavanones or flavanone-rich juice on vascular function observed in a postprandial state, several previous studies suggest other flavonoid-rich beverages have also improved endothelial function. For example, a previous postprandial study by Lorenz et al. (2007) demonstrates that endothelial function measured by FMD at 2 h post consumption of black tea rich in catechins and a standardised breakfast was significantly augmented in healthy postmenopausal women, in relation to baseline FMD, with water as a control drink. However, the improvement in FMD by black tea was completely inhibited by the addition of milk, probably due to the complexes formed between caseins in the milk and catechins in black tea, indicating the important influence of food matrix on vascular effects by flavonoids. Likewise, cocoa drink rich in flavan-3-ols significantly increased FMD in subjects with at least 1 cardiovascular risk factor at 2 h post consumption, compared with baseline FMD whereas cocoa drink low in flavan-3-ols did not alter FMD (Heiss et al., 2003). Taken together, the disparity in previous literature pertaining to the acute impact of flavanone-rich orange juice might be explained by differences in participant profile (age, gender, BMI, healthy or at increased cardiovascular disease risk), inter-individual variations in polyphenol absorption and metabolism, different methods for assessment of vascular function (RH-PAT, FMD, LDF) as the results may be subject to the sensitivity and reproducibility of the technique (Woo et al., 2014; Onkelinx et al., 2012; Dhindsa et al., 2008), measurement conducted in the fasted state or in the postprandial state, differential choices of the control drink (most studies matched for sugar content without equivalent vitamin C) and observation made at different time points.

3.4.3 Comparison of different approaches for assessment of endothelial function

In the literature, a variety of approaches have been used when investigation effects of flavonoid-rich foods/beverages on vascular function. Flow-mediated dilation was first introduced by Celermajer et al. (1992), using high-resolution ultrasound to examine the changes in diameters of the brachial and superficial femoral arteries caused by reactive hyperaemia following occlusion of the blood flow. It has then been widely adopted as a measure of endothelial function for its non-invasive manner. As reviewed in Chapter 1,

it is well accepted that FMD is technically challenging (Corretti et al., 2002) and the competence in performing FMD with good reproducibility requires substantial practice over several months. Furthermore, the variability in FMD is highly operator-dependent. In contrast, finger plethysmography used in the present study is easy to operate and recording of the data is operator-independent, together with a 3-lead ECG module and a pulse transducer, providing information on multiple outcomes such as vascular compliance, peripheral blood flow, cardiac electrical activity, pulse transit time and pulse wave velocity.

Another widely used approach is RH-PAT, which can be used as an indication of atrial stiffness by calculation of the augmentation index (Matsui et al., 2004). In addition, measurements conducted on the contralateral arm can be used as a control for the concurrence of endothelium-independent changes in vascular tone, such as those caused by alterations in the autonomous nervous system tone (Hijmering et al., 2002). Furthermore, significant correlations have been observed between FMD and reactive hyperaemia index (RHI) measured by RH-PAT during the same episode of reactive hyperaemia, irrespective of gender and presence of cardiovascular diseases (Woo et al., 2014; Kuvin et al., 2003). Although RH-PAT is easier to perform and less operator-dependent, also evaluating vascular endothelial function in a non-invasive manner, it is a widely held view that brachial artery FMD is a more sensitive method of assessing endothelial function and cardiovascular risk (Lind, 2013; Onkelinx et al., 2012). No changes in vascular function were observed via RH-PAT, despite potential biological significant concentrations of flavanone metabolites (Schär et al., 2015), highlighting the importance of the sensitivity of the approaches for measurement, which might in part explain the lack of effect in the present study.

3.4.4 Conclusions

The present study suggests substantial variations in the flavanone contents of commercial orange juices, which might have contributed to the conflicting results in the literature. The flavanone content ranges of commercial blond and blood orange juices were comparable. Made-from-concentrate orange juice was found to be more abundant in flavanones. Vascular functions were not acutely altered at 2 h following consumption of

a flavanone-rich orange juice in healthy subjects under the conditions investigated. However, it remains possible that favourable vascular effects of orange juice consumption can be observed using other techniques and/or after a more prolonged consumption.

3.4.5 Limitations and future work

Some limitations of the present study are worth noting. First, the lack of suitable control drink in the acute study. The effect of orange juice would become clearer if compared with a control drink with same sugar content to balance the influence from the sugars in the orange juice and therefore remove the effect from insulin on the vascular function. Second, the experiment time, set to 2 h in the current study, which was considered sufficient for this preliminary study. Changes in vascular function post juice consumption showed tendency for improvement in vascular function but were not significant. It remains possible that more pronounced changes would have been observed at other time points such as 5-7 h, during which the maximal plasma concentrations of flavanone metabolites have been observed (Manach et al., 2005). However, investigation in vascular function in a fasted state made it difficult to conduct measurement at 5-7 h when subjects need to stay fasted for longer hours. Finally, it appears that finger plethysmography for evaluation of endothelial function may not be sensitive enough for detection of small changes. Therefore, in the following Chapter 4, FMD was adopted for investigation regarding effects of flavanone-rich orange juice on endothelial function. In future studies, randomised controlled crossover intervention studies are needed to test the vascular effects of flavanone-rich orange juice with a control drink marched for sugar and vitamin C content, at various time points (0, 2, 5, 7 h) or after a long exposure (several weeks/months) in a more defined population regarding ethnicity, BMI, age. The mechanism by which flavanones modulate resultant FMD should be explored by determining concurrent plasma concentrations of nitrite, nitrate and nitroso species and correlations between FMD and these concentrations.

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Chapter 4 Effects of blood orange juice on vascular function in healthy overweight/obese subjects

4.1 Abstract

Epidemiological studies have indicated that the consumption of citrus fruit is inversely associated with the risk of cardiovascular disease. Acute and chronic effects of citrus juice have been investigated intensively in recent years with mixed results, which might be due to a number of factors including body weight and health status, age and gender. In contrast to blond orange juice, little is known on the effects of daily consumption of blood orange juice on vascular function. The aim of this study therefore was to investigate the effects of blood orange juice consumption for 2 weeks (400 ml/day) compared with a control drink on vascular function and cardiovascular risk factors.

Fifteen overweight/obese men and women (aged 20-45 years) of European origin were enrolled in this randomized controlled crossover trial. Endothelial function (brachial artery flow-mediated dilation: FMD), blood pressure, anthropometric measures, lipid profile and hsCRP were evaluated before and after each intervention. Premenopausal women started the intervention on specific days of the menstrual cycle to minimise the fluctuations in oestrogen levels between four measurements of FMD, which were also monitored in serum samples.

The results demonstrate a significant increase in FMD following consumption of blood orange juice (pre: 8.15% ± 2.92%, post: 10.16% ± 3.31%) compared with the control drink (pre: 8.11% ± 2.52%, post: 7.77% ± 2.43%) (time by treatment interaction: P=0.002). The impact of the juice on FMD did not differ with gender (p=0.922). Notably, this favourable effect on endothelial function was only observed in overweight subjects but not in obese subjects. Baseline blood pressure, lipid profile, high-sensitivity C rective protein (hsCRP) and endothelin 1 (ET-1) were generally within healthy ranges and were not affected by the intervention. Present data indicate that blood orange juice exerts favourable effects on endothelial function in healthy overweight men and premenopausal women of European origin, possibly due to the elevated NO bioavailability by flavonoids.

4.2 Introduction

Numerous epidemiological studies have suggested that consumption of citrus fruit is inversely associated with the risk of cardiovascular diseases (CVD) (Cassidy et al., 2012; Knekt et al., 2002), inflammatory markers and endothelial dysfunction (Landberg et al., 2011). There has been an increasing interest in the potential cardio-protective properties of flavanones in recent years. *In vitro* studies suggest flavanones induce endothelial nitric oxide synthase expression and the resultant nitric oxide (NO) production in endothelial cells (Rizza et al., 2011; Liu et al., 2008), and other genes associated with antioxidant and cytoprotective function as detailed in Chapter 2. However, randomized controlled trials on the chronic effect of blond orange juice (Constans et al., 2015; Asgary and Keshvari, 2013) and/or single compound hesperidin (Salden et al., 2016; Morand et al., 2011) or naringin (Demonty et al., 2010; Jung et al., 2003) on cardiovascular health have shown mixed results. Numerous factors might have contributed to these discrepancies, including subject profile, inter-individual variations, compliance, use of different methods of analysis and controlling for confounding factors.

In contrast with blond orange juice, effects of blood orange juice on cardiovascular health have been much less researched. Compared with blond orange juice, blood orange juice is reported to have higher concentrations of flavanones and hydroxycinnamic acids (Rapisarda et al., 1998), as well as anthocyanins which are responsible for the red colour, together with high amounts of vitamin C, making it a good source of bioavailable antioxidants (Riso et al., 2005).

In terms of the subject profile of previous studies, the majority of previous studies have involved male subjects with increased cardiovascular risk (Constans et al., 2015; Morand et al., 2011) or postmenopausal women (Habauzit et al., 2015). Few studies have investigated the effects of polyphenol-rich food on endothelial function in premenopausal women and have not controlled for the confounding effect of oestrogen, which rendered the results questionable. Hence, little is known on the effects of citrus flavonoids in premenopausal women. Likewise, ethnicity has rarely been controlled for in previous studies. However, ethnicity-associated differences have been observed in the

metabolism of polyphenols (Maskarinec et al., 1998), possibly due to differences in the enzymes involved. For example, lactase phlorizin hydrolase (LPH) plays a pivotal role in the exclusive hydrolysis of some polyphenol glucosides before absorption (Day et al., 2000). It is assumed that LPH deficiency occurs in 5% of European adults and 90% of African and Asian adults. In addition, dietary nitrate exerts important vascular affects through the nitrate-nitrite-NO pathway (Lundberg et al., 2008), mediated partially by aldehyde dehydrogenase (ALDH2). Notably, there is high prevalence of ALDH2-deficient phenotype among Asian populations (Chang et al., 2017; Thomasson et al., 1991) whereas ALDH2 deficiency is believed to very uncommon in Caucasians (Brennan et al., 2004; Shibuya and Yoshida, 1988). Taken together, investigation on vascular effects of polyphenol-rich products in subjects across different ethnic groups will lead to considerable variations. Moreover, higher BMI is reported to be associated with a higher risk of coronary heart disease (CHD) in middle aged women after controlling for confounding factors such as age, smoking, menopausal status and parental history of CHD (Willett et al., 1995). Chronic low-level inflammation and impaired endothelial function have been observed in healthy obese subjects (Woo et al., 2004; Perticone et al., 2001). The observed endothelial dysfunction is associated with reduced NO bioavailability and imbalance between NO and endothelin-1 (ET-1) production (Caballero, 2003). Hence, overweight/obese subjects of European origin were recruited for the present study.

In Chapter 3, acute effect of orange juice on vascular function was investigated using finger plethysmography. In this chapter, a more sensitive approach, flow-mediated dilation (FMD), was used for evaluation of endothelial function. It is assumed that a small FMD (as detailed in Chapter 1) is an indication of low NO bioavailability, associated with increased cardiovascular risk (Inaba et al., 2010). FMD, therefore, has been widely adopted to evaluate endothelial function. However, in previous studies confounding factors do not appear to be controlled for when assessing effects of citrus flavonoids on endothelial function using FMD. One of confounding factors is oestrogen in premenopausal women, based on the fact that oestrogen increases NO production in endothelial cells, mediated partially through oestrogen receptor-α (Muller-Delp et al., 2003). The understanding of variations in endothelial function during the menstrual cycle is crucial to the study design of randomised controlled trials (RCT) investigating any

effects on premenopausal women and the data interpretation. Specifically, brachial artery FMD during late follicular phase has been documented to be significantly higher than that during other phases of the menstrual cycle in healthy premenopausal women (Adkisson et al., 2010; Williams et al., 2001), coincided with highest levels of oestradiol and plasma nitrite/nitrate, whereas no significant changes in FMD, oestradiol and plasma nitrite/nitrate were observed during early follicular phase, early luteal phase and late luteal phase (Adkisson et al., 2010). Therefore, controlling for menstrual cycle phase on the day of FMD testing in premenopausal women is imperative when investigating effects of other factors (such as food and exercise) on endothelial function. However, to our knowledge, menstrual phase on the day of FMD measurement has not been controlled for in previous studies investigating effects of citrus flavonoids on endothelial function in premenopausal women (Salden et al., 2016; Hashemi et al., 2015; Buscemi et al., 2012).

Both volunteer-related and methodology-related factors affect the reproducibility of brachial artery FMD (van Mil et al., 2016). More specifically, the reproducibility of FMD has been reported to be poorer in subjects with CVD, with larger coefficients of variation compared to healthy controls (van Mil et al., 2016, Craiem et al., 2007). Hypertension has a significant influence on the reproducibility of FMD ($P < 0.001$), independent of other factors (van Mil et al., 2016). Moreover, a meta-analysis by Witte et al. (2005) indicates that the association between FMD and cardiovascular risk is stronger in low-risk individuals. In high-risk individuals, it should be noted that the brachial artery FMD might not reflect endothelial function accurately as the measured FMD might be distorted by stiffness in the arteries. In support of this, the association between nitroglycerine-induced dilation and cardiovascular risk is also stronger in individuals at low risk (Witte et al., 2005). Therefore, subjects with hypertension or CVD were excluded in the present study, to reduce the variability in FMD (the primary outcome of the present study), thereby reducing the likelihood of type I and II errors.

The aim of this chapter, therefore, was to investigate the effects of flavanone-rich blood orange juice on endothelial function and cardiovascular risk factors in healthy overweight/obese men and premenopausal women of European origin.

4.3 Methods

4.3.1 Subjects and study design

The study was approved by Biological Sciences Faculty Research Ethics Committee, University of Leeds (Ethics Reference Number: BIOSCI 15-030), in accordance with ethical principles of the Declaration of Helsinki of 1975 as revised in 2013. Subjects were recruited through flyers, emails and a participant database with a similar subject profile generated through the EU funded Pathway-27 project (MEEC 15-015). Interested subjects were asked to contact the research team and were provided with a study information sheet detailing all aspects of the study and what it involved. The eligibility was confirmed following a short health and lifestyle questionnaire, conducted verbally or by email. Potential subjects were given at least 48 hours to consider the participation and to ask any questions.

In total, 296 subjects responded to the advertisement, out of which, 272 male and female subjects, aged 20-60 years, were assessed for screening according to the following criteria: Caucasians (of European origin), generally healthy without any cardiovascular diseases, non-smokers, with BMI over 25 kg/m^2, without use of medications or dietary supplements (vitamins, antioxidants), without lactose intolerance. Thirty subjects met these criteria however some failed to participate for the following reasons: time slots for the study clashed with their work, reluctance about venepuncture, and their unavailability to attend all four appointments. Sixteen subjects provided written informed consent prior to commencement of the study. One subject was excluded because of the use of medications during the intervention. Finally, fifteen subjects completed the study. The subject flow diagram is shown in Figure 4.1.

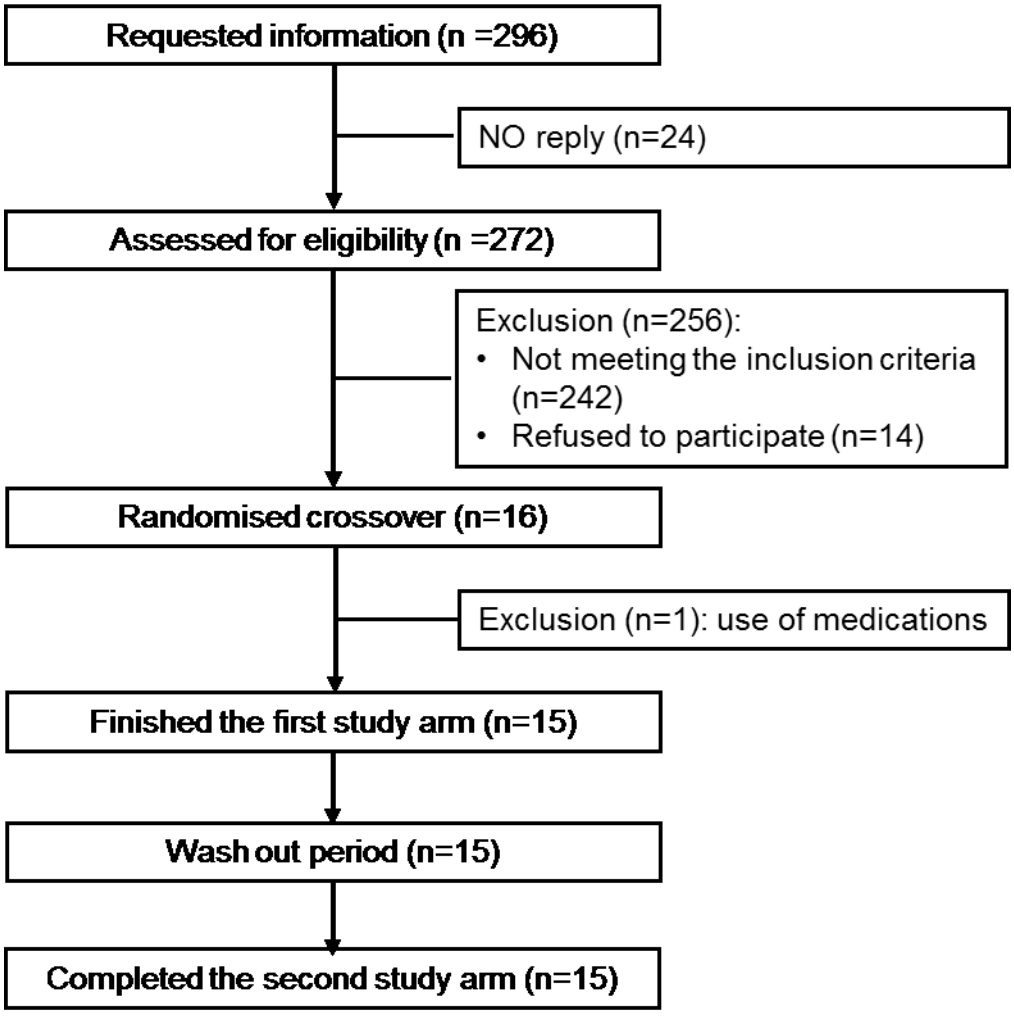

Figure 4.1. Flow diagram illustrating subject recruitment and study implementation.

The aim of the present study was to evaluate the effects of daily consumption of blood orange juice on cardiovascular health in healthy overweight/obese subjects. For this purpose, the study design was a randomized, controlled, crossover trial. A simple randomisation was used to assign A and B to the two juices tested in the study. As a result, blood orange juice and orange juice drink were coded as drink A and B, respectively. Block randomisation was conducted to allocate drink sequences (starting with drink A or B) to subject codes which were assigned to subjects after enrolment. All the data were saved with subject codes and drink A or B so that the researchers who conducted sample processing and data analysis were blind to the study. Through two 2-week periods, subjects were asked to daily consume 400 ml of drink A or B (200 ml with breakfast and dinner) in a randomised order, with a 1-week washout period between each treatment. Throughout the study, subjects were asked to maintain their lifestyle as usual,

including dietary routine and physical activity level. The time of each juice consumption was recorded by the subject in the citrus drink consumption record form which was returned to the researcher after 2-week consumption. During participation in the study, subjects were asked to maintain their dietary habits generally and refrain from high consumption of citrus foods and the consumption of flavonoid-rich beverages (such as coffee, tea, fruit juices and red wine), as suggested by other RCTs (Buscemi et al., 2012; Morand et al., 2011).

Measurements were conducted prior to and following 2-week juice consumption. Subjects were instructed to stay fasted and restrain from exercise for 12 h before measurements in the morning. After the subject was supine and comfortable for 15 min to reach a cardiovascular steady state, blood pressure was measured in triplicate with 2-minute intervals (as detailed in section 3.3.4). Endothelial function was evaluated via brachial artery FMD following the protocol described in detail in section 4.3.2. Each test for a given subject was performed at the same time of the day at the same imaged area of the brachial artery. Following brachial FMD measurement, venous blood samples (total volume: 14 ml) from antecubital vein were collected into vacutainers (Z Serum Sep Clot Activator tubes and K2E K2EDTA tubes, Greiner Bio-One, Austria) to produce serum and plasma. A urine sample (up to 50 ml) was collected at each visit. Anthropometric measures were also conducted before and after each intervention.

Female subjects started the intervention on specific days of the menstrual cycle in order to avoid any measurements during the late follicular phase, thereby minimising the fluctuations in oestrogen levels, which were also monitored in serum samples. Regarding contraception for female subjects, there were three circumstances: subjects without it, subjects taking combined pills (oestrogen and progesterone), and subjects taking mini-pills (progesterone-only). For those who were taking combined pills, they took the pill daily with a 7-day break during menses. All four appointments were arranged on the days when they took the combined pill, to control for confounding effect of the pill on FMD. For those who were taking mini-pill, as they were taking it daily without any breaks, the control for the experiment was the same as those without any contraceptive methods.

4.3.2 Assessment of brachial-artery endothelial function

4.3.2.1 Ultrasound scan protocol

The protocol for assessment of brachial-artery endothelial function via FMD in our lab (Harris et al., 2017; 2014) is in accordance with established guidelines (Thijssen et al., 2011; Harris et al., 2010). Subjects were instructed to stay fasted and avoid exercise for 12 h before the assessment. Examination of vascular function via duplex ultrasonography (Vivid E9 with XDclear, GE Healthcare, US) was conducted in the vascular laboratory which was quiet, dimly lit, and temperature-controlled (21±1°C) to minimise fluctuations in vascular tone. The subject was instructed to wear suitable clothes for the ultrasound scan without any restrictive clothing that might interfere with the blood flow to the arm, for the accurate measurement of FMD. The subject was in a supine position for 15 min to reach a cardiovascular steady state before extending the right arm in a comfortable position for the ultrasound scan of the brachial artery using a 7-MHz linear array probe (9L, GE Healthcare, USA). During the ultrasound examination, the subject was instructed to keep the examined arm as immobilised as possible.

The brachial artery was examined above the antecubital fossa in the longitudinal plane via high resolution B-mode ultrasound to obtain the optimal image of the brachial artery and lumen interface. The probe was locked by a clamp to stay at the same position during the ultrasound scan and the clamp allowed micro movement of the probe if necessary. Subsequently, pulsed-wave Doppler was turned on for simultaneous acquisition of the brachial artery diameter and the pulsed-wave Doppler velocity signals. The insonation angle between the vessel orientation and the Doppler beam was kept as close to 60º as possible across all tests as a compromise for optimal data acquisition of B-mode ultrasound and pulsed-wave Doppler signals. The sample size (gate width) for detection of blood velocity was adjusted to be as wide as possible to cover the lumen without encompassing the arterial walls as recommended (Harris et al., 2010). The same sample size was maintained during the ultrasound scan and across repeated measures on the same subject.

A rapid inflation and deflation pneumatic cuff was placed on the forearm distal to the imaged artery. Before cuff inflation, baseline image of the brachial artery was recorded

for 20 s with 15 images per second using a vascular imaging software (Vascular Imager, Medical Imaging Applications, Iowa, USA). The cuff was inflated to 220 mmHg and maintained at that pressure for 5 min. Post deflation image was recorded for 180 s starting from 30 s before cuff deflation. Figure 4.2 demonstrates the setup for brachial mediated-dilation (FMD) measurement.

Figure 4.2. Assessment of brachial flow-mediated dilation (FMD) with lower cuff placement distal to the imaged artery.

4.3.2.2 Post-test analysis by software

The brachial artery diameter and blood flow were detected off-line using Brachial Analyzer for Research (version 6, Medical Imaging Applications, Iowa, USA), which is currently considered as a reference method in FMD data analysis. This software provides continuous edge-detection for calculation of arterial diameter and blood flow, independent of investigator bias (Faita et al., 2011; Woodman et al., 2001).

Automatic detection of the branchial arterial diameter relies on the change in the grey pixel of the image. The edge is detected by determining the part where the most significant change of the grey pixel intensity happens. After calibration, a region of interest was selected on the part of the artery with best-defined walls as shown in Figure 4.3. Subsequently, the brachial artery diameter was automatically detected and the result was exported to a report (Microsoft Excel Worksheet 2013) for the calculation of FMD.

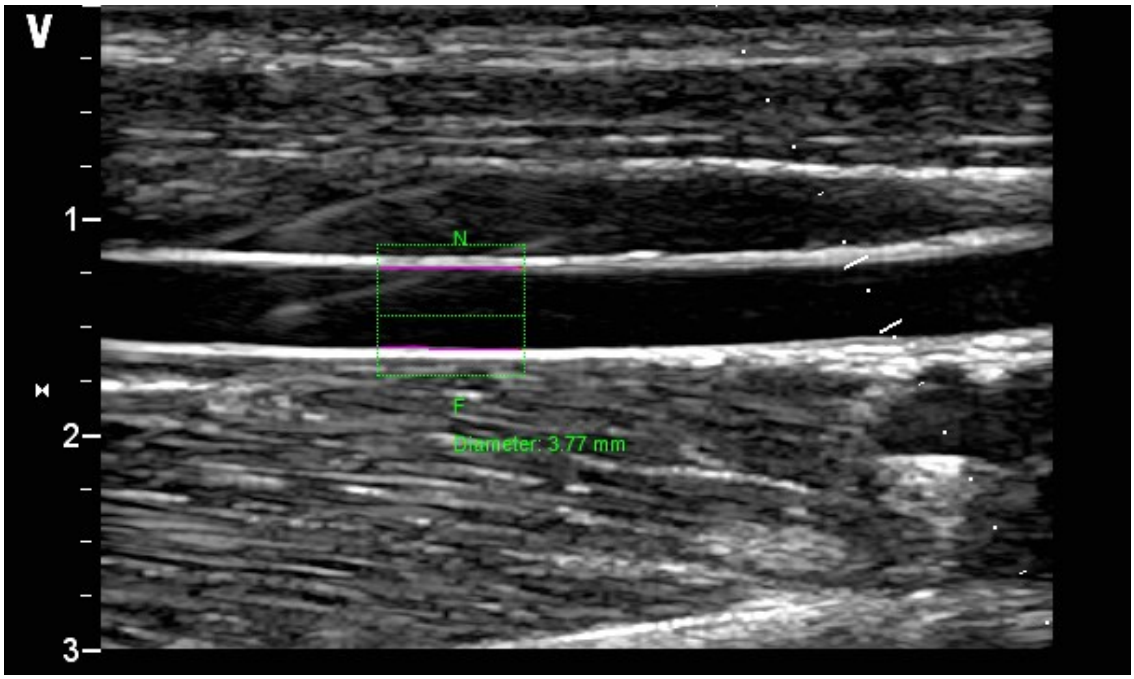

Figure 4.3. The image of the brachial artery with the selection of region of interest.

Similarly, the blood flow analysis started with calibration and the selection of a region of interest which covered the pulsed-wave Doppler signals (Figure 4.4) before the automatic detection. The result was also exported to a report (Microsoft Excel Worksheet 2013) for subsequent calculations.

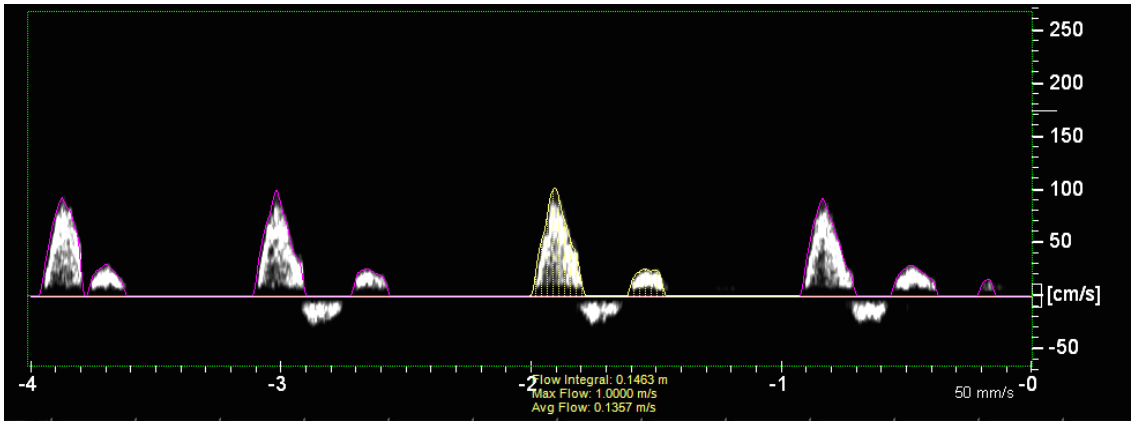

Figure 4.4. The image of the pulsed-wave Doppler signals with the selection of region of interest.

4.3.2.3 Calculation of FMD and shear rate

The raw data calculated by Brachial Analyzer for Research are illustrated in Figure 4.5 (baseline data) and Figure 4.6 (post deflation data). The average of resting brachial artery diameter was calculated from 300 images recorded prior to cuff inflation.

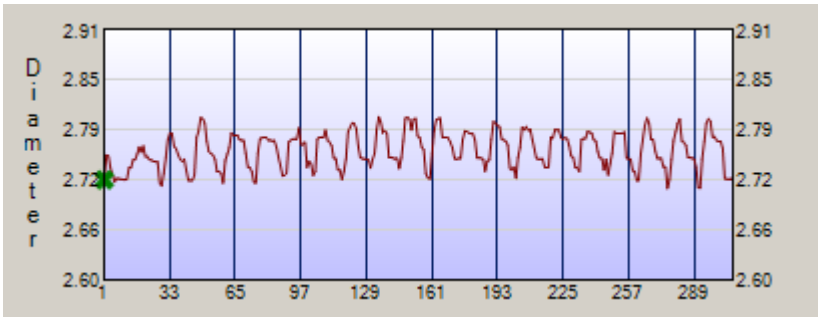

Figure 4.5. Resting brachial artery diameter of a subject from the software (Brachial Analyzer for Research).

Post deflation diameter data were smoothed by making moving averages of 3 consecutive diameters. The post deflation peak diameter was calculated from the maximum diameter of the moving averages. Absolute and relative FMD were calculated as follows:

Absolute FMD (mm) = Peak diameter - resting diameter

$$\text{Relative FMD (\%)} = \frac{(\text{Peak diameter} - \text{resting diameter})}{\text{Resting diameter}} \times 100$$

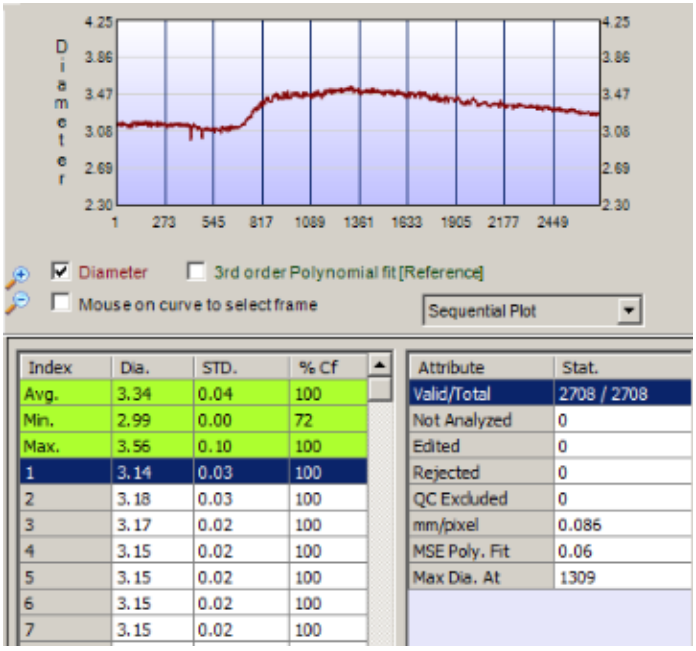

Figure 4.6. Analysis of brachial artery diameter of a subject recorded from 30 s prior to cuff deflation for 180 s, from the software (Brachial Analyzer for Research).

Although shear stress is the established stimulus for FMD response, accurate *in vivo* measurement of it is very difficult. As blood viscosity does not differ considerably between individuals or after interventions (Padilla et al., 2008), shear rate is often calculated as an indication of the stimulus for FMD. Velocity time integral (VTI) for blood flow was exported from the Brachial Analyzer for Research and the average VTI per second were calculated. The area under the shear rate curve (AUC) was calculated as (8 x VIT)/baseline diameter (Rakobowchuk et al., 2012). AUC to peak diameter was calculated as an indication of the stimulus for FMD as recommended (Thijssen et al., 2011). Time to peak diameter was also calculated as the time period starting from deflation to the onset of the maximum diameter of the moving averages.

FMD was not normalised to shear rate in this thesis as the validity of this approach is questionable in the literature (Thijssen et al., 2011; Atkinson et al., 2009). The accuracy of this approach depends on the relation between FMD and shear stress. It is only recommended when this relation is at least moderately strong, linear and consistent between subjects and groups, which is only observed in young subjects but not in other groups (Atkinson et al., 2009; Thijssen et al., 2009). Nonetheless, it is recommended in the expert-consensus guideline that shear rate should be calculated and considered as the stimulus for FMD; the relationship between FMD and shear rate should be investigated if necessary (Thijssen et al., 2011). It should also be noted that the magnitude of FMD is not determined by shear rate per se. Arguably, other factors like endothelial function to produce vasodilators in response to a given shear stress, characteristics of the vessel wall (Lehoux et al., 2006), function of the smooth muscles in response to vasodilators also contribute to the resultant FMD magnitude.

4.3.2.4 Reliability and validity of brachial artery FMD measurement

Although it has been reported that brachial artery FMD is more reproducible than other non-invasive techniques in evaluating endothelial function (Onkelinx et al., 2012), it is noteworthy that FMD is sensitive to methodology-related factors and subject-related factors.

Among methodology-related factors, the method to create shear stress has a crucial impact on the mechanisms of FMD. Distinct shear stress profiles created with different methods (reactive hyperaemia, hand warming, or infusion of vasodilator) yield varied mechanisms of the resultant dilation (Pyke and Tschakovsky, 2005). The duration and position of cuff occlusion both independently determine whether the dilation is NO-dependent. More specifically, FMD in response to reactive hyperaemia created with the wrist occlusion for 5 min was significantly supressed by the infusion of the nitric oxide synthase (NOS) inhibitor N^G-monomethyl-L-arginine (L-NMMA) whereas the dilation was not affected by L-NMMA after the wrist occlusion for 15 min (Mullen et al., 2001). Similarly, FMD response induced by reactive hyperaemia resulting from the distal occlusion was abolished while the response after proximal occlusion was only moderately attenuated (Doshi et al., 2001). Nitric oxide-dependent brachial artery FMD is assumed as an indication of *in vivo* NO bioavailability thereby reflecting vascular function. Hence, the distal occlusion for 5 min was adopted in the present study to create a reactive hyperaemia resulting in a primarily NO-dependent FMD. In addition, continuous measurement of brachial artery diameter across the cardiac cycle, data analysis using automatic edge-detection software, identifying the true peak diameter post deflation, and use of a probe clamp are documented to improve reproducibility (Greyling et al., 2016). Therefore, these methodology-related factors were controlled for according to the guidelines to improve the reliability of repeated FMD measurements.

Regarding subject-related factors, age, hypertension and dyslipidemia were reported to significantly contribute to the variability of brachial artery FMD (van Mil et al., 2016). Even in healthy subjects, several factors still lead to variations in FMD: intake of polyphenols (Lekakis et al., 2005), vitamins (Title et al., 2000), caffeine (Papamichael et al., 2005), medications, high fat intake (Wei-Chuan et al., 2004), high sugar intake (Title et al., 2000), smoking (Celermajer et al., 1993), recent exercise (Clarkson et al., 1999), and menstrual phase (Williams et al., 2001). Moreover, FMD also demonstrates hourly variations (Järvisalo et al., 2006). Hence, these subject-related factors were controlled for when conducting FMD.

In order to access intra-reliability, repeated measures of FMD were conducted on 10 healthy subjects with each individual examined during the same time of the day on two consecutive days. The coefficient of variation (CV; calculated as the standard deviation divided by the mean) of baseline brachial artery diameter was 0.4%. The CV of relative FMD was 6.12%, demonstrating good reproducibility. The 95% confidence interval (CI) for the difference in relative FMD between the means of repeated measures was -0.47% to 0.51%. The reproducibility of repeated FMD measures with a CV of 0-10% was considered as excellent in a meta-analysis study on the reproducibility of repeated FMD measures across different research centres worldwide (van Mil et al., 2016).

4.3.3 Processing and analysis of biological samples

Fasting blood samples were left at room temperate for 30 min to allow a clot to form. Serum was separated immediately following centrifugation (3000 x g for 10 min at 20°C). Plasma was also separated immediately following centrifugation (2000 x g for 10 min at 4°C). Spot urine samples were centrifuged at 2000 x g at 4°C for 15 min. The supernatant of urine samples were filtered through a 0.22 µm CA-CN syringe filter (Gilson Scientific Ltd, Bedfordshire, UK). Thereafter, aliquots of serum, plasma and urine samples were stored at -80°C prior to analysis. Plasma ET-1 was analysed using QuantiGlo ELISA from R&D Systems (Abingdon, UK). Urine samples of 1 ml were freezed drid for future analysis of creatinine and metabolites of citrus flavonoids.

Serum samples were sent to Research and Development (Blood Sciences, Leeds General Infirmary) for analysis of lipid profile, high sensitivity C-reactive protein (hsCRP) and oestradiol. Total cholesterol, HDL cholesterol (HDL-chol), triglycerides (TG) were measured by enzymatic assays (Siemens Healthcare Diagnostics Inc., Erlangen, Germany). Concentrations of LDL cholesterol (LDL-chol) were calculated with the Friedewald equation where all concentrations were in mmol/L ([LDL-chol] = [Total chol] - [HDL-chol] - ([TG]/2.2)). Inflammatory marker hsCRP was analysed using ADVIA Chemistry XPT System (Siemens Healthcare Diagnostics Inc., Erlangen, Germany). Oestradiol was measured using an immunoassay (Siemens Healthcare Diagnostics Inc., Erlangen, Germany).

4.3.4 Test drinks

Three brands of blood orange juice are commercially available in the UK: Tropicana, Innocent and Waitrose. The flavanone content of these samples were analysed by LCMS (as detailed in section 3.3.2) and the juice with highest concentrations of flavanones was selected for the present study. Orange juice drink (Capri-Sun Orange Juice Drinks) with similar carbohydrate content was selected as the control drink and the flavanone content was also analysed by LCMS. The composition of test drinks are shown in Table 4.1. The juice for 2-week consumption was provided to each subject prior to the intervention and stored in their home refrigerators until consumption.

Table 4.1. Composition of test drinks used in the human intervention study.

	Innocent	Capri-Sun
Drink volume (ml/day)	400	400
Energy (kcal)	188	164
Carbohydrate (g)	44	40
Sugar (g)	38	40
Hesperidin (mg)	320.9[a]	25.1[b]
Narirutin (mg)	38[a]	3.9[b]

Different letters indicate significant differences in hesperidin and narirutin contents of test drinks ($p<0.05$).

4.3.5 Statistical analysis

To detect a 2.0 unit increase in %FMD (the primary outcome of the present study), assuming a standard deviation of 2.0 (based on reliability data), with 80% power and at the 5% significance level, a total sample size of 10 subjects was required to complete a two-treatment crossover study.

Data are presented as mean ± standard deviation. Statistical analysis was conducted using Statistical Package for the Social Sciences (SPSS, version 24, IBM Corporation, USA). Data were tested for normality using Shapiro-Wilk test. Data at the end of each treatment were analysed using a linear mixed model for crossover trials, with age, BMI, baseline values as covariates, subjects nested within treatment sequence as the random effect, and gender, dietary treatment, treatment sequence, experimental period, the interaction between treatment and experimental period as fixed effects. The flavanone contents in

different juices were analysed using one-way ANOVA. Significance was defined at $p < 0.05$. Pearson's correlation analyses were conducted.

4.4 Results

4.4.1 Characteristics of test drinks

Quantification of flavanone composition is detailed in Chapter 3. Hesperidin and narirutin were found to be the major flavanones in the blood orange juices analysed. The flavanone compositions of the three commercially available blood orange juices are displayed in Table 4.2. Hesperidin, narirutin and total flavanones in the blood orange juice from Innocent were significantly higher compared with the other two brands. Therefore, blood orange juice from Innocent was selected for the present study.

Table 4.2. Flavanone contents in three commercial available brands of blood orange juice (mg/100 ml). Data are mean ± SD (n=3).

	Hesperidin	Narirutin	Total flavanones
Innocent	80.2 ± 2.7^{a}	9.5 ± 0.1^{a}	89.7 ± 2.6^{a}
Waitrose	58.6 ± 3.0^{c}	5.1 ± 1.2^{b}	63.7 ± 2.6^{c}
Tropicana	70.7 ± 1.7^{b}	5.8 ± 1.5^{b}	76.5 ± 0.4^{b}

Means within the column without the same letter (a, b, c) are significantly different ($P < 0.05$; one-way ANOVA with Tukey's test).

Compared with the flavanones in the blood orange juice (drink A), those in the orange juice drink (drink B) were negligible (Figure 4.7). Regarding intervention compliance, only two subjects missed drinking juice once during the 2-week consumption and all the other thirteen subjects kept drinking juice twice per day during two 2-week periods, according to the citrus drink consumption record forms.

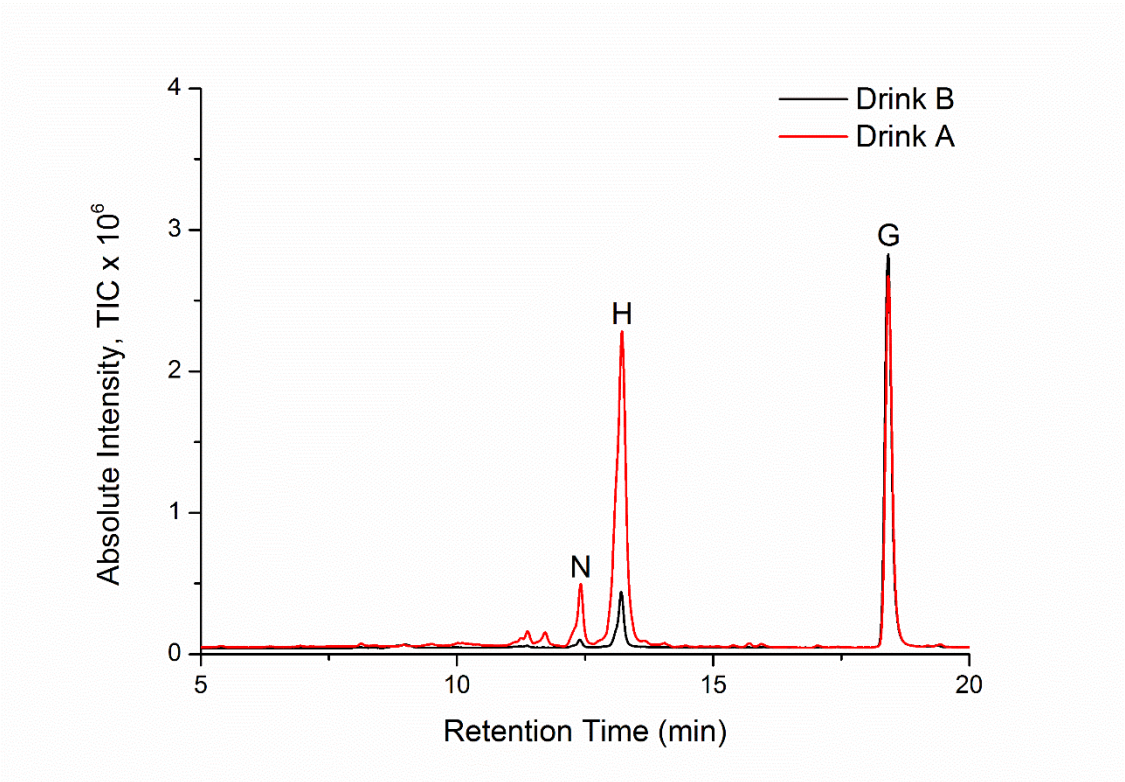

Figure 4.7. LCMS chromatogram of citrus flavonoids extracted from blood orange juice (drink A) and orange juice drink (drink B). N is narirutin, H is hesperidin and G is internal standard genistein.

4.4.2 Baseline subject characteristics

The baseline clinical characteristics of subjects in the present study are summarised in Table 4.3. Fifteen overweight (body mass index 28.3 ± 3.1 kg/m^2) subjects (aged 20-45 y) were enrolled in the study, with ten premenopausal women and five men. The premenopausal women were relatively younger than the men (26.7± 4.7 y and 32.8 ± 8.1 y, respectively), with a slightly lower BMI of 27.8 ± 2.5 kg/m^2 compared with 29.3 ± 4.2 kg/m^2 of men. The subjects were normotensive with average blood pressures of 110 mmHg over 91 mmHg. The averages of lipid profiles were generally within healthy range: total cholesterol < 5.2 mmol/L, HDL-cholesterol close to 1.6 mmol/L, LDL-cholesterol < 3.3 mmol/L, triglycerides < 1.7 mmol/L. The average of inflammatory marker hsCRP was < 3 mg/L, indicating low levels of inflammation in the studied subjects. Taken together, the subjects were generally healthy and at low risk of cardiovascular diseases.

Table 4.3. Baseline clinical characteristics of the study population (n = 15, F= 10, M =5).

	Mean ± SD	Range
Age (y)	28.7 ± 6.5	20-45
BMI (kg/m^2)	28.3 ± 3.1	25.5-36.5
Systolic blood pressure (mmHg)	110.0 ± 12.9	91.0-128.7
Diastolic blood pressure (mmHg)	71.9 ± 9.5	59.3-92.3
Total cholesterol (mmol/L)	4.7 ± 0.6	4.0-5.8
HDL-cholesterol (mmol/L)	1.5 ± 0.5	0.8-2.1
LDL-cholesterol (mmol/L)	2.6 ± 0.4	2.1-3.2
Triglycerides (mmol/L)	1.3 ± 0.6	0.5-2.8
hsCRP (mg/L)	0.9 ± 0.9	0.2-3.4

No changes in diet and lifestyle to change weight were confirmed at each visit. Data from self-reported food records were imcomplete and not robust due to unclear portion sizes and poor compliance in recording, therefore are not shown. However, as shown in Table 4.4 unchanged body weight throughout the study may indicate there were no major changes in the diet and lifestyle.

Table 4.4. Body weight pre and post 2-week consumption of blood orange juice (A) and orange juice drink (B) in a randomised order (n=15).

	PREA	POSTA	PREB	POSTB
Body weight (kg)	83.91 ± 9.53	84.21 ± 9.62	84.70 ± 9.45	84.34 ± 9.68

4.4.3 Effects on brachial-artery endothelial function

Individual variations were observed in baseline brachial artery diameter between subjects, ranging from 2.91 mm to 4.71 mm. Distinct differences in baseline brachial artery FMD were observed between subjects (4.14%-14.78%), as illustrated in Figure 4.8. Baseline brachial artery FMD was significantly inversely associated with resting brachial artery diameter (r=-0.538, p=0.039).

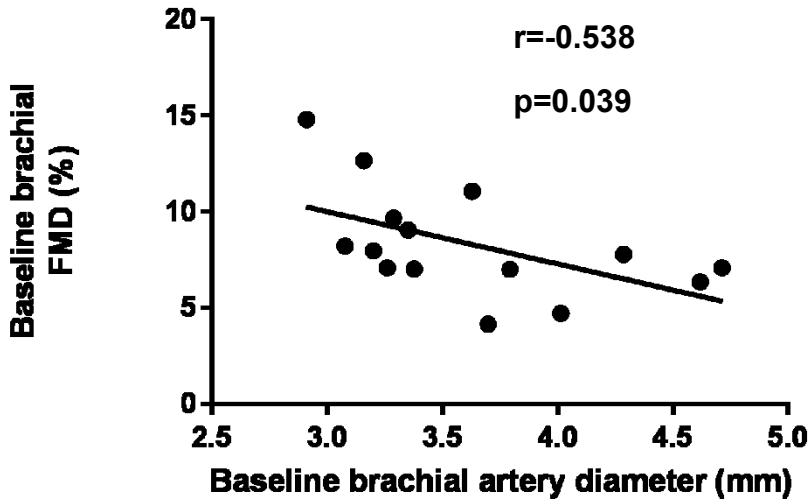

Figure 4.8. The scatterplot of baseline brachial artery diameter (mm) and baseline brachial FMD (%) prior to any treatment (n=15).

Resting brachial artery diameter did not change following the consumption of either juice, indicating no structural changes of the brachial artery (Table 4.5). The baseline brachial artery FMD values before the consumption of blood orange juice and orange juice drink were similar (8.15 ± 2.92% and 8.11 ± 2.52%, respectively), which may indicate good control of the experiment and no carryover effects.

Table 4.5. Brachial artery endothelial function and endothelin 1 (ET-1) (mean ± SD) pre and post 2-week consumption of blood orange juice (A) and orange juice drink (B) in a randomised order.

	PREA	POSTA	PREB	POSTB
Baseline diameter (mm)	3.62 ± 0.56	3.64 ± 0.54	3.63 ± 0.57	3.62 ± 0.56
AUCpeak (a.u.)	55441.37 ± 19120.73	57299.51 ± 22266.82	57430.87 ± 27386.07	56627.79 ± 21020.8
Absolute FMD (mm)	0.29 ± 0.08[b]	0.36 ± 0.09[a]	0.29 ± 0.07[b]	0.27 ± 0.07[b]
Relative FMD (%)	8.15 ± 2.92[b]	10.16 ± 3.31[a]	8.11 ± 2.52[b]	7.77 ± 2.43[b]
Time to peak diameter (s)	47.62 ± 13.55	47.48 ± 14.76	47.51 ± 17.05	46.52 ± 17.66
ET-1 (pg/ml)	1.08 ± 0.18	1.05 ± 0.27	1.15 ± 0.22	1.09 ± 0.29

Means within the row without the same letter (a, b) are significantly different ($P < 0.05$): absolute and relative FMD significantly increased post 2-week consumption of drink A. All n=15 except for ET-1 (n=10).

The absolute FMD increased significantly after 2-week consumption of blood orange juice from 0.29 ± 0.08 mm to 0.36 ± 0.09 mm (time by treatment interaction: $p=0.001$) whereas no changes were observed after the consumption of orange juice drink (pre and post as 0.28 ± 0.07 mm and 0.27 ± 0.07 mm, respectively). Similarly, the relative FMD significantly increased from 8.15 ± 2.92% to 10.16 ± 3.31% after the consumption of blood orange juice with a significant time effect ($p=0.017$) and a significant treatment effect ($p=0.001$), while there were no changes after the consumption of orange juice drink (pre and post as 8.11 ± 2.52% and 7.77 ± 2.43%, respectively), as illustrated in Figure 4.9. There were significant time by treatment interactions in both absolute and relative FMD ($p=0.001$ and $p=0.002$, respectively). The individual baseline relative FMD also had a significant effect on the relative FMD following the juice consumption ($p<0.001$).

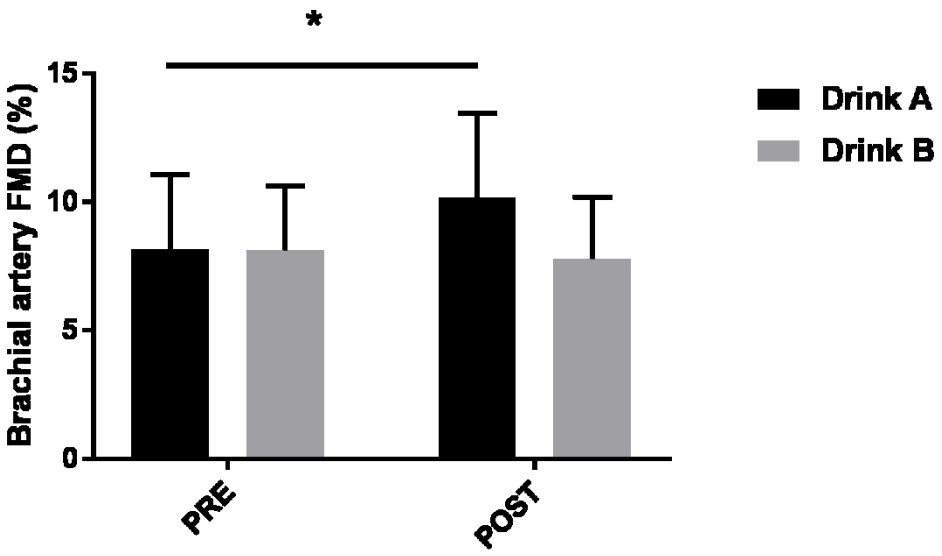

Figure 4.9. Brachial artery relative FMD pre and post of 2-week consumption of blood orange juice (A) and orange juice drink (B) in a randomised order (n=15). Data are mean with SD. *indicates significant differences between pre and post consumption of blood orange juice ($p<0.05$).

Notably, the increase in %FMD after blood orange juice consumption was found to be moderately inversely correlated with baseline brachial artery diameter (r=-0.309, p=0.263) (Figure 4.10).

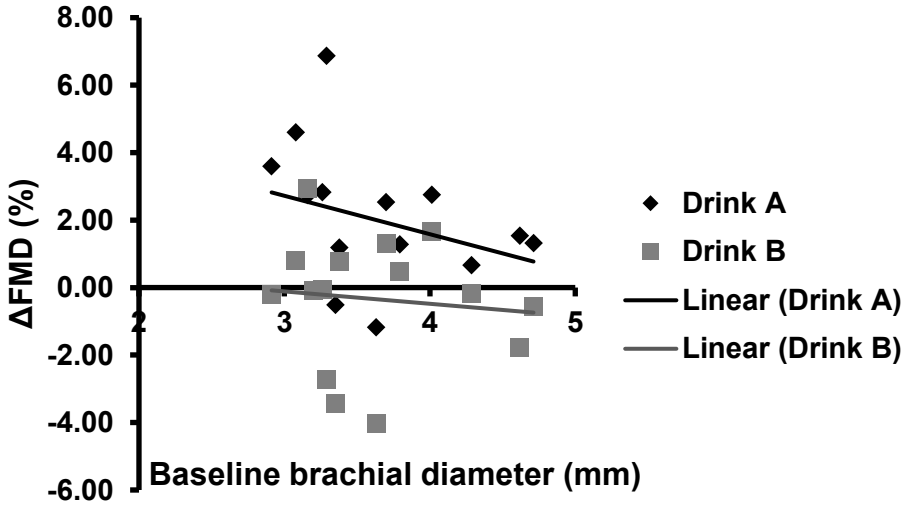

Figure 4.10. The change in relative FMD (%) after 2-week consumption of blood orange juice (A) and orange juice drink (B) in relation to baseline brachial diameter.

No significant changes were observed in the area under the shear rate curve to peak dilation (AUCpeak) as the occlusion to create reactive hyperaemia was controlled to remain the same throughout the study. However, a strong and significant correlation between AUCpeak and absolute FMD was only found after 2-week consumption of blood orange juice ($r=0.603$, $p=0.017$) whereas the correlations were weak and not significant before the consumption of blood orange juice ($r=0.13$, $p=0.658$), before the consumption of orange juice drink ($r=0.229$, $p=0.411$) and after the consumption of orange juice drink ($r=0.259$, $p=0.352$). Similarly, the correlation between AUCpeak and relative FMD was also strong and significant ($r=0.626$, $p=0.013$) while no significant correlations were found before the consumption of blood orange juice ($r=0.273$, $p=0.345$), before the consumption of orange juice drink ($r=0.406$, $p=0.133$) and after the consumption of orange juice drink ($r=0.361$, $p=0.186$). Taken together, it may suggest that the brachial artery responded better to the same stimulus after 2-week consumption of blood orange juice, indicating improved endothelial function. There were no significant changes in time from cuff deflation to peak diameter ($p>0.05$). Additionally, vasoconstrictor ET 1 did not change by the intervention as shown in Table 4.5.

It is a widely held view that FMD is gender and age specific. Therefore, in the statistical analysis, the primary outcome of this study, %FMD was adjusted for age and gender. Age range of the subjects recruited in this study was controlled to minimise the confounding effect of age, in order to investigate the effect of blood orange juice. As a result, the statistical analysis showed that the effect of age on relative FMD (the primary outcome) in this study was not significant ($p=0.619$). The effect of the juices did not differ with gender in this study ($p=0.922$), indicating premenopausal women responded similarly to the juices as men.

Interestingly, favourable effects of blood orange juice on endothelial function were only observed in overweight subjects (n=12) (relative FMD before and following blood orange juice consumption as 7.99% ± 3.23% and 10.49% ± 3.64%, respectively). In contrast, no changes were observed in obese subjects (n=3) following blood orange juice consumption (pre and post as 8.82% ± 1.29%, 8.84% ± 0.95%, respectively) (time by treatment interaction: $p=0.278$). Notably, there was a moderate inverse correlation

between BMI of the subjects and changes in relative FMD following 2-week consumption of blood orange juice (r=-0.416, p=0.123) (Figure 4.11 A). Likewise, changes in relative FMD following 2-week consumption of the control drink were inversely correlated with BMI of the subjects (r=-0.448, p=0.094) (Figure 4.11 B). Taken together, this may indicate that beneficial effects from blood orange juice consumption on endothelial function decreased with an increase in BMI.

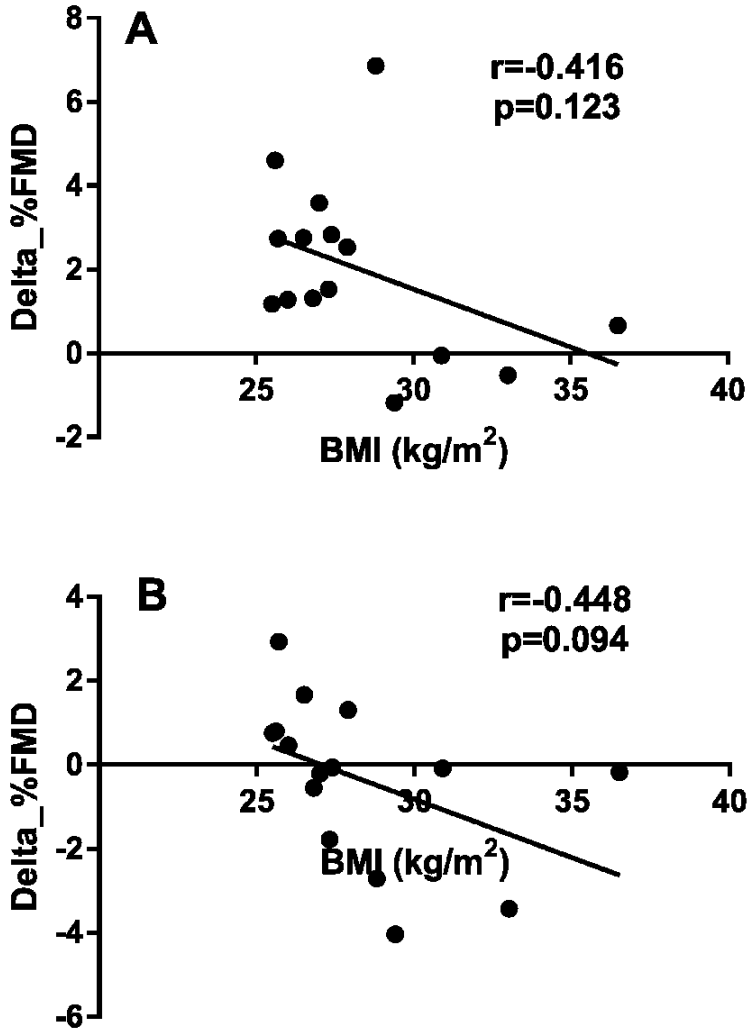

Figure 4.11. Delta of %FMD following 2-week consumption of blood orange juice (A) and the control drink (B) in relation to BMI of subjects.

In addition, the effect of habitual consumption of citrus products on relative FMD changes after juice consumption was not significant (p=0.68).

4.4.4 Menstrual phase control

No significant changes in endogenous oestradiol levels in premenopausal women were observed during the present study ($p > 0.05$) (Table 4.6).

Table 4.6. Oestradiol in premenopausal women pre and post of 2-week consumption of blood orange juice (A) and orange juice drink (B) in a randomised order (n=8).

	PREA	POSTA	PREB	POSTB
Oestradiol (pmol/L)	326.50 ± 269.95	276.25 ± 254.03	266.50 ± 164.89	288.00 ± 213.84

4.4.5 Effect on blood pressure

Systolic blood pressure and diastolic pressure did not change after either blood orange juice consumption or orange juice drink consumption, as shown in Table 4.7.

Table 4.7. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) pre and post of 2-week consumption of blood orange juice (A) and orange juice drink (B) in a randomised order (n=15).

	PREA	POSTA	PREB	POSTB
SBP (mmHg)	108.4 ± 11.5	108.0 ± 10.6	108.4 ± 11.3	107.9 ± 10.7
DBP (mmHg)	70.9 ± 7.5	69.2 ± 7.3	71.6 ± 8.6	69.5 ± 8.1

4.4.6 Effects on lipid profile and inflammation

There were no significant changes in total cholesterol, HDL cholesterol, LDL cholesterol and hsCRP ($p > 0.05$) (Table 4.8). It should be noted that these variables were generally within healthy ranges at baseline.

Table 4.8. Lipid profile and hsCRP pre and post 2-week consumption of blood orange juice (A) and orange juice drink (B) in a randomised order (n=12).

	PREA	POSTA	PREB	POSTB
Total cholesterol (mmol/L)	4.63 ± 0.6	4.68 ± 0.56	4.55 ± 0.57	4.54 ± 0.77
HDL-c (mmol/L)	1.53 ± 0.49	1.57 ± 0.57	1.59 ± 0.57	1.50 ± 0.49
LDL-c (mmol/L)	2.62 ± 0.39	2.60 ± 0.23	2.39 ± 0.46	2.46 ± 0.46
Triglycerides (mmol/L)	1.23 ± 0.77	1.28 ± 0.61	1.38 ± 0.68	1.28 ± 0.61
hsCRP (mg/L)	0.59 ± 0.29	0.58 ± 0.35	0.87 ± 0.82	1.14 ± 1.47

4.5 Discussion

Little is known regarding effects of blood orange juice on endothelial function in the literature. To our knowledge, the crossover study by Buscemi et al. (2012) is the only currently published study to investigate how blood orange juice consumption affects endothelial function as measured by FMD. This previous study demonstrated that consumption of blood orange juice for 1 week resulted in a significant increase in brachial artery FMD and a decrease in inflammatory markers (C-reactive protein, IL-6 and TNF-α) in subjects with augmented cardiovascular risk (Buscemi et al., 2012). Noteworthy, there are several drawbacks in this study. First, baseline FMD was only measured once in this cross-over study, prior to any treatment. The lack of a second baseline FMD measurement before second juice consumption period, together with a relatively short period (3 days) as wash-out, may have resulted in poor control of the study. Hence, carry-over effects could not be ruled out and the drinking sequence might have had an effect on the outcome of this study. Second, subjects enrolled in this study were aged 19-67 years old across both genders. There was no control for the confounding effect of oestrogen in premenopausal women on the day of FMD measurement, therefore, naturally occurring variations in oestrogen in premenopausal women during the 17-day intervention may have contributed to the changes in FMD observed in the study. Exactly how much of the increase in FMD was related to cardio-protective properties of blood orange juice, therefore, is difficult to ascertain in this study. Taken together, caution should be warranted when interpreting these results.

Evidence from previous clinical studies regarding effects of polyphenols on endothelial function in healthy subjects is inconclusive, which might be caused by insufficient control of confounding factors like oestrogen and ethnicity. The present study is the first study to take those factors into study design that showed significantly improved endothelial function in healthy subjects. The main finding were as follows: 2-week consumption of flavanone-rich blood orange juice significantly improved brachial artery FMD, thereby suggesting improved endothelial function, in both men and

premenopausal women; blood pressure, lipid profile and hsCRP were generally within healthy ranges and not affected by the intervention.

4.5.1 Flavanone content

In agreement with previous studies, hesperidin and narirutin were found to be the major flavonoids in orange juice (Vanamala et al., 2006; Mouly et al., 1998). The daily consumption of 358.9 mg total flavanones was ingested from 400 ml of blood orange juice. Extensive analysis of biological samples for the quantification of flavanone metabolites and gut-derived phenolic compounds was beyond the scope of the present study. Although the recovery of flavanones was not determined in the present study, the findings of Pereira-Caro et al. (2014) suggest that hesperidin and narirutin from orange juice are highly bioavailable, when their metabolites and catabolites are both taken into account, with a recovery of over 37% of intake (nearly 100% with hippuric acid taken into calculation). Similarly, anthocyanins from orange juice have been reported to be more bioavailable with a recovery of 44% when taking into consideration metabolites and catabolites in feces (Ferrars et al., 2014; Czank et al., 2013).

4.5.2 Modulation of endothelial function by flavanones

The baseline brachial artery FMD values prior to any treatment observed in the present study are in agreement with other values that have been reported in the literature. A 5-minute ischemia distal to the examined brachial artery by high-resolution ultrasound is associated with 7.3% ± 3.2% FMD response in healthy adults (Thijssen et al., 2008). Baseline brachial artery FMD was significantly inversely associated with baseline brachial artery diameter ($r= -0.538$, $p = 0.039$) in the present study, in accord with earlier research by Celermajer et al. (1992) demonstrating a significant inverse association between the dilation of brachial artery in response to flow increases and baseline artery diameter in healthy subjects.

Substantial variability in shear rate has been reported between individuals (Pyke and Tschakovsky, 2005). Reporting shear rate data and investigating their relationship with %FMD are crucial for accurate interpretation of %FMD and understanding the

mechanisms by which the dilation is elicited, as recommended in the guideline of accurate assessment of FMD (Thijssen et al., 2011). However, little is known on the shear rate in a food consumption intervention whereas only %FMD has been reported in the literature. The present study demonstrated that daily consumption of blood orange juice for 2 weeks resulted in a significant increase in brachial artery FMD while shear rate remained unchanged during the study, indicating the increase in FMD was not due to changes in shear rate. Moreover, strong and significant correlations between shear rate and FMD (both absolute and relative) were only found after blood orange juice consumption. In support of these findings, a significant correlation was only observed in younger adults (27±6 y), but not in older adults (58±4 y) (Thijssen et al., 2009), which may indicate a better preserved endothelial function in younger adults and progressive loss of endothelial function in the aging process. Taken together, consumption of blood orange juice may have contributed to improved endothelial function, thus more dilation was induced in response to unchanged shear rate. Furthermore, another novel finding of the current study is the differential responses of endothelial function to flavonoid-rich food consumption, depending on BMI of the subjects, which to our knowledge, have not been reported in previous studies. However, given the small sample size of the present study, caution should be taken when interpreting those data regarding the effects on obese subjects, which need to be confirmed in future larger cohorts.

The confounding effect of oestrogen was controlled for in the present study resulting in no significant changes in the oestradiol levels in premenopausal women on the day of FMD measurement (before and after either juice consumption). Therefore, the increase in FMD after blood orange juice consumption was not due to changes in oestradiol levels.

Given that vasoconstrictor ET-1 was not altered by the intervention, the significant increase in brachial artery FMD following blood orange juice daily consumption could be attributed to elevated vascular relaxation by high concentrations of flavonoids present in the juice. This favourable effect of blood orange juice on endothelial function accords with our previous *in vitro* study (Chapter 2), which showed that flavanones positively modulated gene expression relevant to NO production and antioxidant status in human endothelial cells. In support of these findings, consumption of blood orange juice for 21

days (600 ml/day) significantly increased plasma antioxidant concentrations (Riso et al., 2005). Consumption of blackcurrant juice rich in polyphenols and vitamin C for 6 weeks (250 ml, 4 times per day) also resulted in significant increase in FMD in healthy adults (Khan et al., 2014).

In contrast, a randomised parallel study by Salden et al. (2016) did not show any significant changes in FMD in healthy overweight individuals (aged 54±15 y, 50% female) after 6-week consumption of hesperidin 2S extract derived from *citrus sinesis* peel. It is noteworthy that the confounding effect of oestrogen in premenopausal women was not controlled for in this previous study. Whereas in a randomised crossover study by Rizza et al. (2011), 3-week consumption of hesperidin resulted in a significant increase in FMD from 8.24±0.88% to 10.26±1.19% (both mean ± SEM), to a similar extent as the present study, but in subjects (aged 52±2 y) with metabolic syndrome. It should be noted that in this previous study, it was unlikely there was a confounding effect of oestrogen on the results given the age range of subjects. Other factors might have also contributed to the discrepancy between these two studies, including different subject profile, inter-individual variations, compliance and co-ingestion of food.

Although the effect of age on %FMD was not significant in this study, it should be noted that there is a decline in %FMD in aging process: in healthy men, FMD is preserved until 40 years of age before a gradual decline, compared with a steeper decline in healthy women after early 50s, as a result of aging-associated progressive endothelial dysfunction (Celermajer et al., 1994). Healthy subjects with relatively preserved brachial artery FMD were selected for this study. Time from cuff deflation to peak diameter was not affected by treatment in the present study, as this variable is more associated with age (Black et al., 2008), with an increase in aging process.

Regarding the effects of flavonoid-rich food on endothelial function, a systematic review of RCTs demonstrated that consumption of flavonoid-rich products resulted in an increase in %FMD both acutely (2.33%) and chronically (0.73%), with a greater magnitude of %FMD increase in an acute manner (Kay et al., 2012). However, studies on flavanone-rich food were not covered in this systematic review due to lack of clinical

data on flavanone-rich food. Caution should be taken when interpreting the acute increase in %FMD after flavonoid-rich food consumption, as %FMD is not measured in a fasted state and therefore may not reflect changes in endothelial function truthfully. FMD as a measure of endothelial function should be measured in a fasted state, according to established guidelines for accurate assessment of FMD (Thijssen et al., 2011; Harris et al., 2010; Corretti et al., 2002).

4.5.3 Modulation of blood pressure by flavanones

Previous human clinical data have shown that chronic consumption of citrus juice (Asgary and Keshvari, 2013; Reshef et al., 2005) or hesperidin (Morand et al., 2011) result in a reduced blood pressure but this is not evidenced by other studies (Habauzit et al., 2015; Rizza et al., 2011). No significant changes in blood pressure were observed in the present study. A reduction in blood pressure after consumption of blood orange juice was not expected in the present study, given the baseline blood pressure values were relatively low (110.0 ± 12.9 mmHg systolic and 71.9 ± 9.5 mmHg diastolic).

4.5.4 Modulation of lipid and inflammation by flavanones

Plasma lipid concentrations are conventional cardiovascular risk factors. High-sensitivity CRP (hsCRP) as the prototypic marker of inflammation is often used as an independent risk marker for prediction of future cardiovascular diseases (Ridker et al., 2001). Chronic effects of flavanones from citrus juice or extract on these markers have been controversial in the literature, probably due to different compositions of juice or extract and differences in baseline levels of lipid and inflammation in the subjects. Specifically, effects of 500 ml daily consumption of blood orange juice for a longer term (12 weeks) moderately reduced total cholesterol and LDL cholesterol in healthy overweight or obese women whereas the effects on inflammatory markers varied according to grade of obesity, favourable effects only observed in subjects with BMI lower than 30 kg/m^2 (Azzini et al., 2017). Similarly, in the present study favourable effects of blood orange juice consumption on relative FMD were also only observed in overweight subjects, with an absence of an effect in obese subjects. However, given the small sample size of the present study, further investigation on more subjects is needed. Taken together, it may

suggest cardiovascular protective effects of blood orange juice are more likely to occur in normal-weight and overweight subjects, but not in obese subjects.

In agreement with these findings, a randomised parallel study by Dallas et al. (2014) demonstrated that consumption of blood orange derived extract for 12 weeks resulted in a reduction in inflammatory markers and oxidative stress in healthy overweight subjects. In contrast, a randomised parallel study suggested that 12-week consumption of orange juice (250 ml/day) in subjects with increased CVD risk resulted in a reduction in triacylglycerol concentrations in subjects with highest initial levels while lipid profile and inflammatory markers were not affected (Simpson et al., 2016). In contrast, chronic favourable effects on lipid profile and inflammation biomarkers were observed in normal weight and overweight subjects after daily consumption of 750 ml of orange juice for 8 weeks (Dourado and Cesar, 2015).

4.5.5 Conclusions

The present study is the first study to show significantly improved endothelial function in healthy overweight but not obese men and premenopausal women of European origin from daily consumption of blood orange juice, compared to an orange juice drink with matched sugar content, with confounding effect of oestrogen levels controlled for. The increase in FMD was not due to a change in shear stimulus, the arterial structure, or ET-1, indicating enhanced NO bioavailability by citrus flavonoids. This effect of blood orange juice did not differ with gender. Baseline blood pressure, lipid profile and inflammation of studied subjects were generally within healthy ranges and no changes were observed in the present study.

Shear rate as the stimulus for FMD is crucial to the interpretation of FMD data and the understanding of endothelial function. However, it has rarely been discussed when investigating effects of polyphenols on endothelial function. In the present study, induced by unchanged shear rate, improved endothelial function was observed. This increase in FMD might be due to elevated NO bioavailability after blood orange juice consumption which is supported by our *in vitro* assay findings.

4.5.6 Limitations and future work

There are several limitations to the present study. First, distinct differences in the colour and taste of the two juices made double-blind impossible. However, the two juices were coded in a randomised manner and these codes were used in all the labelling of biological samples and FMD data. The researchers who conducted the analysis of the biological samples and FMD data were blinded to which juice the subject had been consuming. In addition, it was considered unlikely that the outcomes of the present study (e.g., endothelial function, lipid profile, hsCRP) were influenced by subjects knowing which juice they were consuming. Second, although vitamin C content was not matched in the control drink, it is unlikely the observed enhancement in FMD was due to vitamin C present in the juice for the following reasons: clinical data suggest that doses of vitamin C up to 500 mg did not alter endothelial function, both acutely and chronically (Ashor et al., 2015) whereas vitamin C content in the blood orange juice ingested in the present study was only 84 mg per 200 ml consumption (168 mg daily with breakfast and dinner). In addition, given the short half life of vitamin C, it seems unlikely that vitamin C exerted any effect on the markers determined after a 12 h overnight fast. Finally, blood samples from three subjects were incomplete due to difficulties in venepuncture. These subjects were with higher BMI (29, 33, 36.5 kg/m^2) compared with others enrolled in the study. The information on how consumption of blood orange juice modulated their lipid and inflammation profiles, therefore is lacking.

Although the present data may encourage the consumption of blood orange juice, it should be noted that the favourable effects on endothelial function may not only be subject to the composition of the juice ingested (high or low contents of flavonoids, fibre and sugar), but also potentially subject to age, BMI, baseline inflammation levels of subjects. Randomised controlled intervention studies are needed to test the effects of long term consumption of blood orange juice on vascular function in larger number of individuals, as well as in other ethnic groups. In addition, the novel finding in the differential responses of endothelial function to blood orange juice consumption depending on the BMI of the subjects is valuable for future RCTs and warrants further investigation in more subjects. It would also be interesting to see if structural vascular changes occur after long term consumption of flavonoid-rich foods or beverages.

Although functional vascular changes (indicated by %FMD) induced by polyphenols have been intensively investigated in recent years, structural vascular changes by polyphenols have not been investigated in previous studies yet are of great importance in better understanding the mechanisms underlying chronic consumption of a certain food, before recommendations for the general population can be made.

Chapter 5 General discussion

5.1 Overview of thesis

The incidence of cardiovascular disease (CVD) is not only the leading cause of death worldwide, but is also increasing globally, especially in developing countries (World Health Organisation, 2016). Consequently, CVD causes huge economic loss and diminishes the quality of life. As a well known modifiable factor of CVD, a healthy diet is highly recommended as a balanced combination of low levels of saturated fat, salt, sugar as well as abundant fibre and wholegrain foods, fruits and vegetables.

In recent years, there has been increasing research on flavonoids, in light of an inverse association between flavonoid intake and the risk of CVD suggested by numerus epidemiological studies (Rienks et al., 2017; Tresserra-Rimbau et al., 2016). Among flavonoid-rich products, citrus fruits and juices are not only prevalently consumed worldwide, but also have high bioavailability, the consumption of which, therefore is of great significance. However, the effect of citrus products are actually less researched than other flavonoid-rich products such as green tea, soya, cocoa and dark chocolate. Furthermore, epidemiological studies have suggested that higher habitual intake of flavanones is significantly associated with a lower risk of ischemic stroke (Cassidy et al., 2016) in healthy men and coronary heart disease in postmenopausal women (Mink et al., 2007).

However, results from *in vivo* human studies are conflicting. Additionally, *in vitro* studies investigating the mechanisms by which citrus flavonoids may exert beneficial effects on endothelial cells are limited. Therefore, the aim of this thesis was to investigate effects of citrus flavonoids on cardiovascular health, with a focus on endothelial function.

The present data indicate brachial artery NO-dependent FMD was significantly increased following 2-week consumption of blood orange juice in healthy overweight subjects of European origin, whereas there were no significant changes in the brachial artery structure, shear stimulus, oestrogen levels or vasoconstrictor ET-1 (Chapter 4),

suggesting a functional improvement in endothelial function, likely due to elevated NO bioavailability by citrus flavonoids. This is in agreement with improved vascular compliance following orange juice consumption (Chapter 3) as well as enhanced NO production and elevated mRNA levels of eNOS and antioxidant genes induced by citrus flavonoids (as illustrated in Figure 5.1, data from Chapter 2). It should be noted that only nobiletin was tested for its effect on NO production by DAF-2 assay. It remains possible other citrus flavonoids and their metabolites may also exert favourable effects on NO production. Increased NO bioavailability might be due to ROS reducing capacity by citrus flavonoids to preserve BH_4 as well as NO induction and increased antioxidant status by citrus flavonoids.

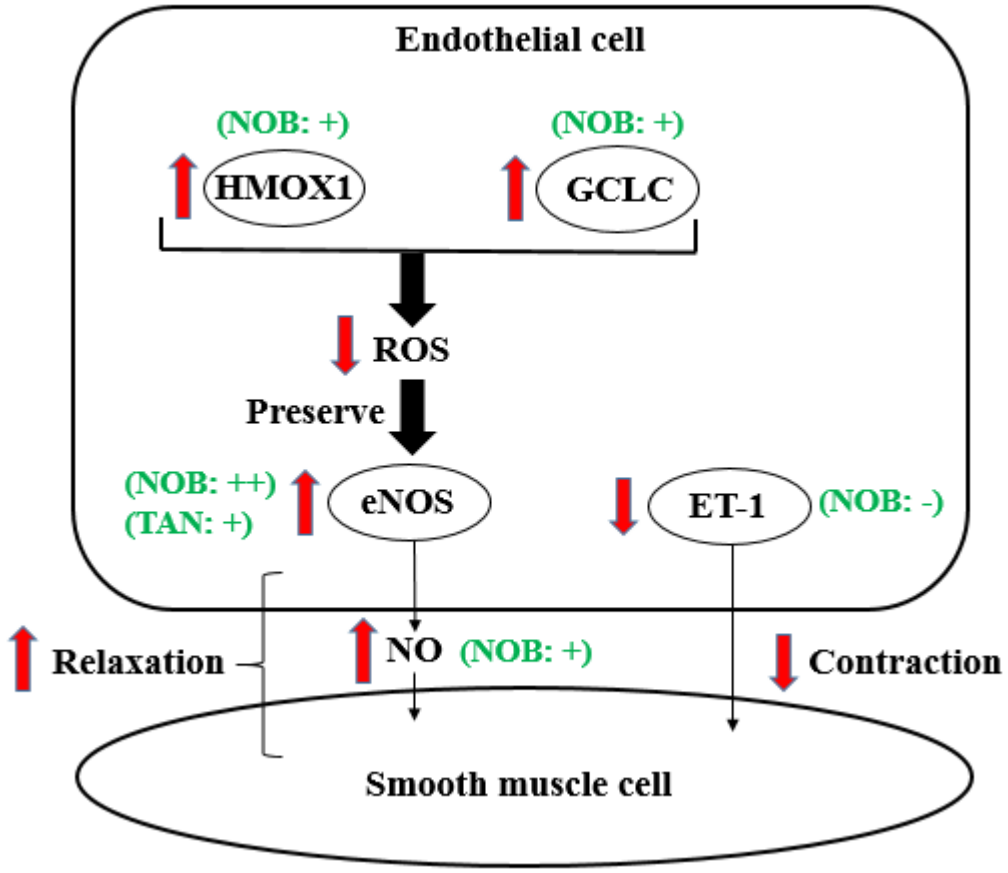

Figure 5.1. Effects of citrus flavonoids on relaxation and contraction pathways. eNOS, endothelial nitric oxide synthase; ET-1, endothelin 1; GCLC, gamma glutamyl cysteinyl synthetase; HMOX1, heme oxygenase-1; NO, nitric oxide; NOB, nobiletin; ROS, reactive oxygen species; TAN, tangeretin; +, significant increase; -, significant decrease.

Given the key role of NO in modulating vascular tone, inhibiting inflammation and platelet aggregation, how citrus flavonoids regulate its synthesis therefore is of great interest and was investigated in Chapter 2 in EA.hy926 cells. In addition, excessive amounts of superoxide not only reacts with NO, reducing its bioavailability, but also leads to eNOS uncoupling. As a result, eNOS is switched from producing NO to generating superoxide, contributing to oxidative stress in endothelial cells by this vicious cycle as detailed in Chapter 1. Hence, the effect of citrus flavonoids on gene expression related to antioxidant defence and cell protection against oxidative stress is also important, in order to maintain normal eNOS function and therefore normal endothelial function. Hesperetin, naringenin, nobiletin, tangeretin were tested for their potential in regulating mRNA expression of NOS3, HOMX1 and GCLC. Most abundant in citrus peels, the effect of nobiletin on NO production and upregulation of the expression of cytoprotective genes was most potent among tested citrus flavonoids in the present work. In support of this novel finding, a randomised controlled parallel study by Hashemi et al. (2015) demonstrated that endothelial function as measured by FMD in overweight and obese participants (6-18 y) significantly increased following consumption of extracts of lemon and sour orange peels. Although hesperetin and naringenin were not effective in upregulating eNOS, HOMX1, GCLC expression in the present study, it remains possible their metabolites might exert favourable effects by upregulating the expression of these protective genes or by other mechanisms, given that hesperidin significantly improved microvascular reactivity acutely (6 h) and also led to a significant reduction in diastolic blood pressure post 4-week consumption in healthy overweight men (50-65 y) (Morand et al., 2011). Specifically, there are alternative mechanisms by which citrus flavonoids may exert beneficial effect on cardiovascular health such as downregulation of ROS-forming enzymes and inhibition of platelet aggregation and leukocyte adhesion, which have been observed with some flavonoids and warrant further investigation on flavanones.

Since *in vitro* findings may not reflect *in vivo* conditions truthfully, the effects of flavanones have been actively studied in animal models, which have suggested their beneficial effects on endothelial function (Herrera et al., 1996), lipid profile (Cho et al., 2011), blood pressure (Ikemura et al., 2012) and platelet reactivity (Jin et al., 2007). However, evidence from human *in vivo* studies is limited and contradictory. Hence,

scientific evidence demonstrating a favourable impact of dietary flavanones on cardiovascular health in humans has not yet been established. Therefore, the effect of flavanone-rich juices on cardiovascular health was investigated in an acute manner (Chapter 3) and post regular consumption (Chapter 4).

Regarding the selection of citrus product to test *in vivo* effects, flavanone-rich juice was selected instead of citrus supplement or extract, in light of their poor bioavailability. Specifically, plasma concentrations of flavanone aglycones and metabolites are significantly increased post orange juice consumption whereas the same dose of flavanones in the form of supplement results in only a negligible amount of flavanone aglycones and metabolites in a crossover study by Schär et al. (2015). Likewise, the lack of bioactive effect of other flavonoid supplement has been often reported in the literature in RCTs whereas in the same trial flavonoids in the form of foods or beverages exert significant effects on same subjects (Bondonno et al., 2017; Kerimi et al., 2017), highlighting the importance of food matrix on absorption and metabolism of flavonoids. For selection of a flavanone-rich orange juice, the flavanone content of nine commercial orange juice, including blond and blood orange juice, were determined in Chapter 3. The flavanone composition of orange juice was generally in agreement with previous data: hesperidin and narirutin were the predominant flavanones in both blond and blood orange juice; higher total flavanone content was found in made-from-concentrate orange juice in comparison to not-from-concentrate orange juice.

In the present study flavanone-rich blond orange juice did not significantly improve cardiac electrical activity, blood pressure, vascular compliance, peripheral blood flow, pulse transit time and pulse wave velocity in an acute manner, which is in agreement with the findings of a randomised controlled crossover study investigating acute effects of orange juice and hesperidin (Schär et al., 2015). However, it should be noted that the approaches used in these two studies (plethysmography and RH-PAT, respectively), although validated, are not as sensitive as FMD. Therefore, favourable effects on endothelial function was observed acutely (2-7 h post blond orange juice consumption) using FMD under the challenge of double high-fat meal (Rendeiro et al., 2016).

Given that the favourable effect of flavonoid-rich products on endothelial function is relatively small (Kay et al., 2012), a more sensitive approach might be better to detect this small but clinically meaningful difference post consumption. Hence, FMD was used for the following *in vivo* investigation on regular consumption of flavanone-rich products. A flavanone-rich blood orange juice was selected for this intervention, given that blood orange juice has been less studied than blond orange juice and its effect on cardiovascular health is largely unknown. With additional anthocyanins, there might be additive or synergistic activity of anthocyanins and flavanones, resulting in enhanced effect on outcomes of interest.

One of the novelties in this study design is that only subjects of European origin were recruited, in light of distinct genotypes observed between different ethnic groups, which play a crucial role in absorption and metabolism of flavonoids (Cassidy and Minihane, 2016). Specifically, LPH is a key enzyme in the initial hydrolysis of flavonoid glycosides and therefore is of great importance to the following absorption and metabolism of flavonoids. However, LPH deficiency is prevalent in African and Asian adults but very rare in European adults. Furthermore, gut microbiome also plays an important role in the biotransformation of not only flavonoids but also their phase I and II metabolites (Williamson and Clifford, 2010). Indeed, large variability in microbial-derived metabolite profiles has been observed between Western and Asian populations. For instance, it is estimated that 50-70% of Asians can produce the microbial-derived metabolite equol following consumption of soya products, whereas there are only 25-30% of equol producers in Western populations (Setchell et al., 2013). Therefore, investigation regarding the effect of blood orange juice on subjects of European origin, instead of subjects from mixed ethnic groups, narrows down the genotype-associated variations in the bioavailability of flavonoids, thereby reducing potential confounding factors in bioactivity of flavonoids and their metabolites. Another novelty in this study is the consideration of female hormone impact on evaluation of endothelial function by FMD in premenopausal women, thereby reducing confounding effects of high oestrogen levels on endothelial NO production.

In this study (Chapter 4), given that healthy overweight/obese subjects were recruited, their baseline endothelial function (primary outcome), blood pressure, lipid profile and hsCRP were generally within healthy ranges. Following 2-week regular consumption of blood orange juice, a significant increase was observed in endothelial function (pre: 8.15% ± 2.92%, post: 10.16% ± 3.31%) (time by treatment interaction: P=0.002), whereas endothelial function did not change following consumption of the control drink (pre: 8.11% ± 2.52%, post: 7.77% ± 2.43%). In addition, no significant changes were observed in oestrogen levels and shear rate (AUCpeak), indicating the increase in FMD was not due to those factors. Furthermore, a strong and significant correlation between AUCpeak and absolute FMD was only observed post 2-week consumption of blood orange juice (r=0.603, p=0.017), further indicating improved endothelial function. Moreover, the effects of the juices on FMD did not differ with gender (p=0.922), suggesting premenopausal women responded similarly to blood juice and control drink as men. Interestingly, this favourable effect of blood orange juice on endothelial function was only observed in overweight subjects but not in obese subjects. Other outcomes, such as blood pressure, lipid profile and hsCRP, were generally within healthy ranges during the intervention and were not altered.

5.2 Impact and implications of this thesis

The work from this thesis investigated how selected citrus flavonoids modulated the expression of eNOS and cytoprotective genes in human endothelial cells to explore the mechanisms by which flavanones impact endothelial function, flavanone content in commercial orange juices, the acute effect of a flavanone-rich blond orange juice on vascular function in healthy subjects as well as effect of regular consumption of a flavanone-rich blood orange juice on endothelial function, blood pressure, lipid profile, inflammation in overweight/obese subjects of European origin. The *in vitro* and *in vivo* methods established in this thesis can be used to explore the effect of other flavonoids and metabolites on endothelial function. The results provide novel insights into how citrus flavonoids regulate endothelial function, both *in vitro* and *in vivo*, as well as other cardiovascular biomarkers in healthy subjects. The investigations regarding the effects of citrus flavonoids on cardiovascular health in this thesis, both *in vitro* and *in vivo,* were

conducted under healthy conditions, given that CVD can be prevented to some extent and prevention is better than treatment.

Among citrus flavonoids tested, nobiletin was most potent at inducing NO production and cytoprotection as well as inhibiting vasoconstrictor ET-1 mRNA expression at higher concentrations. This novel finding, together with its anti-inflammatory effect (Chen et al., 2017) and neuroprotective effect (Zhang et al., 2016), makes it a promising supplement. Given that the citrus peel and seeds are abundant in numerous flavonoids, including narirutin, naringenin, hesperidin and nobiletin, utilisation of these by-products into functional supplements may be of great interest as it will not only reduce substantial amounts of agricultural and food by product waste, but also provide functional supplements from natural sources.

Shear rate (AUCpeak) as the stimulus for FMD is crucial to the interpretation of FMD data and the understanding of endothelial function. Importantly, if shear rate is not carefully controlled by reactive hyperaemia created with accurate cuff occlusion, it will give rise to variations in the resultant FMD, which might be mistaken as a 'functional change' after an intervention (a type I error). This might to some extent account for the conflicting evidence in the literature. However, shear rate has rarely been calculated and discussed in the literature when investigating effects of polyphenols on endothelial function. In the present study, unchanged shear rate confirmed the increase in FMD was not due to increased stimulus, thereby indicating improved endothelial function. Future studies investigating effect of polyphenols or any given factors (for example continuous or interval exercise) should calculate and report shear rate, together with resting branchial artery diameter and FMD (absolute and relative values). Caution should be taken when examining FMD data, given that 1% decrease in FMD is associated with 13% increase in CVD risk. Therefore, associations between shear rate and FMD should also be explored to provide additional information on endothelial function. Furthermore, confounding factors such as oestrogen levels in premenopausal subjects and recent physical activity should be controlled and monitored when conducting FMD for evaluation of endothelial function, in order to examine the effect of an applied factor in an intervention study. This applies to any research area that involves FMD measurement.

With increasing understanding of flavonoid absorption and metabolism, its complexity has now been more recognised, due to numerous factors such as age, gender, genotype, gut microbiota and lifestyle. Investigations into a specific subject group might narrow down variations and provide information on the effect of flavonoids on this specific group. The recruitment of overweight/obese subjects of European origin, as performed in this thesis, is a good example. Controlling ethnicity in order to narrow down variations in genotypes is important, in light of the wide inter-individual variability observed between different ethic groups, probably due to distinct habitual dietary patterns and genetic variations. Otherwise, recruitment of subjects with mixed characteristics might obscure an existing effect following flavonoid intake in a responsive subgroup but not in others. For instance, equol has been recognised to be more bioactive than its food precursor daidzein. Beneficial effects of equol can only be seen in equol producers but not in non-producers. Therefore, future RCTs should be focused on specific subject groups (based on age, gender, ethnicity/genotype, BMI, CVD risk).

Another novel finding of this thesis is the differential responses in endothelial function observed between overweight and obese subjects following blood orange juice consumption, again highlighting the importance of characterising subjects into specific subgroups. Although the sample size in the present study is small and present results warrant further confirmation in larger cohorts, the differential responses based on BMI in the present study might not be a simple coincidence, as supported by other studies where favourable effects in inflammatory markers were only observed in overweight subjects but not in obese subjects following consumption of blood orange juice for 12 weeks (Azzini et al., 2017). The lack of favourable effects in obese subjects on endothelial function observed in the present study and inflammatory markers observed in the previous study by Azzini et al. (2017) might be explained by obesity-induced imbalance between the NO pathway and the vasoconstrictor ET-1 (Iantorno et al., 2014) as well as obesity-induced imbalance between pro-inflammatory and anti-inflammatory adipokines, which contributes to the development of chronic inflammation and therefore increases the risk of systemic metabolic dysfunction and CVD (Nakamura et al., 2014).

Fruit and vegetable juices contribute to five daily portions of fruit and vegetables. Importantly, it is recommended that daily consumption of juice should be limited to 150 ml (National Health Service, 2015), given the lack of fiber and abundance of sugar in juice. The intake of free sugars should be less than 10% of total energy intake and a further reduction to below 5% might provide additional benefits to maintain healthy body weight and reduce dental caries (World Health Organization, 2015). Excessive sugar intake has been linked with dental caries (Peres et al., 2016), obesity (Gulati and Misra, 2014) and type 2 diabetes mellitus (Malik et al., 2010). In the present study, subjects were asked to consume 200 ml of blood orange juice or a control drink with breakfast and with dinner (400 ml/day), which was higher than the recommendation but below the doses (500-1000 ml/day for 4 weeks) supplemented in some RCTs (Constans et al., 2015; Giordano et al., 2012; Morand et al., 2011). No adverse effects were observed in any outcomes measured following overnight fasting in the present study. In contrast, endothelial function in overweight subjects significantly improved following 2-week consumption of blood orange juice whereas no changes in endothelia function were observed in obese subjects, which might be explained by obesity-induced endothelial dysfunction. Furthermore, the beneficial effects of flavonoids might not outweigh the detrimental effects of high sugar content on obese subjects. Whether juice consumption could be beneficial for obese subjects still warrants future investigation in larger cohorts.

Taken together, the present work will have strong potential to impact the field of polyphenols and health research, in providing insight into mechanisms of action and *in vivo* evidence of the effect of flavanone-rich orange juice on cardiovascular health in a acute manner and following regular consumption, highlighting the importance of examining effect of polyphenols on specific subject groups as well as establishing *in vitro* and *in vivo* methods to evaluate polyphenols for their potential in improving cardiovascular health. Furthermore, suggestions on FMD measurement, data calculation and interpretation go beyond the field of polyphenols and health research and apply to any research area that involves FMD.

5.3 Limitations and future perspectives

There are some limitations to the present work. With flavanone metabolism better understood in recent years, the effect of flavanone metabolites should also be examined on the expression of eNOS and cytoprotective genes. Plethysmography utilised in the acute study might not be sensitive enough for detection of small changes in vascular function following orange juice consumption. In addition, control drinks matched for all the macro- and micro-nutrients except flavanones in both *in vivo* studies would have been better to distinguish the effects of flavanones. However, having a control drink matched for all the macro- and micro-nutrients except flavonoids has been difficult in RCTs in the literature, given the availability of facilities to manufacture such control drinks and the additional cost.

Despite emerging evidence in absorption and metabolism of citrus flavonoids, this is still not fully understood. Ideally, isotopically labelled flavanones can be used for identification and quantification of flavanone phase II metabolites and gut-derived phenolic derivatives. The next step is to synthesise the major phase II metabolites and gut-derived phenolic derivatives at physiologically achievable concentrations to examine how they modulate the gene expression of eNOS, ET-1 and cytoprotective genes, as well as different pathways in inflammation and cytoprotection. In addition, the impact of age, gender, BMI and the microbiome on absorption and metabolism of flavonoids is largely unknown and requires further investigation in clinical studies. Furthermore, distinct variability in bioavailability of flavonoids from different sources (for example, orange juice and hesperidin supplement) has been observed. However, the mechanisms underlying this remain to be resolved in future studies before the consumption of flavonoid extract or supplement can be recommended.

As discussed, long term RCTs on flavanone-rich products with macro- and micro-nutrients matched control products are required to fully understand how flavanones impact endothelial function and other cardiovascular biomarkers according to subject profile (age, gender, BMI, ethnicity/genotype, CVD risk). Urine samples need to be collected for at least 24 h for quantification of metabolites and catabolites post

consumption. Association between the observed effects and concentrations of metabolites and catabolites should also be explored.

Additionally, it would be interesting to see if chronic consumption of flavanones results in not only functional changes but also structural changes in endothelial function. The randomised, placebo-controlled, cross-over study by West et al. (2014) demonstrated that prolonged consumption of flavonoid-rich food increases resting brachial artery diameter and basal blood flow. Specifically, in this previous study, baseline brachial artery diameter and basal blood flow significantly increased after 4-week consumption of dark chocolate and coca drink (total flavanols=814 mg/d) in healthy overweight and moderately obese subjects (BMI 25–37 kg/m^2, aged 40–64 y). Notably, peak diameter post cuff deflation also significantly increased following 4-week consumption of dark chocolate and coca drink, resulting in unchanged FMD (West et al., 2014). However, further studies are needed to confirm changes in resting brachial artery diameter and basal blood flow, induced by regular consumption of flavonoid-rich foods or beverages.

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Appendices

Appendix 1: Ethics approval for Chapter 3

Performance, Governance and Operations
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David Hauton

School of Food Science and Nutrition

University of Leeds

Leeds, LS2 9JT

MaPS and Engineering joint Faculty Research Ethics Committee (MEEC FREC)

University of Leeds

4 June 2018

Dear David

Title of study	**SUPPLEMENTS AFFECTING DAILY ACTIVITIES (The effect of food supplements on breathing, blood pressure and heart rate in daily activities)**
Ethics reference	**MEEC 14-028**

The above project was reviewed by the MEEC Faculty Research Ethics Committee at its virtual meeting of 11th May 2015. The following documentation was considered:

Document	Version	Date
MEEC 14-028 Ethical_Review_Form_V3-D Hauton-Supplements & Activities-version Ia.doc	1	14/04/15
MEEC 14-028 Risk assessment-Breathing altered gas mixtures-D Hauton.doc	1	14/04/15
MEEC 14-028 Risk assessment-Supplements&Blood Collection-D Hauton.doc	1	14/04/15
MEEC 14-028 Draft-subject recruiting poster-Supplements&Activities.pptx	1	14/04/15
MEEC 14-028 Draft Subject Information Sheet-Diet&Activities-D Hauton.docx	1	14/04/15
MEEC 14-028 Draft-Consent form-Supplements& Daily Activity-D Hauton.docx	1	14/04/15

On the basis of the information provided, the Committee requested further information/ clarification on the following matters before approval can be granted:

Application section	**Comment**	**Response required/ amended application required/ for consideration**
A9	Please could you confirm the safety of a dose of 70g per day of ketone esters? This seems very high.	Response required
A10	Regarding the question about naming the phlebotomist this is not necessary as long as a trained phlebotomist is used.	For consideration
Participant information sheet	Adverse reaction to cold/ Reynaud's should probably be added to the list of exclusion criteria on the participant information sheet (it is listed on the application).	Amended participant information sheet required
Consent form	The Committee suggests adding a statement to the consent form asking participants to confirm they are not aware of having any food allergies and they do not have any of the conditions, on medication etc. that are listed on the information sheet?	Amended consent form required
General comment	A9 is supposed to give a short summary, this was too long and too much detail which could have been summarised and included in other section e.g. C1 and C2 which were also overly long.	For consideration

A response should be sent to the Committee which addresses each of these points, and further consideration will be given to your response. Please highlight or use a different colour font to indicate the changes to your application form and supporting documents.

The Committee is not able to approve your application at this stage so you are unable to begin your research. Please do not hesitate to contact us if you have any questions.

Yours sincerely

Jennifer Blaikie

Senior Research Ethics Administrator, Research & Innovation Service

On behalf of Professor Gary Williamson, Chair, MEEC FREC

Appendix 2: Ethics approval for Chapter 4

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UNIVERSITY OF LEEDS

Biological Sciences Faculty Research Ethics Committee

University of Leeds

Lu Li

PhD student

School of Food Science and Nutrition

University of Leeds

LS2 9JT

4 June 2018

Dear Lu Li

Title of study: **The impact of citrus products on cardiovascular health**

Ethics reference: **BIOSCI 15-030 amendment Nov 16**

I am pleased to inform you that the application for the ethical review of an amendment to the above project has been reviewed by the Chair of the Biological Sciences Faculty Research Ethics Committee and I can confirm a favourable ethical opinion on the basis of the information provided in the following documents:

Document	Version	Date
BIOSCI 15-030 amendment Nov 16 Ethical review LL V4.doc	1	14/11/16
BIOSCI 15-030 Amendment_form_LL .doc	1	10/11/16
BIOSCI 15-030 flyer LL V3.pdf	1	10/11/16
BIOSCI 15-030 info sheet LL V3.docx	1	10/11/16
BIOSCI 15-030 consent form LL V3.doc	1	10/11/16
BIOSCI 15-030 citrus drink consumption record form LL V1.docx	1	10/11/16

BIOSCI 15-030 Ethical review LL V3.doc	3	11/07/16
BIOSCI 15-030 info sheet LL V2.docx	2	11/07/16
BIOSCI 15-030 consent form LL V2.doc	2	11/07/16
BIOSCI 15-030 Participant Screening Questionnaire LL V2.docx	2	11/07/16
BIOSCI 15-030 summarry of clarifications.docx	1	11/07/16
BIOSCI 15-030 flyer LL V1.pub	1	18/05/16
BIOSCI 15-030 vascular assessment 2016.doc	1	18/05/16
BIOSCI 15-030 Food frequency questionnaire LL V1.docx	1	18/05/16
BIOSCI 15-030 food record LL V1.docx	1	18/05/16

Please notify the committee if you intend to make any further amendments to the original research as submitted at date of this approval, including changes to recruitment methodology. All changes must receive ethical approval prior to implementation. The amendment form is available at http://ris.leeds.ac.uk/EthicsAmendment.

Please note: You are expected to keep a record of all your approved documentation, as well as documents such as sample consent forms, and other documents relating to the study. This should be kept in your study file, which should be readily available for audit purposes. You will be given a two week notice period if your project is to be audited. There is a checklist listing examples of documents to be kept which is available at http://ris.leeds.ac.uk/EthicsAudits.

We welcome feedback on your experience of the ethical review process and suggestions for improvement. Please email any comments to ResearchEthics@leeds.ac.uk.

Yours sincerely

Jennifer Blaikie

Senior Research Ethics Administrator, Research & Innovation Service

On behalf of Prof Edward White, Chair, BIOSCI Faculty Research Ethics Committee

CC: Student supervisor(s)

Appendix 3: Participant information form

UNIVERSITY OF LEEDS

Information for participants
The impact of citrus products on cardiovascular health

You are being invited to take part in a research study at the University of Leeds. Before you decide to take part, it is important that you understand the purpose of the study and what it will involve. Please take time to read through the following information carefully. If you are unclear about anything or would like more information, please feel free to ask us. Take time to decide whether or not you wish to take part.

What is the purpose of the study?
Consumption of citrus products (for example, grapefruit, orange juice and supplements) has been associated with benefits for cardiovascular health. The aim of the study is to test whether blood orange juice compared to a control drink will improve your blood vessel function and other health indicators (such as blood pressure and blood lipids).

Am I suitable to take part?
We want to study female and male adults aged between 20 and 60 years who are generally fit and healthy with a body mass index (the body mass divided by the square of the body height) over 25 kg/m^2. Smokers and anyone with health problems, such as diabetes, liver or kidney disease will not be able to take part. Women who are pregnant or breastfeeding will be excluded. We will ask about your health in a short questionnaire. Suitable volunteers should not be taking dietary supplements (e.g. vitamins, extracts from plants) on a regular basis. If you are suitable based on the questionnaire, you will be invited to take part in the study.

What will the study involve?
You will be asked to consume either blood orange juice or juice drink together with breakfast and dinner on a daily basis for 2 weeks, and after 3-7 days you will be asked to consume the other drink for 2 weeks. You will be invited to the research centre on four separate days (altogether four visits) for the assessment of your cardiovascular health. For the measurement of blood vessel function, you will be lying down on your back. A blood pressure cuff will be put around your lower arm and inflated for 5 minutes. This is a standardized and safe procedure but will cause a little discomfort towards the end of the 5 minutes. This discomfort will disappear when the cuff is released. We will hold a

probe on your upper arm to assess vessel function using ultrasound (like checking an unborn baby in the womb).

At the first session, we will explain the study and all procedures in detail with you. You will complete some questionnaires regarding your health status and diet. The measurement of blood vessel function will be conducted before and after 2 weeks' consumption. You'll be asked to make a tick in the Citrus Drink Record Form each time you consume the drink. A small blood sample (15 ml which is about a tablespoon) will be taken from the crook of the arm at each visit. You will be asked to provide one urine sample (50 ml) at each visit. Blood and urine samples will be used to analyse metabolic changes following citrus consumption and how they impact on cardiovascular health.

What are the benefits and risks of taking part in the study?

Benefits

You will be provided with citrus drinks each for 2 weeks and your cardiovascular health will be examined. Your participation in this study will allow a better understanding of how to improve cardiovascular health through diet, which together with future studies may help people better prevent cardiovascular diseases in the future. In order to compensate for your time, you will receive a £40 voucher on completion.

Risks

There might be a risk of bruising and infection associated with blood collection; to minimize this risk, blood will be taken by a qualified phlebotomist and all blood safety measures will be in place. Participants who are allergic to tape or cotton wool will be provided with tissue and alternative tape.

Can I withdraw from the study at any time?

Yes, you will be able to withdraw at any point before completion of the study without consequences. If you withdraw before you finish all the sessions, the data already collected will be removed from the data set, with your urine samples and blood samples destroyed. On completion of all the sessions, it will not be possible to exclude your data from the study.

How confidential are the results?

All personal information and results are kept strictly confidential. Once you decide to participate, you will be given a participant code and this code will be linked to data. Therefore the data will not be identifiable to researchers. Anonymized findings will be published in scientific journals and presented at scientific conference.

Do I have to change my diet?

You will be requested to maintain your dietary habits generally but try to refrain from high consumption of citrus foods and the consumption of flavonoid-rich beverages (coffee, tea, cocoa, fruit juices and wine). Consumption of other supplements before or during this study is not recommended. We do ask you to inform us if you are ill and are prescribed any medication during the study.

What will happen to the results of the research study?

The results of this study will be analysed and presented at conferences and in peer reviewed journals. Remember that your own results are confidential and that your name will not be associated with any information published from this study. All data will be kept for 5 years and then destroyed.

Who is organising and funding the research?

This research is being organised by Dr Christine Bosch and Dr Karen Birch of School of Food Science and Nutrition and the Centre for Sport and Exercise Sciences, respectively. The funding for this research has been made available from China Scholarship Council and the University of Leeds.

Who has reviewed this study?

This research study has been reviewed by the University of Leeds Research Ethics Committee and ethical approval granted.

Contact for further information:

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Appendix 4: Consent form

Consent to take part in the impact of citrus products on endothelial function	Please tick next to the statement if you agree
I confirm that I have read and understand the participant information sheet explaining the above research project and I have had the opportunity to ask questions about the project.	
I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason and without there being any negative consequences*.	
I understand that the information generated will be anonymous and that no individual results will be published.	
I agree for the data collected from me to be stored and used in relevant future research in an anonymised form.	
I understand that the DNA isolated from my blood samples will be destroyed after the study is finished and the results have been analysed.	
I understand that relevant sections of the data collected during the study, may be looked at by auditors from the University of Leeds or from regulatory authorities where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.	
I agree to take part in the above research project and will inform the lead researcher should my contact details change.	
I understand that I will receive £40 voucher on completion of the study (at the final session) as compensation of my time and participation.	

Name of Participant		Name of Researcher	
Participant's Signature		Researcher's Signature	
Date		Date**	

* You can contact Lu Li via email (fslli@leeds.ac.uk) if you have any further questions. If you decide to withdraw from this study before you finish all the sessions, your data are going to be deleted permanently. On completion of all the sessions, it will not be possible to exclude data from the study.

**To be signed and dated in the presence of the participant.

Once this has been signed by all parties the participant should receive a copy of the signed and dated participant consent form, the letter/ pre-written script/ information sheet and any other written information provided to the participants. A copy of the signed and dated consent form should be kept with the project's main documents which must be kept in a secure location.

This study (BIOSCI 15-030) has been approved by Faculty Research Ethics Committee of University of Leeds on 22/11/16.

Appendix 5: Participant Screening Questionnaire

Participant Screening Questionnaire

Participant Code: Date:

Please provide brief information about yourself by filling in the form or ticking the appropriate answer and provide additional information where necessary.

Section 1: Basic information

Name		Gender	Male	Female
Age		Ethnicity		
Mobile number		Email address		
Height (metres)		Weight (kg)		

Section 2: About your lifestyle and health status

	Yes	NO
Do you smoke?		
Have you ever been diagnosed with a disease of the heart, lungs or blood vessels?		
Females only: Are you pregnant, lactating or actively trying to conceive?		
Females only: Are you currently using any contraceptive treatment or hormone replacement therapy?		
Females only: Do you have a regular menstrual cycle?		
Are you currently taking any medication?		
If **YES**, please give details		
Do you take dietary supplements (vitamins, antioxidants)?		
If **YES**, please give details		
Do you drink coffee regularly?		
If **YES**, how much		/day
Are you on any kind of diet?		
Do you regularly play sport or exercise?		
If **YES**, do you take part in sports or exercise on more than 2 days per week?		
Do you have lactose intolerance?		
Do you have other food intolerance or allergy?		
If **YES**, please give details		

Thank you for your time

Appendix 6: Food Frequency Questionnaire

Food Frequency Questionnaire

Date: Participant code:

Please indicate **how often** you have consumed certain types of food and drink **during the last 12 months** by ticking the appropriate answer and provide additional information where necessary.

	Daily			Weekly			Monthly				
(1=whole fruit)	1	2	3	1-2	3-4	5-6	<1	1	2	3	Never
Frequency of fruit consumption in general											
The most frequently consumed fruits No.1:											
The most frequently consumed fruits No.2:											
The most frequently consumed fruits No.3:											
Orange											
Grapefruit											
Lemon											
Lime											
Tangerine											
Clementine (seedless)											
Other (please specify):											
Fruit juice: (1=100 ml)											
Orange juice											
Grapefruit juice											

Other (please specify):											
Canned fruit: (1=1/3 regular sized can, or 1 small can)											
Type (please specify):											
Marmalade: (1=amount thinly spread on 1 piece of toast)											
Orange marmalade											
Grapefruit marmalade											
Other (please specify):											
	Daily			Weekly			Monthly				
	1	2	3	1-2	3-4	5-6	<1	1	2	3	Never
Tea (1=1 cup)											
Coffee											
Coffee (decaffeinated)											
Cocoa, hot chocolate											
Dark chocolate (1=50 g)											

Thank you for your time!

Appendix 7: Citrus drink consumption record form

Citrus drink consumption record form

Participant code: Date:

Please tick in the following boxes each time you consume **200 ml** of a citrus drink and also record the time you consume it.

Week 1	Mon	Tues	Weds	Thur	Fri	Sat	Sun
citrus drink with breakfast							
citrus drink with dinner							

Week 2	Mon	Tues	Weds	Thur	Fri	Sat	Sun
citrus drink with breakfast							
citrus drink with dinner							

Appendix 8: Food record form

Food record (3 days)

Participant code: Date:

Please record the food and drink directly after consumption during the last 2 days before the measurement and during the day of the measurement.

time	meal type	where	food or drink	amount	brand name
6 am to 8 am					
8 am to 12 noon					
12 noon to 3 pm					
3 pm to 5 pm					
5 pm to 8 pm					
8 pm to 12 am					
12 am to 6 am					

-**Meal type:** breakfast, lunch, dinner, snack…

-**Where:** home, bar, restaurant…

-**Amount:** description of portion size of food or drink consumed, e.g. 2 slices (white bread)…