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In vivo Human Acute Cardiovascular Effects of Dietary Flavanones – Underlying Mechanisms of Action and Impact of Flavanone Metabolism

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Abstract

A body of epidemiological evidence suggests beneficial cardiovascular (CV) effects of dietary citrus flavanones. However, as systematically reviewed, there is currently only limited supporting evidence from randomised controlled trials (RCT); in particular, relatively little is known about which flavanone-derived circulating metabolites might be the underlying mediators of potential beneficial CV effects of flavanones.

In an acute crossover RCT, male participants at a mild to moderate CV disease (CVD) risk received dietary interventions (in random order): orange juice (hesperidin dose: 320 mg) or control, whereby both were matched for sugar and vitamin C. Markers of CVD risk and plasma concentrations of previously identified and novel flavanone / phenolic metabolites were simultaneously assessed at baseline and 5 h post dietary intervention (5 h is the anticipated time of peak plasma concentration of flavanone metabolites).

At 5 h post intervention, the orange juice intervention resulted in significantly elevated plasma concentrations of 8 flavanone metabolites (mean ± SD: 1.60 ± 1.33 µM and 0.02 ± 0.01 µM, respectively; $P < 0.0001$) and 15 phenolic acid metabolites (mean ± SD: 19.59 ± 7.46 µM and 5.69 ± 1.70 µM, respectively; $P < 0.0001$) compared with the control intervention. However, these elevated metabolite plasma concentrations did not result in acute improvements in digital endothelial function, central arterial stiffness, CV autonomic function, platelet activation, nitric oxide production and NADPH oxidase gene expression.

In summary, relatively high plasma concentrations of metabolites (predominantly phenolic acids metabolites) were detected 5 h after a single dose of flavanones in men at a mild to moderate CVD risk. Simultaneously assessed markers of CVD risk were not beneficially affected and further acute RCTs with a longer time course (up to 24 h) and short-term to chronic (weeks up to years) RCTs are required to determine the potential beneficial CV effects of dietary flavanones.

Table of contents

Table of figures

Table of tables

List of acronyms

%CV	Coefficient of variation
ADME	Absorption, distribution, metabolism and excretion
ADP	Adenosin-diphosphate
ANS	Autonomic nervous system
APC	Allophycocyanin
BAEC	Bovine aortic endothelial cells
BHS	British Hypertension Society
BMI	Body mass index
BP	Blood pressure
BRS	Baroreflex sensitivity
cAI	Central augmentation index
CAS	Chemical abstract service number
cfPWV	Carotid to femoral pulse wave velocity
CHD	Coronary heart disease
CI	Confidence interval
CLD88	Chemiluminescence detector 88
C_{max}	Maximal plasma concentration
CMC	Carboxymethyl-cellulose
CRP	C-reactive protein
CRP	Collagen related peptide
CV	Cardiovascular
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DTPA	Diethylenetriaminepentaacetic acid
EDHF	Endothelial derived hyperpolarising factor
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
ET-1	Endothelin-1
FDOC	Florida Department of Citrus
FITC	Fluorescein isothiocyanate
FMD	Flow mediated dilatation
gp IIb/IIIa	Activated fibrinogen receptor
HDL-C	High density lipoprotein cholesterol
HPLC	High performance liquid chromatography
HPLC	High performance liquid chromatography
HUVEC	Human umbilical vein endothelial cells
I/R	Ischaemia reperfusion
LDL-C	Low density lipoprotein cholesterol

MD	Mean difference
MESH	Medical subject heading
MFI	Median fluorescence intensity
mRNA	Messenger ribonucleic acid
MS^2	Tandem mass spectrometry
NADPH	Nicotinamide adenine dinucleotide phosphate
NEM	N-ethylmaleimide
NO	Nitric oxide
NOx	Nitric oxide metabolites
PAT-RH	Peripheral artery tonometry during Reactive hyperaemia
PE	Phycoerythrin
PGI_2	Prostacyclin
PI	Pulse interval
PMA	Phorbol 12-myristate 13-acetate
PSA	Power spectral analysis
RCT	Randomised controlled trial
RR	Relative risk
RT	Retention time
RAAS	Renin angiotensin aldosterone system
SBP	Systolic blood pressure
SD	Standard deviation
SEM	Standard error of the mean
SFA	Saturated fatty acids
sICAM	Soluble intracellular adhesion molecule
sMRM	Scheduled multiple reaction monitoring
SOP	Standard operating procedure
sVCAM	Soluble vascular cell adhesion molecule
$T_{1/2}$	Half-life
TC	Total cholesterol
T_{max}	Timepoint of maximal plasma concentration
TxA_2	Thromboxane A_2
VSMC	Vascular smooth muscle cells
WISP	Weighted Intake Software Package
xBRS	Cross-correlation baroreflex sensitivity
αBRS	α-index of baroreflex sensitivity

Acknowledgments

Chapter 1.   Literature review on dietary Flavanones: Vascular Bioactivity, Occurrence and Bioavailability

1.1   Cardiovascular disease mortality, underlying pathology and risk factors

One third of all deaths in the UK are caused by cardiovascular disease (CVD) [1] with coronary heart disease (CHD) and stroke being the main CVD events, and atherosclerosis constitutes the main underlying pathology [2, 3].

The early stages of atherosclerosis involve accumulation and oxidation of low density lipoprotein cholesterol (LDL-C) and inflammation in the vasculature, forming lesions known as fatty streaks (Figure 1.1) [3]. Further progression leads to atheroma, characterised by a core of dead cells and a fibrotic cap which narrows the vessel lumen (Figure 1.1) [3]. Cardiovascular (CV) events often result from plaque rupture-induced thrombosis, which completely occludes the artery [3, 4] (Figure 1.1).

Figure 1.1 – Atherosclerosis timeline

| Foam Cells | Fatty Streak | Intermediate Lesion | Atheroma | Fibrous Plaque | Complicated Lesion / Rupture |

Endothelial Dysfunction

| From First Decade | From Third Decade | From Fourth Decade |
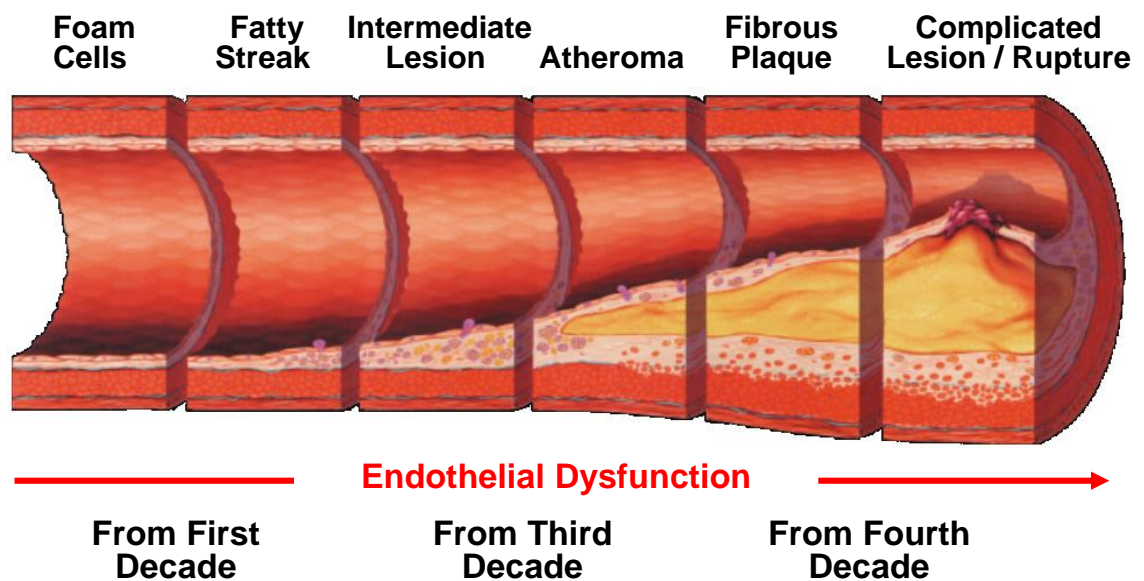
Figure adapted from [5]

An increased absolute risk of CVD is associated with a range of factors including age [6], male gender [6, 7], elevated blood pressure (BP) [8], unfavourable blood lipid profile (i.e. low plasma levels of high density lipoprotein cholesterol (HDL-C) and high levels of total cholesterol, LDL-C and triglycerides) [9], platelet hyperactivity [10], hyperglycaemia [11], smoking [12], obesity [11, 13, 14], positive familial history of premature (<65 years for women and <60 years for men) CVD [15] and a number of diet related risk factors [16]. Prior to manifestation of CVD events, persistent exposure to risk factors results in abnormalities in clinical markers of CVD, including endothelial dysfunction [17], CV autonomic dysfunction [18], central arterial stiffness [19, 20] and increased platelet reactivity [21].

Section 1.2 provides an introduction to the relationship between dietary intake patterns and CVD risk, followed by the potentially CV protective role of dietary flavanones, which are the focus of this PhD work.

1.2   Dietary intake patterns and CVD risk

Dietary components are modifiable risk factors that have been shown to influence the development of CVD [22]. Whilst most dietary guidelines recommend restrictions of foods rich in saturated fatty acids (SFA), there remains limited robust scientific evidence for a causal relationship between decreased intake of SFA and lower risk for CVD [23-25]. However, several reviews have indicated that replacing dietary intakes of SFA with poly- or mono-unsaturated fatty acids, is associated with a decreased risk of CVD [23-25] and that the effects of certain foods cannot be explained simply by differences in their fatty acid content [24].

Numerous studies suggest that diets rich in fruits, vegetables and plant-based food components [26-30], fish [31], olive oil [32], nuts [33], garlic [34] and low in sugars (i.e. monosaccharides) [16], salt [35], processed meat [36] and alcohol [23] are associated with a reduced risk of CVD. A diet rich in fruit was recently reported to be particularly effective in reducing CVD mortality and morbidity having the 3rd greatest impact amongst modifiable risk factors; behind lowering BP or smoking cessation [37]. However, further research is required to examine the bioactive constituents responsible for the observed cardioprotective effects, to establish optimal intakes and inform future dietary guidelines. Flavonoids, which are bioactive constituents widely distributed in fruits, vegetables and plant-based foods, are the focus of this PhD project due to their high potential to contribute to the suggested cardioprotective benefits [38].

This literature review will present an introduction to the flavonoid family of phytochemicals and, with a focus on the sub-group of flavanones, will outline the potential effects of flavanones on CV health and identify research gaps that need to be addressed.

1.3   Flavonoids; chemical structure, food sources and dietary intakes

Flavonoids are a group of compounds synthesised by plants which act as antioxidants, provide colour to attract pollinators and defence mechanisms against harmful insects and microbes [39]. Flavonoids share a common structure of two phenolic rings (A and B) connected by a three carbon bridge (C) (Figure 1.2) [40], and the 6 traditional subclasses of flavonoids are flavonols, flavones, flavan-3-ols, anthocyanidins, isoflavones and flavanones, which differ in substituents on the C ring (Figure 1.2) [38].

Figure 1.2 – Flavonoid subclasses
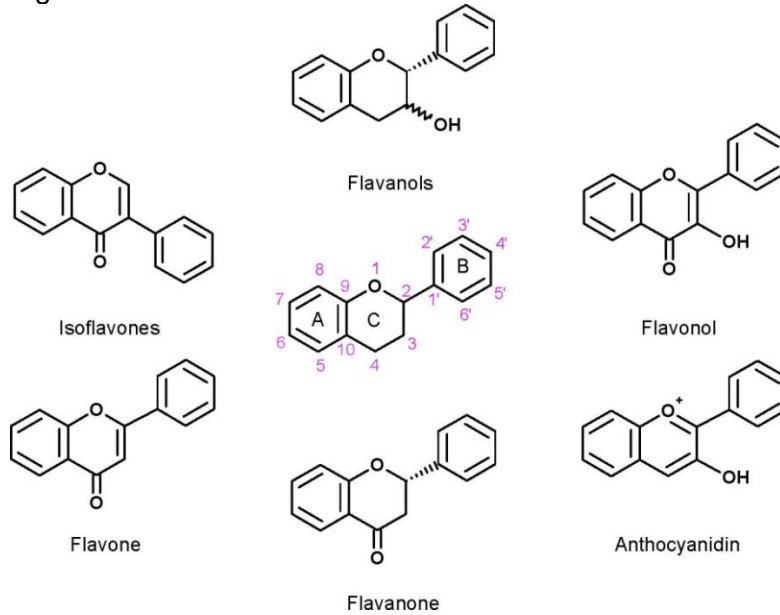
Chemical structure of the 6 traditional flavonoid subclasses with their common basic structure as shown in the centre (adapted from [41])

Flavonoids are generally found as monomers or polymers in fruits, vegetables and plant based food products such as tea, wine and cocoa [40]. Whilst there are limited data about flavonoid intake in the UK, French mean flavonoid intake has been estimated as 1193 mg/d [± 510 standard deviation (SD)] [42]. Based upon a usual diet, flavonoids reach plasma concentrations of nano- to low micromolar in plasma which is suggestive that their potential anti-atherogenic activity may be attributed to modulation of cell signalling pathways (through protein and/or lipid interaction), rather than via classic antioxidant effects [41].

1.4 The potential cardiovascular benefits of flavanone consumption

1.4.1 Epidemiological evidence: flavanone intake and associated relative CVD risk reduction

Cohort studies provide evidence to suggest that comparing the highest versus lowest flavanone/citrus fruit intakes, there is a lower relative risk (RR) of stroke (range: 0.29-0.84) [43-48] and CHD (range: 0.64-0.85) [49-52]. This broad variance of RR may be partly due to differences in gender, age (range 39.3 to 61.5 years), cohort size (range 1950 to 126339 participants), number of years of follow-up (range 3.9 to 28 years), mean body mass index (BMI) (range 24.8 to 26.9 kg/m^2) and variations of habitual intakes of flavanones between different nationalities (i.e. European, Japanese and US populations) of the cohorts (Table 1.1 & 1.2) [43-47, 49-52]. Moreover, reported intakes for both, flavanones and citrus fruit show considerable variance from as low as 0.4 mg/d to as

much as 93.4 mg/d and of <1 serving per month to 1 serving per day, respectively (Table 1.2) [43-53].

However, the lower relative CVD risk associated with flavanone intake has not been universally demonstrated (Table 1.1 & 1.2). In a study by Mursu et al., [52], increased flavanone intake was not associated with a reduction in stroke incidence; which was also the case for a cohort study by Mink et al., [49]. In agreement, the studies of Knekt et al., [45], Yamada et al., [47] and Cassidy et al., [53] did not report an association of lower relative CHD risk with increased flavanone and citrus fruit intake. Additionally, McCullough et al., [54] did not observe a significant inverse association between intake of flavanones and CVD mortality.

While four cohort studies consistently suggested no association between lower RR of haemorrhagic stroke and flavanone/citrus fruit intake, the findings of total and ischemic stroke are inconsistent [45-48] (Table 1.1 & 1.2). A possible explanation might be that flavanones have a protective effect on ischaemic stroke by reducing atheroma, whereas for haemorrhagic stroke, which is more dependent on BP levels [48], increased flavanone intake may not be associated with a large reduction in hypertension [55].

Inconsistencies in cohort characteristics (e.g. baseline CVD risk, number of participants and follow-up years), data collection methods (i.e. 4 d food record or food frequency questionnaire including 30 up to 152 food items), and food constituent databases used to estimate flavanone and citrus fruit intake may account, in part at least, for discrepancies in the associations between increased flavanone and/or citrus fruit intake and lower RR of CVD [56, 57].

Taken together, a body of epidemiological evidence generates the hypothesis that a diet rich in flavanones may reduce CVD risk. However, the findings among existing cohort studies were not fully consistent and the methods used to estimate flavanone/citruc fruit intakes contain a substantial amount of error and imprecision [58]. Hence, well-designed human randomised controlled trials (RCTs) are required to examine whether or not a diet rich in flavanones is causally linked to improved CV health.

Table 1.1 – The relationship between flavanone intake and stroke/CHD incidence based on data from cohort studies

Cohort study	Nationality / gender / age[a] / BMI[a]	# participants / years of follow up	Mean lower and upper intakes	RR (95 % CI) of stroke / CHD incidence comparing upper to lower intakes of flavanone [b]	
Knekt et al., 2002 [45]	Finnish / men and women / 39.3 ± 15.8years / 24.8 ± 4.1kg/m^2	10 054 persons/ 28 years	Hesperetin:1.5mg/d lower and 20.8 mg/d upper quartiles	**Total stroke:** 0.80** (0.64, 0.99) **Ischaemic stroke:** 0.74* (0.55, 1.00) **Haemorrhagic stroke:** 0.62 (0.32, 1.18)	**CHD:** 0.95 (0.76, 1.19)
			Naringenin: 0.4mg/d lower and 6.1 mg/d upper quartiles	**Total stroke:** 0.79** (0.64, 0.98) **Ischaemic stroke:** 0.73** (0.54, 0.98) **Haemorrhagic stroke:** 0.56 (0.29, 1.09)	**CHD:** 0.98 (0.78, 1.22)
Mink et al., 2007 [49]	US / postmenopausal women / 61.5 ± 4.2years, 26.9 ± 5.0kg/m^2	34 492 women/ 16 years	Quintile flavanones: 7.6mg/d lower and 93.7mg/d upper	**Total stroke:** 0.94 (0.69, 1.27)	**CHD:** 0.78** (0.65, 0.94)
Mursu et al., 2008 [52]	Finnish / men / 52.5 ± 5.3years / BMI not reported	1 950 men/ mean 15.2 years	Mean flavanones: 3.1mg/d	**Ischaemic stroke:**0.89 (0.49, 1.63)	n/a
Cassidy et al 2012., [48]	US women / range 30-55 years / 25.7kg/m^2	69 622 women/ 14 years	Flavanones: 18.8mg/d lower and 44.8mg/d the upper quintiles	**Total stroke:** 0.89 (0.77, 1.04) **Ischaemic stroke:** 0.81* (0.66, 0.99) **Haemorrhagic stroke:** 0.82 (0.55, 1.22)	n/a
McCullough et al., 2012 [54]	US / men and women / 69 ± 6.1years / BMI not reported	38 180 men and 60 289 women / 7 years	Flavanones: 3.5 mg/d lower and 49.9 mg/d the upper quintiles	**CVD:** 0.90 (0.80, 1.01)	
Cassidy et al., 2013 [53]	US women / range 25-42 years / 24.6 ± 5.2kg/m^2	93 600 women/ 18 years	Flavanones: 6.6mg/d lower and 71.1mg/d upper quintiles	n/a	**MI:** 0.91 (0.66-1.26)

Abbreviations: BMI, body mass index; RR, relative risk; CI, confidence interval; CHD, coronary heart disease; MI, myocardial infarction[a]; mean ± SD unless stated; [b] after multivariate adjustment; *P-value<0.05; ** P-value<0.01

Table 1.2 – The relationship between citrus fruit intake and stroke/CHD incidence based on data from cohort studies

Cohort study	Nationality / gender / age[a] / BMI[a]	# participants / years of follow up	Mean lower and upper intakes	RR (95 %CI) of stroke / CHD incidence comparing upper to lower intakes of citrus fruit [b]	
Joshipura et al., 1999 [44] and 2001 [51]	US / men and women/ 48.6 years / 24.7 kg/m^2	75 596 women / 14 years and 38 683 men / 8 years	Citrus fruits: 0.1 servings/d lower and 1.8 servings/d upper quintiles	**Ischaemic stroke:** 0.72 (0.47, 1.11)	**CHD:** 0.88* (0.77–1.00)
		84 251 women / 14 years and 42 148 men / 8 years	Citrus fruit juices: 0 servings/d lower and 1 serving/d upper quintiles	**Ischaemic stroke:** 0.65* (0.51, 0.84)	**CHD:** 1.06 (0.85–1.32)
Johnsen et al., 2003 [43]	Danish / men and women / 56.1 years (range 51.2-63.3) / 26±4.1 kg/m^2	54 506 persons / median: 3.09 years	Citrus fruits: 2.9 g/d lower and 100.0 g/d upper quintiles	**Ischemic stroke:** 0.63* (0.41, 0.92)	n/a
Dauchet et al., 2004 [50]	French and Northern Irish / men / 54.7±2.9 years / not reported	8 087 / 5 years	Citrus fruits: ≤0.07 fruits/d lower and ≥0.5 fruits/d upper tertiles	n/a	**CHD:** 0.64* (0.41, 0.99)
Mink et al., 2007 [49]	US / postmenopausal women / 61.5±4.2 years, 26.9±5.0 kg/m^2	34 492 women / 16 years	Orange juices in servings/wk: <1.00 lower and >1.00 upper tertiles	**Total stroke:** 0.91 (0.75, 1.12)	n/a
			Oranges in servings/wk: <1.00 lower and >1.00 upper tertiles	n/a	**CHD:** 0.96 (0.84,1.09)
			Grapefruits in servings/wk: <1.00 lower and >1.00 upper tertiles	n/a	**CHD:** 0.85* (0.74, 0.98)
Mizrahi et al., 2009 [46]	Finnish men and women / 51.9 years / 26.5 kg/m^2	3 932 persons / 24 years	Citrus fruits: men 0 g/d lower and 37-740 g/d upper tertiles; women 0-8 g/d lower and 69-1040 g/d upper tertiles	**Total stroke:**0.77** (0.63, 0.93) **Ischaemic stroke:** 0.79 (0.60, 1.03) **Haemorrhagic stroke:** 0.54 (0.29, 1.01)	n/a
Yamada et al., 2011 [47]	Japanese / men / 54.8±12.1 years / 22.9±2.9 kg/m^2	10 623 persons / mean 10.7 years	Citrus fruits: <1/month lower and almost 1/day upper quintiles	**Total stroke:** 0.40** (0.20, 0.81) **Ischemic stroke:** 0.28* (0.11, 0.72) **Haemorrhagic stroke:** 0.71 (0.24, 2.11)	**CHD:** 0.99 (0.34, 2.80)
	Japanese / women / 55.2±11.4 years / 23±3.2 kg/m^2			**Total stroke:** 0.47* (0.26-0.87) **Ischemic stroke:** 0.39 (0.15, 1.00) **Haemorrhagic stroke:** 0.55 (0.24, 1.23)	**CHD:** 0.67 (0.11, 4.15)

Abbreviations: BMI, body mass index; RR, relative risk; CI, confidence interval; CHD, coronary heart disease; [a] mean ±SD unless stated; [b] after multivariate adjustment; *P-value<0.05; ** P-value<0.01

1.4.2 Human randomised controlled trials examining potential effects of flavanones on markers of CVD risk

Whilst in Chapter 2, RCTs have been systematically reviewed that examined the potential effects of flavanones on a broad range of markers of CVD risk, this chapter specifically focuses on RCTs with BP, endothelial function, autonomic function and platelet function endpoints to identify research gaps and introduce the conducted acute flavanone human RCT described in Chapters 3-5.

To date, only two RCTs have examined the effects of a short-term (3 - 4 weeks) flavanone intervention on BP and endothelial function (Table 1.3) [59, 60]. Whilst Morand et al., [59] conducted a 4 week intervention feeding 292 mg/d hesperidin (a class of flavanones) given as a food source (i.e. orange juice) or a supplement to 23 overweight men, the 500 mg/d hesperidin supplement intervention by Rizza et al., [60] lasted 3 weeks and was performed in 24 adults with metabolic syndrome. Morand et al., [59] found a decrease in diastolic BP (DBP) induced by both, the orange juice and the hesperidin supplement, but no improvement in endothelial function (assessed by microvascular reactivity) or increase in plasma nitric oxide (NO) metabolites (NOx, i.e. nitrate, nitrite and nitroso species) (Table 1.3). In contrast, Rizza et al., [60] did not show a decrease in DBP, but observed an improvement in endothelial function (assessed by flow mediated dilatation (FMD) of the brachial artery) (Table 1.3). In addition, neither of the two studies observed an effect of hesperidin on systolic BP (SBP) [59, 60]. Possibly due to differences in study design (e.g. hesperidin dose, study duration and baseline CVD risk of study population) and different methods used to assess endothelial function, the two studies reported equivocal findings. A possible explanation for a DBP but not a SBP lowering effect of flavanones (as observed by Morand et al., [59]) may indicate that flavanones reduce total peripheral resistance rather than cardiac output [61]. Both of these cross-over designed RCTs in 23 - 24 participants were adequatly powerer to detect clinically relevant potential effects of short-term flavanone intake (i.e. 3-4 week) on blood pressure and endothelial function [59, 60], with Rizza et al., [60] reporting that n = 20 participants were needed to detect a 2% change in FMD with a power of 80 % and a significance level of 5 %.

Within the same study, Morand et al., [59] also examined potential acute effects of flavanones on endothelial function 6 h following a single dose of 292 mg hesperidin (Table 1.3). While microvascular reactivity was improved by both consumption of the orange juice and the hesperidin supplement, no acute changes in NOx plasma concentrations were found [59]. These acute improvements in microvascular reactivity were associated with the significantly elevated mean plasma concentrations of hesperetin of 0.86 ± 0.10 μM following the orange juice (Pearson's correlation: r = 0.698, *P* = 0.0001) and 0.77 ± 0.16 μM following the hesperidin supplement intervention (Pearson's correlation: r = 0.434, *P* =

0.039) compared to no detection after the control intervention. Plasma vitamin C concentration was also significantly elevated 6 h after the orange juice intervention relative to 6 h after the hesperidin supplement and control interventions (i.e. mean ± SEM: 70.5 ± 4.6 µM compared to 52.8 ± 4.6 µM and 50.8 ± 4.6 µM, respectively; $P < 0.001$).

Table 1.3 – Randomised controlled trials examining the effects of flavanones on blood pressure and endothelial function

Study	Participants/ age (mean ± SD)	duration/ study intervention/ dose	Mean effect[1] [95% CI]
Short-term flavanone interventions:			
Morand et al., 2011 [59]	23 overweight men/ 56 ± 5 y	4 wk/ 0.5 L orange juice/ 292 mg hesperidin + 47 mg narirutin per day	**SBP**: -3.0 mm Hg [-8.3, 2.3] **DBP**: -5.5 mm Hg* [-9.0, -2.0] **Microvascular reactivity[2]**: 52 % [-8, 113] **NOx**: 12.4 µM [1.4, 23.4]
		4 wk/ supplement/ 292 mg hesperidin per day	**SBP**: 0.6 mm Hg [-8.3, 2.3] **DBP**: -3.2 mm Hg* [-9.0, -2.0] **Microvascular reactivity[2]**: 17 % [-44, 77] **NOx**: -3.2 µM [-15.0, 8.6]
Rizza et al., 2011 [60]	24 adults with metabolic syndrome/ 52 ± 2 y	3 wk/ supplement/ 500 mg hesperidin per day	**SBP**: 2.7 mm Hg [-1.3, 6.7] **DBP**: 0.6 mm Hg [-2.2, 3.4] **FMD[2]**: 2.5 %* [0.4, 4.6]
Acute flavanone interventions:			
Morand et al., 2011 [59]	23 overweight men/ 56 ± 5 y	6 h/ 0.5 L orange juice/ 292 mg hesperidin + 47 mg narirutin	**Microvascular reactivity[2]**: 105 %**[55, 155] **NOx:** no mean effect (data not reported)
		6 h/ supplement/ 292 mg hesperidin	**Microvascular reactivity[2]**: 48 % [-1, 98] **NOx:** no mean effect (data not reported)

Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure; NOx, plasma levels of nitric oxide metabolites; FMD, flow mediated dilatation; y, year; *P-value<0.05; **P-value<0.01 [1]Difference between control and experimental intervention; [2]methods to assess endothelial function

Across the findings of Morand et al., [59] the orange juice intervention consistently showed greater effects in comparison to the hesperidin supplement intervention (Table 1.3). Whilst these results provide evidence that hesperidin partly mediates the cardioprotective effects of orange juice, other potentially bioactive compounds in orange juice (e.g. narirutin, flavones, vitamin C, β-carotenes, folate and fibre) are possibly exerting additive and/or synergistic effects [23].

In addition to beneficial effects of flavanones on endothelial function and BP, an RCT reported flavanones to acutely improve CV autonomic function [62]. Measuring heart rate variability, 1 h after administration of 500mg glucosylhesperidin (a synthesised from hesperidin with a short absorption time) to 11 healthy women (age range: 18-22 years), a decrease in cardiac sympathetic nervous activity and an increase in cardiac parasympathetic nervous activity was reported compared to the control intervention [62]. Hence, hesperidin-induced modulations of the autonomic nervous system (ANS) might be a further mechanism by which flavanones exert cardioprotective effects [18].

Altogether, these three recent RCTs suggest that flavanones exert beneficial CV effects through a reduction in DBP and an improvement in endothelial function [59, 60, 62]. However, there are insufficient data available to provide scientific evidence and a number of limitations need to be addressed.

Whilst intake of orange juice had more profound vascular effects than intake of an equivalent dose of hesperidin in a supplement compared to an energy matched control intervention [59], little is known about the potential additive/synergistic effects of the range of bioactive compounds found in orange juice and the importance of consuming these bioactive compounds within a natural food matrix as compared to a flavanone extract.

The *in vivo* phase II metabolism of flavanones in humans has previously been established in a number of bioavailability studies and limited data are suggesting bioavailability of phenolic acid colonic breakdown metabolites (Section 1.6). However, Morand et al., [59] related acute flavanone vascular bioactivity to achieved total plasma concentrations of hesperetin (i.e. following enzymatic deconjugation of potential glucuronides/sulfates) independent of phase II metabolism and did not quantify plasma concentrations of phenolic acid metabolites. Hence, associations of flavanone vascular bioactivity to individual plasma hesperetin and naringenin phase II metabolites and phenolic acid metabolites remain to be examined.

Research to date is particularly limited regarding the potential mechanisms by which flavanones exert effects on markers of CVD risk such as vascular function and BP. Whilst *in vitro* and animal studies suggest that flavanones may beneficially modulate a range of CV related cellular pathways (see Section 1.4.3 and 1.4.4), Morand et al., [59] is the only study to date that has explored potential effects of hesperidin on endothelial NO production suggesting no significant increase in plasma total NOx following acute or 4 weeks daily orange juice or hesperidin supplement intake compared to control. However, as reported in the review of [63], plasma total NOx may be a biomarker of limited value because it does not solely originate from endogenously produced vasoactive NO from the endothelium, but also from dietary intakes, the gut microflora and inhaled NO. Thus, further studies are warranted which control for dietary NOx intakes and examine potential flavanone-induced increases of more closely related endothelial NO breakdown products; e.g. nitrite or S-nitrosothiols [64, 65]. In addition, a wider range of vascular related biomarkers need to be explored to further understand the mechanisms by which flavanones mediate beneficial CV effects.

Whilst beneficial effects of 500 mg glucosylhesperidin on cardiac autonomic function have been reported as a mechanism by which flavanones are cardioprotective [62], it remains to be tested whether naturally occurring flavanones (e.g. hesperidin) at a dose achievable within a normal diet may also exert these effects. Furthermore, in addition to the HR variability methodology which [62] used, further potential effects of flavanones on cardiac autonomic function could be assessed using baroreflex sensitivity (BRS), which takes both HR and BP changes into account [18].

In conclusion, flavanones may decrease DBP and improve endothelial function after a 3-4 week intervention (i.e. short-term) [59, 60] and acutely improve endothelial function [59] and autonomic function [62]. However, there are insufficient data to provide scientific evidence about the potential CV protective effects of flavanones and the underlying mechanisms are unknown. Thus, further well-designed RCTs in the field of flavanone research are required. Whilst previously shown beneficial CV effects and their underlying mechanisms of action of flavanones need to be corroborated using complementary methodologies (e.g. peripheral arterial tonometry during reactive hyperaemia (PAT-RH) to test endothelial function, BRS to test autonomic function and plasma nitrite to test vascular NO production), additional potential beneficial CV effects of flavanones need to be assessed using unexplored markers of CVD risk such as arterial stiffness, and platelet activation. To further support the necessity of human RCTs, Sections 1.4.3 and 1.4.4 review the findings of flavanone bioactivity in cell culture and animal models.

1.4.3 In vitro studies suggesting potential mechanisms of action of flavanones on cardiovascular related pathways

Reviews of *in vitro* studies in endothelial [66] and platelet cells [67] suggested that plant polyphenols, including flavanones, may beneficially modulate cellular pathways related to CV health. Whilst in cultured vascular endothelial cells, polyphenols have been suggested to enhance NO and prostacyclin and inhibit endothelin-1 (ET-1) production, thereby improving endothelial dependent vasodilatation and vascular homeostasis (Figure 1.3) [66], polyphenols have also been reported to inhibit platelet aggregation through downregulation of signalling cascades activated by platelet agonists [e.g. collagen, thrombin and adenosine-diphosphate (ADP)] [67] (Figure 1.4). For certain polyphenols, antiplatelet effects have been observed in similar magnitude to aspirin, a drug widely used in CVD prevention [67].

Following treatment of cultured endothelial cells with hesperetin, four studies reported a 1.3 to 2.5 fold increase in NO production using various experimental settings; i.e. hesperetin in different forms (i.e. aglycones, glycosides or glucuronides), arterial or venous endothelial cell lines, incubation times ranging from 5min to 24h and doses ranging from 10µM to 100µM [60, 68-70]. Whilst Rizza et al., [60] also found a 1.8 fold increase in eNOS phosphorylation after hesperetin treatment at a physiological dose (i.e. 1µM) in bovine aortic endothelial cells (BAEC) suggesting an increase in NO production, Chiou et al., observed no change in NO production in human umbilical vein endothelial cells (HUVEC) after incubation with 1µM hesperetin [68]. Yet, elevated eNOS phosphorylation was observed in HUVEC cells with hesperetin treatment at higher doses (10-100 µM) [68].

Several underlying mechanisms by which hesperetin may elevate NO productions of endothelial cells have been suggested. Whilst hesperetin has been shown to stimulate NO synthesis through activation of endothelial nitric oxide synthase (eNOS) via upregulation of the PI3K/Akt/eNOS pathway [60, 68] and increased eNOS gene expression [69], hesperetin has also been observed to decrease NO inactivation through inhibition of superoxide generation partly via down-regulation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and xanthine oxidase [68, 70].

Figure 1.3 – Mechanisms by which flavanones mediate vasodilatation
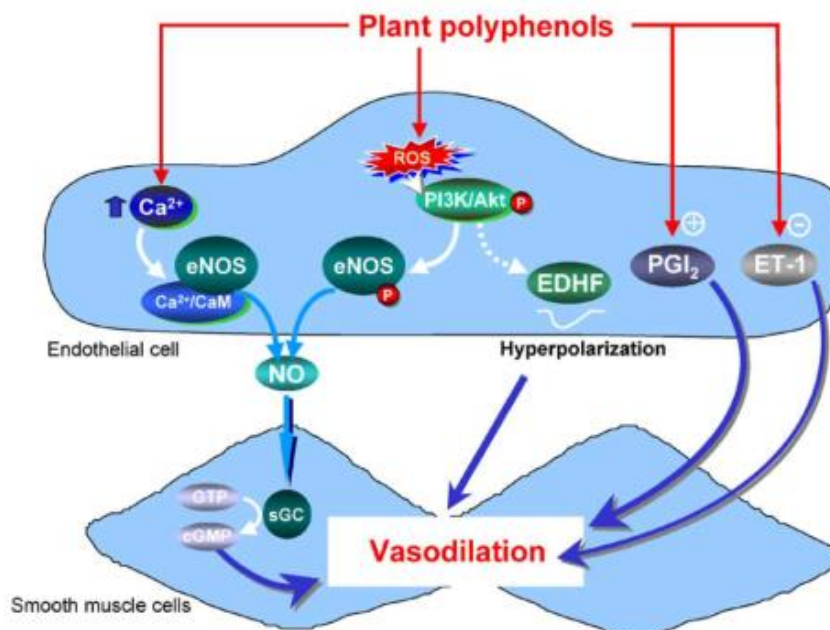
Flavanones may potentially increase endothelial production of relaxing factors [i.e. nitric oxide (NO), prostacyclin (PGI_2) and endothelium-derived hyperpolarising factor (EDHF)] and decrease contracting factors [i.e. endothelin-1 (ET-1)]; Abbreviations: eNOS, endothelial nitric oxide synthase; CaM, calmodulin; Ca^{2+}, calcium; ROS, reactive oxidative species; PI3K, phosphatidyl-inositol 3 kinase; GTP, guanosine triphosphat; cGMP, cyclic guanosine monophosphat; sGC, soluble guanylyl cyclase (adapted from [66])

In addition to hesperetin-induced increases in NO production, further experiments related to potential beneficial cardiovascular effects of flavanones have been conducted using cultured endothelial cells. Hesperetin has been reported to inhibit strain-induced ET-1 production mediated partly through upregulation of the NO and protein kinase G pathways [68]. Moreover, Liu et al., [69] not only examined hesperetin but also naringenin bioactivity; however, naringenin did not enhance NO production using the same cell model, treatment dose (i.e. up to 100 µM) and duration that showed an effect for hesperetin. Across all performed NO and superoxide experiments of Takumi et al., [70], hesperetin was observed to be more bioactive in its aglycone form than when bound to a glucuronide, the main flavanone metabolite absorbed in humans following flavanone intake [71, 72].

In addition to beneficial modulation of endothelial cells, inhibitory effects of flavanones on platelet aggregation have also been suggested in animal platelet cells [73, 74]. This might be a further mechanism by which flavanones could decrease CVD risk because platelets play an important role in atherothrombotic risk and atherosclerosis progression [21]. Following *in vitro* treatment of platelets with hesperetin (aglycones or diglycosides; 10-1600 µM), narirutin (i.e. naringenin-diglycoside; 170-1700 µM), or a citrus fruit extract (3-10 mg/mL) and various platelet agonists (i.e. arachidonic acid, collagen, thrombin and ADP), decreased platelet aggregation has been observed in a dose dependent manner [73-75]. Whilst both naringenin and hesperetin inhibited thrombin-induced platelet aggregation and only hesperetin, but not naringenin, inhibited ADP-induced *ex vivo* platelet aggregation, hesperetin exerted particularly potent inhibition of collagen- and arachidonic acid mediated *ex vivo* platelet aggregation [74] (Figure 1.4). Inhibition of phospholipase C-γ2 phosphorylation, an enzyme specific to the collagen activation pathway, and inhibition of cyclooxygenase, which catalyses the reaction of arachidonic acid to vasoactive prostanoids, have been suggested as the responsible underlying mechanisms of action of hesperetin [73], in addition to a decrease in intracellular Ca^{2+}, which is downstream of the ADP, collagen and thrombin activation pathways [76] (Figure 1.4).

Figure 1.4 – Schematic presentation of the known pathways leading to platelet activation and aggregation [1, 2]
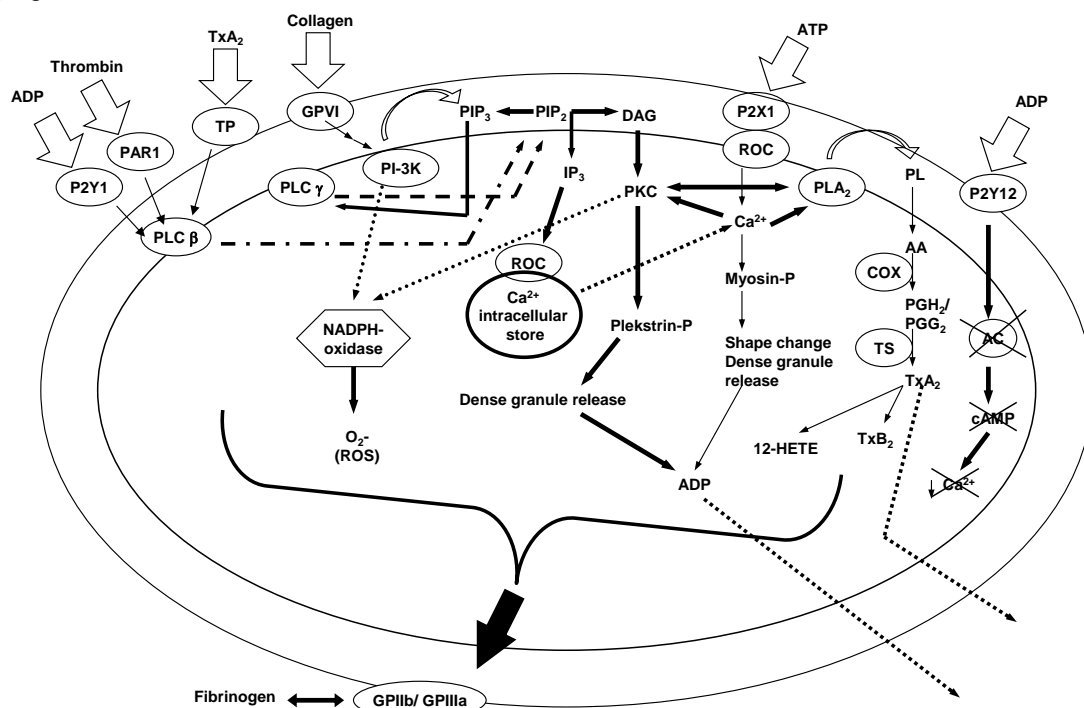
[1]The main platelet agonists include collagen, thrombin, ADP and thromboxane A_2 (TxA$_2$). [2]Platelet activation results in surface expression of the activated fibrinogen receptor (i.e. gpIIb/IIIa) and P-selectin (i.e. as a result of dense granule release). Figure adapted from [76]

A review by Kroon et al., [77] emphasised that *in vitro* research, which assesses flavonoid modulation of cellular pathways related to vascular function, should examine the effects of

polyphenol metabolites as they occur *in vivo*, at physiological doses (i.e. <10µM) and in a cell model which is affected by the pathology to be examined. However, whilst the majority of *in vitro* studies examined the effects of flavanone aglycones and glycosides on endothelial [60, 68, 69] or platelet cells [73-75], the study of Takumi et al., [70] examined the effects of hesperetin-7-glucuronide, a flavanone metabolite that has been identified in human bioavailability studies [71, 78]. Furthermore, only one study used endothelial cells of arterial origin, albeit from bovine and not human, and reported increased eNOS phosphorylation at 1 µM, a concentration that can be achieved from a portion of a flavanone rich food [71], whereas all other studies have used venous endothelial cells and observed beneficial vascular effects of flavanones only at concentrations ≥10 µM [68, 69]. It could be hypothesised that arterial endothelial cells, which have been reported to be an appropriate cell model to study atherosclerosis [79], are more responsive to flavanone treatment compared to venous endothelial cells. Hence, effects of hesperetin on endothelial NO and ET-1 production observed in HUVEC at supraphysiological concentrations, might be present at physiologically relevant concentrations (i.e. 1 µM) in arterial endothelial cells.

In addition to potentiation of endothelium dependent vasodilatation, flavanones have also been reported to exert endothelium-independent vasodilatation. Following incubation of endothelium-denuded rat aortic rings with naringenin and hesperetin, vasodilatation has been observed; however only at doses several magnitudes above physiological concentrations (i.e. 100 µM) [80-83]. Elevations of cGMP and cAMP in vascular smooth muscle cells (VSMC) [81, 82] and activation of calcium dependent potassium channels [80, 83] have been proposed as the underlying mechanisms. However, it has been suggested that these observed effects on endothelium-independent vasodilatation might not occur *in vivo* due to the required supraphysiological concentrations [77].

Flavonoid subclasses other than flavanones have been studied provide a more comprehensive understanding of the mechanisms by which flavonoids may increase NO in the vascular endothelium. There is strong evidence to suggest that (-)-epicatechin, a flavonoid predominantly found in cocoa, is stored and methylated in HUVEC and that methylated (-)-epicatechin acts as a potent inhibitor of NADPH oxidase resulting in decreased superoxide production and, in turn, reduced elimination of endothelial NO [84-86]. Mono-methylation of the catechol B-ring is of major importance making (-)-epicatechin structurally similar to apocynin, a pharmalogical NADPH oxidase inhibitor, and testing 45 polyphenols, including flavanones, the authors conclude that inhibition of NADPH oxidase is an important acute mechanism by which flavaonoids increase endothelial NO [85, 86]. Another study reported that treatment of endothelial cells or coronary artery rings with a physiologically relevant dose of flavonoids and polyphenols from red wine stimulated eNOS activity by mild intracellular ROS production leading to Akt dependent

phosphorylation of eNOS at Ser1177 and dephosphorylation of eNOS at Threonine 495 resulting in increased endothelial NO production [87]. Similarly to red wine polyphenols, equol, a metabolite of isoflavones mainly found in soy, has also been reported to stimulate eNOS activity in HUVEC even at concentrations as low as 100 nM, and exploring underlying mechanism revealed that equol enhances mitochondrial ROS production leading to increased Akt and ERK1/2 phophorylation upstream of the eNOS phosphorylation signalling pathway [88].

In summary, flavanones could potentially exert cardioprotective effects through endothelium dependent vasodilatation [60, 68, 69] and inhibition of platelet aggregation [73, 74] (leading to lower BP, slower atherosclerosis progression and reduced risk of thrombosis), but not through endothelium independent vasodilatation [77]. Further studies are required treating human platelets and arterial endothelial cell models, which are considered to be the most appropriate to study atherosclerosis [79], with flavanone metabolites at physiological concentrations [77], so that these data can inform human studies on the potential underlying cellular mechanisms by which flavanones exert beneficial CV effects. Ultimately, findings from *in vitro* cell culture studies first need to be translated to *in vivo* animal and human studies before firm conclusions can be made, as *in vitro* models can only simulate *in vivo* environments to a limited extent [89].

1.4.4 Animal studies exploring the potential protective effects of flavanones on CVD and possible underlying mechanisms

In addition to cell culture studies, animal studies further substantiate potential cardioprotective effects of flavanones. These protective effects have been observed on primary endpoints, including stroke and coronary heart disease, as well as markers of CVD risk such as BP, endothelial function, CV autonomic function and platelet aggregation.

Pretreatment of rats with 30 to 100 mg/kg/d of hesperidin for 7-15 days protected against ischemia/reperfusion (I/R) induced cerebral [90, 91] and cardiac damage [92] compared to control pretreatment. Following I/R induced damage of heart tissue leading to arrhythmia production and a drop in BP, Gandhi et al., [92] reported maintenance of mean arterial BP and a protective effect against arrhythmias owing to pretreatment with 100 mg/kg/d hesperidin.

Hesperidin has been reported to acutely reduce BP in hypertensive rats [93]. Whilst no acute effects have been shown in normotensive rats that ingested 50 mg/kg hesperidin, hypertensive rats responded to oral administration of glucosyl-hesperidin (30 and 50 mg/kg, but not 10 mg/kg) and intraperitoneal injection of 50 mg/kg hesperetin with a significant decrease in mean SBP by 7.9 %, 8.6 % and 12.3 %, respectively [93].

Following treatment of isolated aortic rings of rats *ex vivo* or *in vivo* with hesperidin or naringin, improved endothelial function has been observed, which indicates for an enhancement in endothelial NO production [93-95]. Vascular protective effects of hesperidin have further been attributed to a decrease in inflammation and oxidative stress [90, 92]. Underlying mechanisms responsible for these effects of flavanones include eNOS phosphorylation [91], an increase in endogenous antioxidants (i.e. glutathione, superoxide dismutase and catalase) [90, 91, 93, 96] and reduced activity/gene expression of superoxide-generating enzymes (i.e. NADPH oxidase [95] and xanthine oxidase [96]).

One study also reported hesperidin to inhibit platelet aggregation in mice and rats [74]. Performing the venous tail bleeding assay in mice to examine platelet aggregation, a single dose of either 10mg/kg hesperidin or 30mg/kg of a citrus fruit extract containing hesperidin has been shown to increase the bleeding time by 4.5 times compared to no treatment [74]. Following oral treatment of rats with hesperidin (10mg/kg) or citrus extract (100mg/kg), the same study also observed decreased *ex vivo* platelet aggregation when induced with collagen, arachidonic acid, ADP or thrombin. In accordance with *in vitro* findings [73, 74], hesperidin more potently inhibited collagen- or arachidonic acid-induced platelet aggregation compared to when induced with ADP or thrombin [74]. In terms of magnitude of effect, in both experiments, hesperidin and citrus extract inhibited platelet aggregation comparable to oral treatment with 50mg/kg aspirin.

Overall, whilst the majority of studies examined hesperidin bioactivity and only one tested potential effects of naringin, flavanones may exert cardioprotective effects through reduced damage of ischemic tissue [90-92] and may decrease CVD risk through mediating a decrease in BP [93], an improvement in endothelial function [93, 95] and a reduction in platelet reactivity [74]. In order to see whether similar beneficial CV effects of flavanones, in particular hesperidin, occur in humans, further clinical RCTs are required.

1.5 Properties and dietary aspects of flavanones

Flavanones are characterised by a ketone substituent on the C ring and according to substituents on the A and B rings are sub-grouped to distinct types (Figure 1.5.1) [40]. As part of the human diet, hesperetin and naringenin (Figure 1.5.2) are the two most commonly consumed types of flavanones. Richly found in a range of citrus fruits and herbs (Table 1.4), they are mostly conjugated to a diglycosides and called hesperidin, narirutin or naringin (Figure 1.5.3) with naringin providing the bitter taste to grapefruits [97].

Historically, for the first time in 1949, flavanones have been associated with beneficial CV effects in which hesperidin was suggested to reduce capillary leakiness, oedema and leg swelling [74]. Furthermore, grapefruit (a rich source of naringin) has been a large area of

research due to its drug interaction [98]. This is attributed to naringin which is a potent modulator of transporter proteins and cytochrome p450 3A4 enzymes that influence both absorption and pharmacokinetics of compounds including flavonoids and drugs [98].

Figure 1.5 – Flavanones; classification and diglycosides occurring in citrus fruits

1. flavanone common structure

3. flavanone diglycosides

Narirutin:

2. flavanone aglycones

hesperidin:

naringenin:

eriodictyol:

narirutin:

hesperetin:
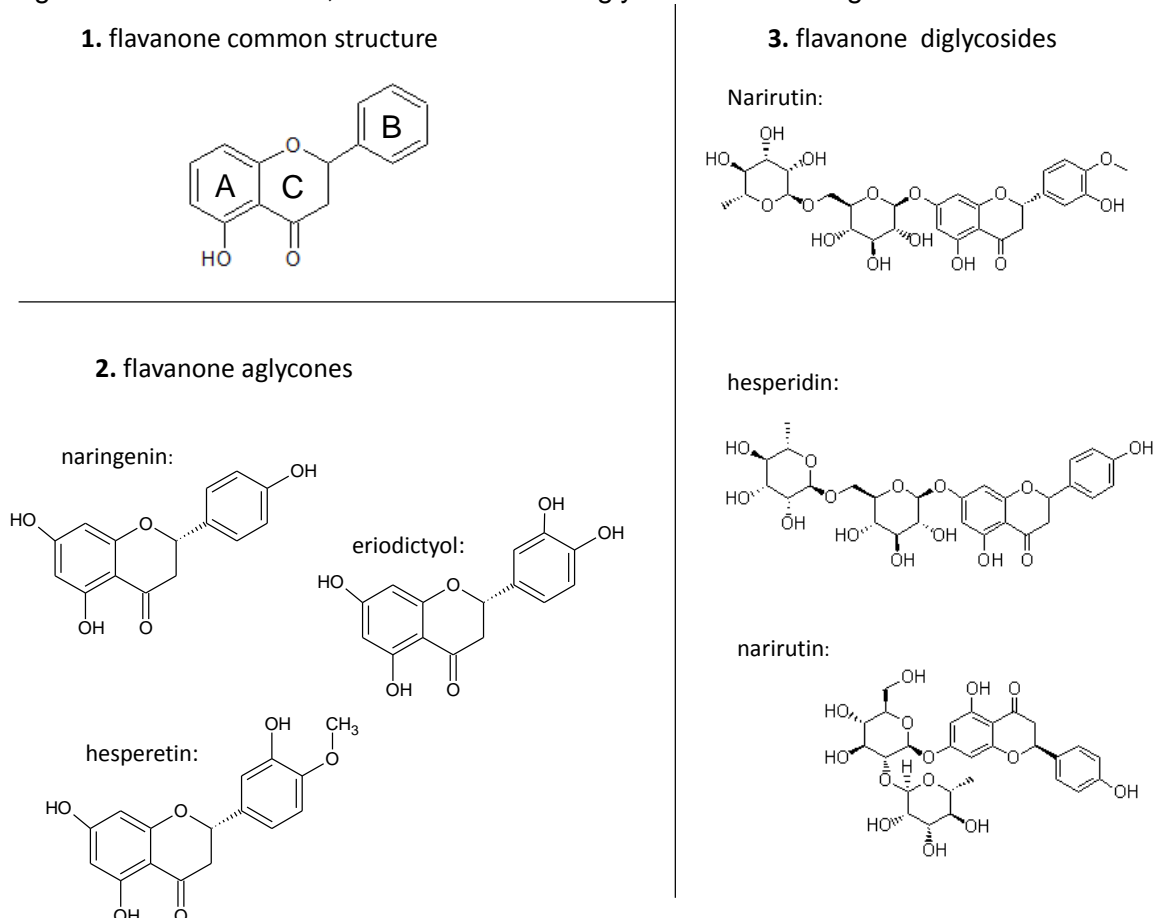
1.5.1 Mean intakes and dietary sources of flavanones

In a study across ten European countries, the mean dietary flavanone intake has been estimated as 34mg/d (±65 SD) and in the general UK population as 43mg/d (±65 SD) with citrus fruits and citrus derived juices and jams forming the main dietary sources [99]. Although flavanone intakes are a magnitude lower compared to intakes of most commonly consumed subclasses (i.e. anthocyanins and flavan-3-ols) [99], the high SDs suggest that a substantially higher flavanone intake can be achieved following a diet rich in citrus fruits. Sourced from the Phenol-explorer database, a summary of dietary components with highest flavanone content [100] can be found in Table 1.4. Taking the generally consumed portion sizes into account, citrus fruits are the main contributors to flavanone intake. Whilst 100 ml orange juice from concentrate contains on average 53 mg hesperidin and 6 mg narirutin and 100 ml of grapefruit juice from concentrate 38 mg naringin, 2 mg hesperidin and 9 mg narirutin, whole citrus fruits can have up to 5 times more flavanones due to the high content in the solid parts, in particular the white pulp [101]. In contrast,

although herbs contain a high concentration of flavanones, their contribution to dietary flavanone intakes is negligible as only small amounts are generally consumed.

Table 1.4 – Flavanone food sources and content[1]

	Flavanone	Mean content ± SD
Citrus fruit juices (mg/100ml)		
Grapefruit, juice from concentrate	Hesperidin	1.6 ± 0.9
	Naringin	37.8 ± 16.9
	Narirutin	9.2 ± 4.1
Grapefruit, pure juice	Hesperidin	0.7 ± 0.8
	Naringenin	1.6 ± 2.2
	Naringin	30.8 ± 15.1
	Narirutin	9.7 ± 4.8
Grapefruit/Pummelo hybrid, pure juice	Naringin	45.1 ± 7.9
	Narirutin	15.0 ± 6.8
Lemon, juice from concentrate	Hesperidin	25.0 ± 9.0
Lemon, pure juice	Hesperidin	17.8 ± 13.7
	Narirutin	0.6 ± 0.1
Lime, pure juice	Hesperidin	13.4 ± 4.2
	Narirutin	0.4 ± 0.2
Orange [Blond], juice from concentrate	Hesperidin	52.7 ± 5.8
	Narirutin	6.3 ± 1.2
Orange [Blond], pure juice	Hesperidin	25.9 ± 12.2
	Naringin	0.1 ± 0.5
	Narirutin	5.4 ± 3.1
Orange [Blood], juice from concentrate	Hesperidin	51.3 ± n/a
	Narirutin	7.3 ± n/a
Orange [Blood], pure juice	Hesperidin	43.6 ± 18.0
	Narirutin	4.8 ± 1.3
Pummelo, pure juice	Naringin	8.5 ± 4.8
Tangerine, juice from concentrate	Hesperidin	36.1 ± 11.2
	Narirutin	6.0 ± 6.0
Herbs (mg/100mg)		
Marjoram, dried	Eriodictyol	3.0 ± n/a
Mexican oregano, dried	Eriodictyol	85.3 ± 11.6
	Naringenin	372.0 ± 42.2
Peppermint, dried	Hesperidin	480.7 ± 316.3
	Narirutin	127.5 ± 240.2
Rosemary, fresh	Naringin	55.1 ± 2.8

[1]Data sourced from the Phenol-explorer database [100]

1.5.2 Impact of food processing on flavanone content and solubility

Mechanical and thermal processing has been shown to affect the flavanone content in oranges [102]. For example following storage of blended oranges for 20 days at 4°C, a 8.1 % decline in hesperidin content was observed [102].

In the citrus juice food matrix, flavanones are either soluble or precipitate to form insoluble aggregates [103]. As described by bioavailability studies (Section 1.6), the soluble flavanones are the most bioavailable [78]. Thus, flavanone solubility in orange juice following processing and storage has been examined [104]. Findings suggest that domestic squeezing of oranges resulted in a 27.2 % higher content of soluble flavanones compared to industrial squeezing and that freezing of orange juice for 1 month at -40 °C

decreased the soluble flavanones by 43.4 %; in contrast, pasteurisation and concentration only had a slight impact on flavanone solubility [104].

1.6 Bioavailability, pharmacokinetics and metabolism of flavanones

Flavanone absorption, distribution, metabolism and excretion (ADME) in human has been an extensive area of research [105] and provides important information for the design of bioactivity studies [106].

Following intake of citrus containing mainly hesperidin, narirutin or naringin (Figure 1.5.3) at doses ranging from 103-441mg, flavanones were detected in plasma between 2 and 24 h and were reported to have a half-life ($T_{1/2}$) of 2.2 to 3.8 h, reaching a mean maximal plasma concentration (C_{max}) of 0.19-5.99 µM between 4.4-6.8 h (T_{max}) after intake [71, 72, 78, 107, 108] (Table 1.5).

The relatively late T_{max} suggests that the site of major flavanone absorption occurs in the distal ileum and colon after hydrolysis of the ingested flavanone-diglycosides to flavanone aglycones (Figure 1.5.2) by the gut microflora [109]. In support, studies which measured the bioavailability of flavanone aglycones (i.e. small amounts occurring in tomatoes) [110] and enzymatically treated flavanone glucosides [111, 112] reported a decreased T_{max} between 2 and 3.7 h. For orange juice, a mouth to colon transit time of 2.1 h (±1.3) was observed in the fasted state, which might explain the detection of trace amounts of flavanones in plasma already 2 h after intake (Figure 1.6.) [71, 72].

The observed differences in bioavailability data (Table 1.5) could be dependent on the characteristics of the flavanone foods, the fasting state of participants and differences in intestinal microflora and genetics between human individuals (Figure 1.6.2 & 3) [71, 72, 78, 107, 108].

Figure 1.6 – Bioavailability of dietary flavanones in humans
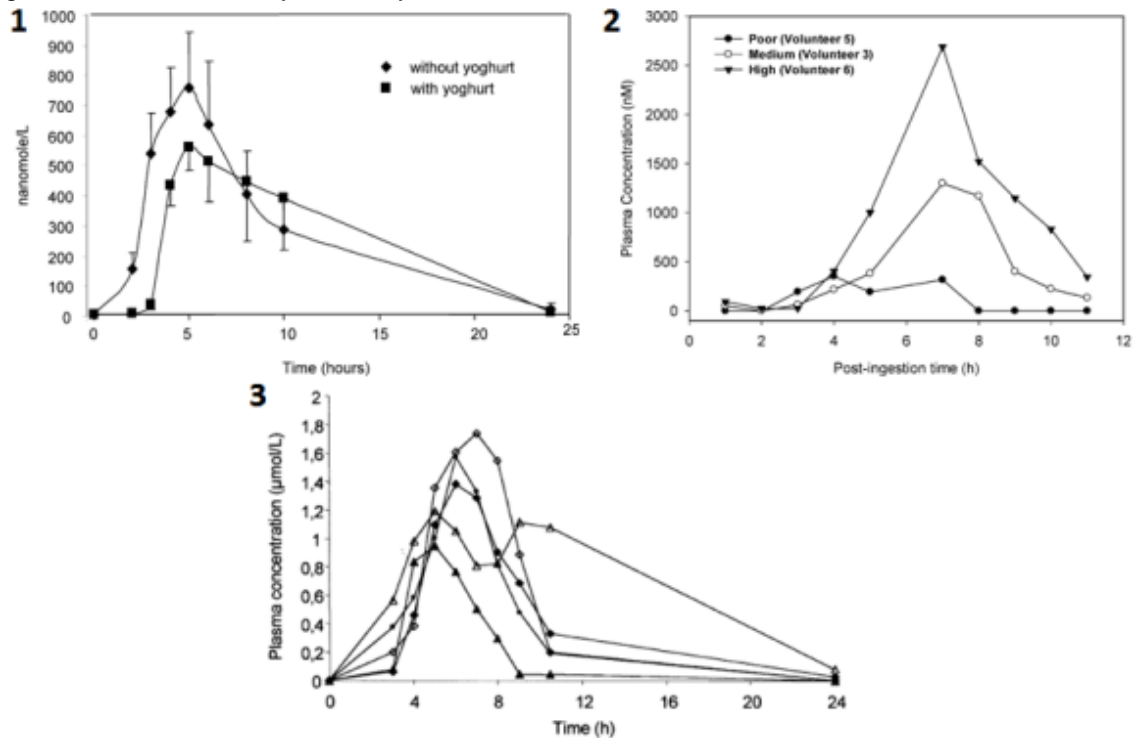
(1) mean hesperetin plasma concentration – time curve following ingestion of orange juice containing 103 mg hesperidin (adapted from [71]); (2 & 3): inter-individual differences in flavanone pharmacokinetics with each recording corresponding to one individual (adapted from [78] and [108], respectively)

Comparing flavanone subclasses and their dietary sources, naringenin (i.e. the major flavanone in grapefruit) has been observed to be more bioavailable than hesperetin (i.e. the major flavanone in oranges) [72, 107]. This discrepancy may be explained by differences in functional groups on the B-ring. Whilst a greater bioavailability can be achieved when increasing the flavanone dose [108], comparing several orange juices, Vallejo et al., [78] have shown that the amount of ingested soluble flavanones correlate better with flavanone bioavailability than the total amount of flavanone intake (correlation coefficient: 0.92 and 0.35, respectively). In contrast, Brett et al., [72] reported little impact of the food matrix on flavanone bioavailability because no differences in pharmacokinetic parameters were found following ingestion of either orange fruit or orange juice providing a similar flavanone dose (Table 1.5).

In respect to the fasting state of participants, Mullen et al., [71] reported that orange juice intake in combination with yoghurt tended to delay the mean T_{max} from 4.4 to 5.1 h and to decrease the mean C_{max} by one third (Figure 1.6.1) [71]. In support, studies in non-fasted participants reported an even higher mean T_{max} ranging from 5.3 to 6.8 h [72, 78, 108].

Table 1.5 – Flavanone pharmacokinetics following intake of dietary citrus flavanones

Study	Intervention	Dose (mL)	flavanone intake (mg)	C_{max} (µM)	$T_{1/2}$ (h)	T_{max} (h)	AUC_{0-24} (µmol*h/L)	Relative urinary excretion (% of intake)
Erlund et al., 2001 [107]	Orange juice[1]	400-760 (8 mL/kg BW)	103.6-196.9	2.8 ± 1.9	2.2 ± 0.1	5.4 ± 1.6	12.9 ± 9.8	6.4 ± 3.9
	Grapefruit juice[1]		137.2-260.7	6.0 ± 5.3	2.2 ± 0.8	4.8 ± 1.1	27.7 ± 26.3	30.2 ± 25.5
Manach et al., 2003 [108]	Orange juice	500	132.6	0.5 ± 0.1		5.4 ± 0.4	4.6 ± 1.3	4.1 ± 1.2
		1000	265.1	1.5 ± 0.2		5.8 ± 0.37	10.6 ± 2.3	6.4 ± 1.3
Mullen et al., 2008 [71]	Orange juice[1]	250	182.5	0.9 ± 0.2	3.6 ± 1.3	4.4 ± 0.5	4.1 ± 2.9	7.0 ± 2.3
	Orange juice and 150 ml full fat yoghurt[1]			0.7 ± 0.2	3.8 ± 0.8	5.1 ± 0.4	3.0 ± 2.6	7.1 ± 2.3
Brett et al., 2008 [72]	Orange fruit[2]	150 g	90.5	0.18 ± 0.12		6.4 ± 3.0	2.1 ± 1.4	8.5 ± 7.0
	Orange juice[2]	300	81.2	0.15 ± 0.90		5.3 ± 2.3	1.8 ± 0.9	7.4 ± 4.9
Vallejo et al., 2010 [78]	Orange juice from concentrate[2]	400	116.8	0.3 ± 0.6		4.5 ± 0.7	1.22 ± 0.3	8.0 ± 1.7
	Orange juice from concentrate[2]	400	215.2	0.7 ± 0.2		6.8 ± 0.6	1.53 ± 0.3	2.4 ± 0.6
	Pulp enriched orange juice[2]	400	281.2	0.2 ± 0.0		6.5 ± 0.5	0.70 ± 0.1	1.5 ± 0.7
	Flavanone extract enriched orange juice from concentrate[2]	400	440.8	1.5 ± 0.3		6.7 ± 0.3	5.83 ± 1.0	7.1 ± 1.5
	Orange flavonoid extract dissolved in water[2]	400	207.2	1.6 ± 0.4		5.9 ± 0.7	5.00 ± 1.0	14.3 ± 4.4

Mean ± SD

Abbreviations: AUC, area under the curve; BW, body weight; C_{max}, maximal plasma concentration; $T_{1/2}$, half life
[1] consumed by fasted participants unaccompanied by a meal; [2] consumed by fasted participants together with a controlled breakfast

Figure 1.7 – Flavanone metabolites identified in human biological samples after oral intake of dietary flavanones

1. flavanone conjugate phase 2 metabolites

2. flavanone derived phenolic acid metabolites

Hesperetin-7-O-glucuronide

Naringenin-7-O-glucuronide

Hesperetin-3'-O-glucuronide

Naringenin-4'-O-glucuronide

Hesperetin-3'-O-sulfate

Hesperetin-3',7-O-diglucuronide

3-Methoxy-4-hydroxyphenyl hydracrylic acid

3-Hydroxyphenyl hydracrylic acid

Dihydroferulic acid

3-Hydroxyhippuric acid

3-Hydroxyphenyl acetic acid
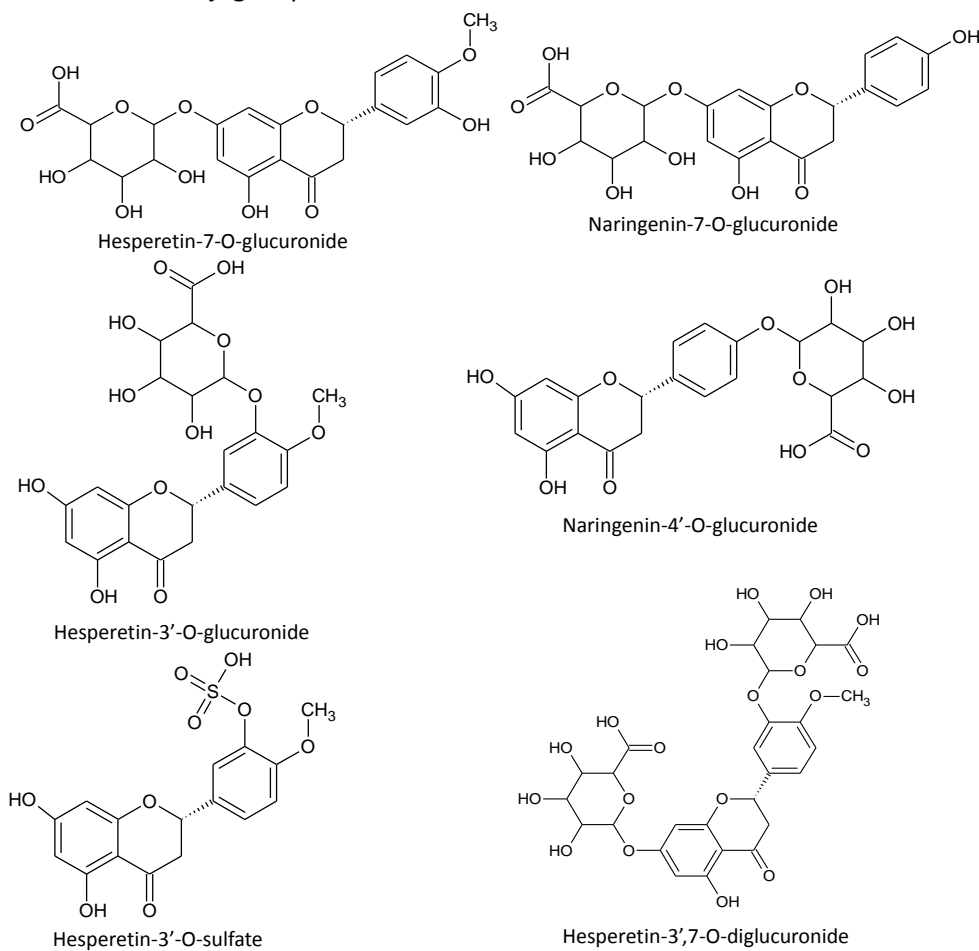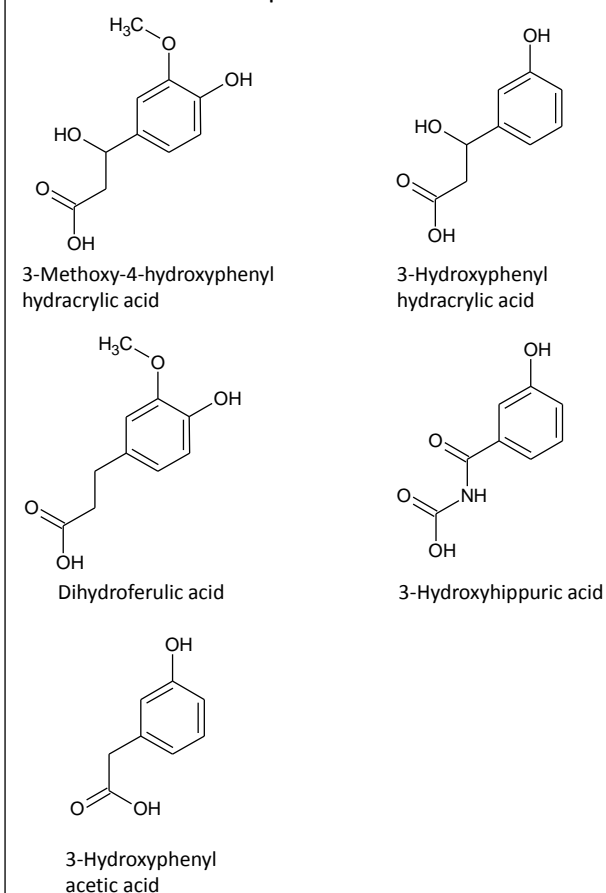
1. flavanone phase II metabolites that have been detected in human plasma following intake of orange juice [71, 72, 78, 107, 108]; 2. five flavanone derived phenolic acids that have been identified in urine following intake of orange juice [113].

Across all conducted studies, a high variance of the maximal flavanone plasma concentration has been reported between participants, which might be attributed to differences in the intestinal microflora and genetics of human individuals (Figure 1.6.2+3) [71, 72, 78, 107, 108]. To a small extent, flavanone bioavailability decreases with increasing age [72], whereas no influence of sex and BMI has been observed [72, 78].

Although no human data are available, the tissue distribution of flavanones has been examined in rats gavaged with radioactively-labelled naringenin [114]. The study demonstrated that flavanones can pass the blood brain barrier and are retained in the brain, heart, lungs, liver, spleen and kidneys for at least 18h where they potentially elicit bioactivity [114].

Whilst none or only trace amounts of flavanone aglycones have been detected in plasma, most flavanones are subject to phase II metabolism (i.e. flavanone-glucuronides, sulfates and -sulfoglucuronides) [71, 72] and further colonic metabolism to phenolic acids [78, 113] (Figure 1.7). Following intake of orange juice, while Vallejo et al., [78] identified phenolic acids in plasma and urine, Roowi et al., [113] observed a mean increase of five out of nine tested potential urinary phenolic acids in urine collected 5-24 h after orange juice intake compared to water intake (Figure 1.7.2). Although a complete pharmacokinetic profile for flavanone-derived phenolic acids remains to be established, total relative phenolic acid urinary excretion accounted for as much as 37 % of the ingested flavanones [113].

Regarding the excretion of flavanone conjugate phase II metabolites, collection of urine for 36 h following flavanone intake has demonstrated that most of the urinary excretion occurs between 6 and 24 h. The highest rate of 40 to 56 % of total urinary excretion was observed between 6 and 11 h and a residual excretion of 0.7 to 2.2 % between 24 and 36 h [108]. Whilst a mean relative urinary excretion for flavanone-phase II metabolites (i.e. % of the flavanone intake) ranging between 1.5 and 30.2 % has been reported (Table 1.5) [71, 78, 107, 108], the remaining fraction is either not absorbed, subject to further metabolism to phenolic acids [78, 113] and eventually carbon dioxide [115] or eliminated through biliary excretion [109].

How the diverse microorganisms in the gut (i.e. gut microbiome) and their genomes (i.e. the metabolome) functionally contribute to human physiology are not well understood and have only recently become an extensive area of research [116]. For example, there is evidence to suggest that the gut microbiome of obese individuals includes more genes responsible for the capacity to extract energy from the diet compared to lean individuals [116, 117]. Similarly, the gut microbiome plays an important role in ADME of flavanones (and polyphenols), because the gut microbiome explains, to a large extent, the inter-individual variability of flavanone absorption (Figure 1.6.2) and by which extent flavanones

are metabolised to phenolic acids [118]. Conversely, a diet rich in flavanones (and polyphenols) may also affect the composition of the gut microbiota and, in turn, influence the functional contribution of the microbiome [118].

In summary, flavanones are a relatively well absorbed flavonoid subclass with phase II flavanone metabolites reaching nano- to micromolar plasma concentrations between 4.4 to 7 h after intake of citrus (Table 1.5) and a substantial fraction of ingested flavanones is metabolised by the gut microbiome and bioavailable in form of circulating phenolic acids [105, 113]. However, the pharmacokinetic parameters of these flavanone-derived phenolic acid metabolites remain to be established and acute human RCTs are required that relate flavanone bioactivity to circulating levels of flavanone phase II conjugates and phenolic acid metabolites.

1.7 Established markers of CVD risk used in acute RCTs to examine cardioprotective effects of dietary compounds such as flavanones

Potential beneficial effects of dietary components are commonly assessed through markers of CVD risk [119]. Since the conducted human RCT examined the potential acute effects of flavanones on vascular function (Chapters 3 & 4), this section introduces complementary as well as novel clinical markers of CVD risk and related blood biomarkers which might be acutely affected by dietary flavanones.

1.7.1 Endothelial function plays a key role in vascular homeostasis and may be improved by dietary flavonoids

Endothelial cells, which form the inner monolayer of the arterial wall, have an important regulatory role maintaining vascular homeostasis and conversely endothelial dysfunction substantially contributes to the development of atherosclerosis [4, 120]. In response to physical and chemical stimuli, endothelial cells secrete a range of factors to regulate vascular homeostasis (i.e. vascular tonus, structure, inflammation and thromboresistance) (Figure 1.8) [4, 17]. Whilst under physiological conditions, a balance is maintained between relaxing factors (e.g. nitric oxide and prostacyclin) and contracting factors (e.g. endothelin-1 (ET-1), thromboxane A_2 and prostaglandin H_2), persistent exposure to CVD risk factors causes endothelial dysfunction [17]. The consequences are an increase in vascular tonus, smooth muscle cell proliferation, vascular inflammation and platelet reactivity [17].

Figure 1.8 – Endothelium and vascular homeostasis
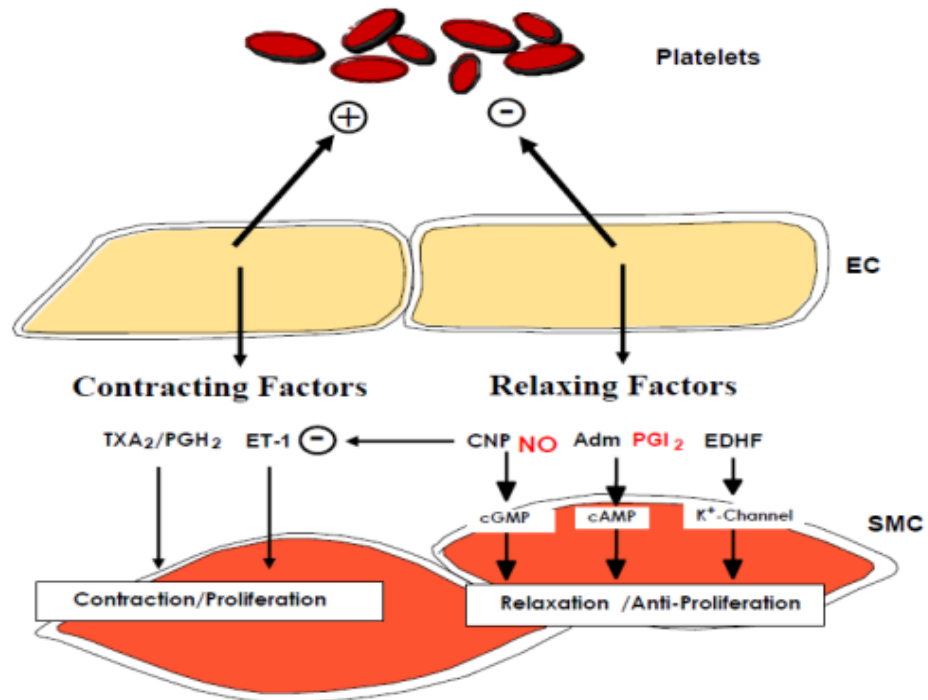
Range of factors released by endothelial cells which regulate vascular tonus, smooth muscle cell proliferation and platelet reactivity; abbreviations: EC, endothelial cells; SMC, smooth muscle cells; TXA_2, thromboxane A_2; PGH_2, prostaglandin H_2; ET-1, endothelin-1; CNP, C-type natriuretic peptide; NO, nitric oxide; Adm, adrenomedullin; PGI2, prostacyclin; EDHF, endothelium derived hyperpolarising factor, cGMP, cyclic guanosinmonophosphate, K^+, potassium (adapted from [130])

While most clinical methods to assess endothelial function are invasive (i.e. acetylcholine induced changes in coronary artery or microvascular blood flow) or technically demanding [i.e. brachial artery flow mediated dilatation (FMD)], digital vasodilator function measured after reactive hyperaemia by peripheral artery tonometry (PAT-RH) is a non-invasive and operator-independent method to assess endothelial function [121]. PAT-RH is a strong predictor of CVD risk as suggested by a 7 year prospective study in 270 outpatients reporting a significantly higher incidence of cardiovascular adverse events in patients with baseline endothelial dysfunction (i.e. PAT-RH < 1.5) [122], and correlations of PAT-RH and multiple CVD risk factors such as obesity, diabetes and hypertension were found in the Framingham Heart Study [123]. Even though two studies observed a significant correlation between PAT-RH and coronary microvascular endothelial function (i.e. r = 0.41, P < 0.001; in 94 CAD patients) [124] and FMD (i.e. r = 0.55, P < 0.0001; in 89 healthy subjects) [125], others found no correlation of PAT-RH with the Framingham CVD risk score and FMD in a community based US cohort (i.e. r = -0.01, P = 0.70, n = 1843) [126] or 222 middle-aged participants (i.e. r = 0.12, P = 0.06) [127]. Overall, and despite these inconsistent findings regarding the validation of PAT-RH against other measures of endothelial function, PAT-RH may provide useful information about peripheral endothelial function and was chosen for the present study due to its operator-independencdy.

As outlined in Section 1.4.2 and also documented in a systematic review [128], flavonoids, including flavanones [59, 60], have been suggested to improve clinical surrogate markers of endothelial function and this effect is partly mediated by an increase in endothelial NO bioavailability [129]. However, specifically for the flavanone subclass, more studies are required to provide scientific evidence.

Blood biomarkers are commonly assessed to examine the potential underlying mechanism of action responsible for an improvement in endothelial function. Increased endothelial NO production, a key mediator of vasodilatation, was suggested to be responsible for approximately 60 % of the PAT-RH response [129], and can be measured indirectly through quantification of plasma nitrate, nitrite and/or nitroso species (e.g. S-nitrosothiols) [65, 131]. Further available blood biomarkers that play a role in endothelial function include markers related to endothelial NO production (e.g. superoxide generating NADPH oxidase [132] and asymmetric dimethylarginine [133]), vasodilators (e.g. PGI_2 [17] and hydrogen disulfide [134]), vasoconstrictors (e.g. ET-1 [135] and the renin-angiotensin-aldosterone system (RAAS) [5]) and markers of vascular integrity (e.g. micro particles [136] and endothelial progenitor cells [137]).

In summary, the PAT-RH is a useful method to test potential acute effects of dietary flavanones on endothelial function, and changes in related blood biomarkers need to be explored to understand the potential underlying mechanisms of action.

1.7.2 Baroreflex sensitivity as a marker of CVD risk

The autonomic nervous system (ANS), which consists of parasympathetic and sympathetic pathways, regulates most organ systems including control of short-term BP fluctuations [138].

In response to beat-to-beat changes in BP, the baroreceptors, which are stretch receptors located in the carotid sinuses and the aortic arch, reflexly elicit inverse changes in heart rate and arterial tonus via the ANS [139] (Figure 1.9). CV autonomic dysfunction, which is characterised by sympathetic overactivity and parasympathetic hypoactivity, is a prognostic marker of CVD events and has been associated with CVD risk factors such as hypertension and obesity [18, 139-141]. Furthermore, CV autonomic dysfunction has been closely linked with endothelial dysfunction [142] and a correlation between parasympathetic autonomic function and arterial stiffness was observed in healthy men [143].

Figure 1.9 – The baroreceptor reflex in response to an increase in blood pressure[1]
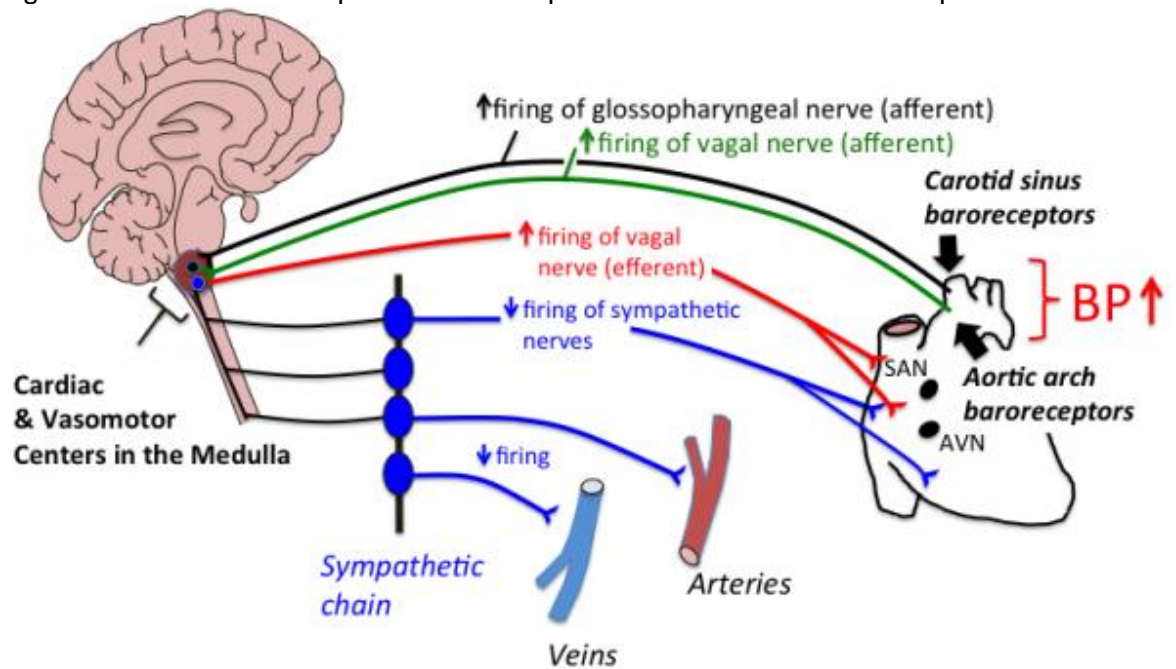
[1] The reflex also responds in the opposite way to a decrease in blood pressure.
Abbreviations: AVN, atrioventricular node; SAN, sino-atrial node (Figure adapted from [61])

Although the pathophysiology of autonomic dysfunction is not fully understood, decreased NO bioavailability [144, 145], increased levels of ET-1 [135] and superoxide-generating NADPH oxidase [146] in the brain may cause autonomic dysfunction. Moreover, at sites of the baroreceptors, impaired PGI_2 production, decreased arterial distensibility, platelet aggregation and increased levels of oxidative stress were associated with autonomic dysfunction [147].

Baroreflex sensitivity (BRS) is a widely used technique to measure CV autonomic function which involves the assessment of changes in BP followed by ANS induced changes in the heart rate [139]. Whilst the gold standard method to quantify BRS involves injection of vasoactive drugs, there are non-invasive methods such as recording BP and heart rate changes that occur spontaneously or during simple physical tasks, e.g. strained breathing (i.e. Valsalva manoeuvre) [139].

Previous studies reported non-dietary flavanones to beneficially affect autonomic function [62, 148]. Assessing heart rate variability, intake of 500 mg non-dietary glucosyl hesperidin acutely decreased cardiac sympathetic and increased cardiac parasympathetic nerve activity in 11 healthy women compared to intake of control treatment [62]. Similarly, in rats, administration of 60 mg glucosylhesperidin has been shown to acutely decrease cutaneous sympathetic nervous activity [148]. To support this limited evidence, further studies are warranted that examine whether flavanones naturally occurring in our diet can also beneficially modulate autonomic function assessed through BRS, in participants at a higher risk of CVD and using a dose that can be achieved in a normal diet.

Flavanones may not only beneficially affect the arterial baroreceptors but also brain regions responsible for cardiac autonomic function, given the ability of flavanones to cross the blood brain barrier [114]. Hence, considering the pathophysiology of CV autonomic dysfunction, the mechanisms by which flavanones may improve CV autonomic function require examination through assessment of changes in blood biomarkers such as NO [144, 145], PGI_2 [147], ET-1 [135], NADPH oxidase [146] and platelet activation [147].

1.7.3 Arterial stiffness as a predictor of CVD events and the beneficial effects of dietary flavonoids

Stiffening of large central arteries (i.e. aorta and carotid arteries), respectively an increase in carotid to femoral pulse wave velocity (cfPWV) or central pulse wave reflection, are recognised markers of CVD risk and strong predictors of CVD events and mortality [20, 149, 150]. Arterial stiffness is characterised by structural changes and hypertrophy of the arterial wall, in particular changes in vascular composition of scaffolding proteins (i.e. increase in collagen and decrease in elastin) and increased VSMC tone and proliferation [151, 152]. A range of factors have been suggested to contribute to the progression of central arterial stiffening, such as age, sex, smoking status, diabetes, chronic renal disease, elevated angiotensin II and endothelin-1 activity, salt intake, endothelial dysfunction and CV autonomic dysfunction [19, 143, 152, 153].

Since the pulse speed along the aorta strongly correlates with central arterial stiffness, cfPWV is considered the best surrogate marker of arterial stiffness and is an independent CVD risk marker for which reference values from a large healthy European population are available (Table 1.6) [153]. Using an oscillometric technique [i.e. the Vicorder equipment (Skidmore Medical, Bristol, UK)] with a cuff based system at the femoral and carotid arteries, cfPWV can be measured non-invasively and with little operator training dependence [20, 154, 155]. Regarding the method's reliability, in a comparison with cfPWV measured by applanation tonometry, high repeatability of the oscillometric technique was reported by Hickson et al., [154], but not by van Leeuwen-Segarceanu [155].

Table 1.6 – cfPWV reference values from a healthy European population[1]

Age category in years	Mean cfPWV (± 2 SD) in m/s	Median cfPWV (10-90 pc) in m/s
<30	6.2 (4.7-7.6)	6.1 (5.3-7.1)
30-39	6.5 (3.8-9.2)	6.4 (5.2-8.0)
40-49	7.2 (4.6-9.8)	6.9 (5.9-8.6)
50-59	8.3 (4.5-12.1)	8.1 (6.3-10.0)
60-69	10.3 (5.5-15.0)	9.7 (7.9-13.1)
≥ 70	10.9 (5.5-16.3)	10.6 (8.0-14.6)

[1] n = 1455; Abbreviations: cfPWV, carotid to femoral pulse wave velocity; 10pc, the upper limit of the 10th percentile; 90 pc, the lower limit of the 90th percentile; Table adapted from [153]

A recent study demonstrated that central pulse wave reflection, another surrogate marker of arterial stiffness, can be accurately estimated as the central augmentation index (cAIx) using the same oscillometric technique [156]. The method involves estimation of central BP derived from non-invasive measures of brachial BP and has been validated against invasive and applanation tonometry measurements of central BP [156].

In a systematic review, Pase et al., [157] stated that whilst pooled data from four isoflavone interventions giving 80-118mg isoflavones/d for 5 to 12 weeks in healthy participants suggested a significant decrease in arterial stiffness, there were insufficient data for other flavonoid subgroups, with no studies examining flavanones. Nevertheless, two acute studies have reported beneficial acute effects on arterial stiffness following a single dose of flavan-3-ols provided as dark chocolate [158] or as tea [159] in healthy individuals. Endothelium dependent dilatation of resistant arteries, resulting in a decrease in pulse wave reflection, has been proposed as the underlying mechanism responsible for the acute beneficial effects of flavan-3-ols on arterial stiffness [158, 159]. Whether flavanones also improve arterial stiffness and what the underlying mechanisms might be remain areas of future research.

1.7.4 Increased platelet activation plays a key role in atherothrombotic risk and may be decreased by dietary constituents

Whilst platelets are important for primary haemostasis repairing vascular injuries, they also play a key role in the development of thrombi leading to coronary occlusion and stroke, and increased platelet activation and aggregation contributes to the progression of atherosclerosis [21].

In a pathophysiological state, there is an impaired interaction between endothelial cells and platelets [160]. Dysfunctional endothelium lacks its thromboresistant ability due to increased permeability, secretion of pro-coagulant compounds such as von Willebrand factor and collagen and decreased secretion of platelet-inhibitory mediators such as NO and PGI_2, thereby increasing platelet reactivity [160]. Conversely, activated platelets promote endothelial dysfunction and atherosclerosis through secretion of soluble CD40L, P-selectin and matrix metalloproteinase [21, 161].

Whilst turbidimetric measurement of *ex vivo* platelet aggregation is the historical gold standard for the assessment of platelet function, quantification of P-selectin (i.e. a protein indicating platelet degranulation) and activated fibrinogen receptor (gpIIb/IIIa) expression on *ex vivo* stimulated platelets using whole blood flowcytometry is another recognised method to test platelet function [162]. Furthermore, circulating levels of soluble CD40L, P-selectin, prothrombin fragments 1+2 or urinary levels of TXA_2 metabolites are further available *in vivo* blood biomarkers of platelet function [163].

Platelet-inhibitory effects may be a potential mechanism by which dietary constituents such as flavonoids contribute to cardiovascular health. Whilst flavanones have been suggested to inhibit platelet aggregation in platelets *in vitro* [73-75] and in animals *in vivo* [74] (see Sections 1.4.3 & 4), and although a critical review of human RCTs found sufficient evidence to suggest that flavan-3-ols inhibit platelet aggregation [164], the potential platelet-inhibitory effects of flavanones in humans remains to be examined.

1.8   Summary and concluding remarks

The present literature review describes current knowledge on flavanones, their dietary occurrence, human ADME and potential CV benefits. Furthermore, remaining limitations on existing data that need to be addressed in future research were discussed and some of these gaps were addressed to some extent in the present work.

In summary, CVD is one of the main causes of mortality in the UK [1] and intake of dietary components is a modifiable risk factor with an impact on the development of CVD [22]. Flavanones may be an important dietary component, given the high content of flavanones in citrus fruits and juices, both of which are highly consumed worldwide [99], and flavanones being a relatively well-absorbed flavonoid subclasses [109]. Indeed, a body of epidemiological evidence suggests an inverse association of flavanone/citrus fruit intake and RR of CVD [43-52] and *in vitro* and animal studies reported beneficial effects on underlying vascular function and BP.

However, there are insufficient *in vivo* human data to determine if dietary flavanones have beneficial CV effects and the potential underlying mechanisms by which flavanones may exert these beneficial CV effects remain poorly understood. To date, there are two RCTs suggesting beneficial acute or short-term (i.e. 3-4 weeks) effects of orange juice or hesperidin (doses ranging between 292mg and 500mg) intake on DBP and endothelial function in different study populations (i.e. healthy overweight men and adults with metabolic syndrome).

In conclusion, further human RCTs are warranted to corroborate previously reported beneficial effects of dietary flavanones on endothelial function, assess potential benefits on unexplored markers of CVD risk (e.g. CV autonomic function, arterial stiffness and platelet activation) and relate findings in these markers of CVD risk to potential changes in blood biomarkers to explore the underlying mechanism of action. Moreover, potential additive/synergistic beneficial CV effects of flavanones with other bioactive compounds found in the food matrix of orange juice and associations with plasma concentrations of absorbed individual flavanone metabolites require further examination. The aim of this PhD research project was to systematically review existing human RCTs on flavanones and CVD risk markers (including vascular, metabolic and inflammatory) (Chapter 2), and

to conduct an acute human dietary RCT (Chapters 3-5) to address research gaps identified in the literature and systematic review (Chapter 1+2).

1.9 PhD hypothesis

It is hypothesised that a single dose of flavanones, given as a supplement or within orange juice, acutely improves markers of CVD risk at times of anticipated peak plasma flavanone concentration (i.e. 5 h after intake) in men at mild to moderate CVD risk (10 to 20 % over 10 years based on the British Hypertension Society (BHS) risk calculator [38]). Flavanones reach (sub)micromolar plasma concentrations between 4 and 8 h after their intake and may, in turn, exert acute cardioprotective effects over a prolonged period. However, the present study was constrained to examining potential effects only at 5 h after intake of flavanones because of limited hours of study nurse cover and concurrent implementation of 2 further acute flavonoid intervention RCTs by the same research group.

Specific PhD objectives:

1. To systematically review existing human RCTs on flavanones and markers of CVD risk.

2. To examine potential acute effects of hesperidin on markers of CVD risk; specifically, digital endothelial function, CV autonomic function, central arterial stiffness and platelet activation.

3. To assess CV plasma biomarkers to explore potential underlying mechanisms of action by which flavanones may improve markers of CVD risk.

4. To investigate potential additive/synergistic effects of bioactive compounds in the orange juice matrix; in particular testing the effect of orange juice *versus* matched hesperidin and vitamin C *versus* matched vitamin C.

5. To quantify flavanone-derived phenolic acid metabolites in plasma and potentially identify novel metabolites.

6. To explore associations between acute changes in CVD risk markers with plasma concentrations of individual flavanone metabolites.

Chapter 2. Flavanone extracts, flavanone-rich foods and CVD risk markers; a systematic review of randomised controlled trials

2.1 Introduction

Epidemiological evidence suggests an association between an increased intake of flavanones and citrus fruit and a lower relative risk of coronary heart disease (CHD) [49-51] and stroke [43, 45-48], with support from *in vitro* and animal studies [60, 94, 165], yet a causal relationship in human has not been established. In a previous systematic review, two studies on flavanones [166, 167] were included in a systematic review of flavonoids and cardiovascular (CV) disease (CVD) risk (papers search up to July 2007 [128]) and since this date, a number of additional RCTs have been published relating to the effect of flavanones on markers of CVD risk. However, there is no systematic review of randomised controlled trials (RCTs) examining the effects of flavanones and citrus fruit on markers of CVD risk.

The aim of this systematic review, therefore, was to provide an updated assessment of RCTs examining potential cardioprotective effects of flavanones. To do this, the methodology of this systematic review has followed the Cochrane handbook of systematic reviews [168] and built upon our group's previous systematic review [128]. A systematic review protocol was developed, to include a flavanone specific search strategy, study selection criteria, data collection method and data analysis strategy to answer the primary research question.

2.2 Objectives

This systematic review was conducted to examine the effects of acute as well as chronic flavanone intake on CVD risk biomarkers. The primary aim was to explore potential effects of flavanones on related clinical markers of CVD risk, including blood pressure (BP), endothelial function, autonomic function and arterial stiffness and associated blood biomarkers (e.g. nitric oxide metabolites (NOx) and endothelin-1). Secondary outcome measures included biomarkers of lipid status, metabolism, inflammation, platelet aggregation and venous insufficiency.

Secondary study aims were to explore how the specific constituents of flavanones (e.g. hesperetin, naringenin and eriodictyol), intervention dose, intervention duration, studied participants (i.e. healthy, at elevated baseline CVD risk or with CVD) and food matrix (i.e. flavanone extracts or flavanone-rich foods) might influence the benefits of flavanones in reducing CVD risk.

2.3   Methodology

2.3.1 Criteria for study inclusion

This systematic review was restricted to RCTs of either parallel or crossover design that were conducted in adults (> 18 years old) and gave a dose of flavanones orally as an extract, as a pure compound or within a food source (i.e. citrus fruits) which must have been compared with a control group receiving a closely matched control intervention. Study endpoints had to include markers of CVD risk, i.e. systolic and diastolic BP (SBP and DBP), endothelial function, autonomic function, arterial stiffness, biomarkers of vascular function, plasma lipids, inflammatory biomarkers, biomarkers of platelet aggregation (e.g. thrombin and von Willebrand Factor), metabolic biomarkers (e.g. plasma glucose and insulin) and venous insufficiency.

Exclusion criteria also included:

- No translation to English, German, French or Spanish
- Multifactorial intervention in which the effect of flavanones could not be isolated
- Study populations with pregnant or breastfeeding women
- Participants with renal problems, inflammatory conditions (e.g. rheumatoid arthritis) or terminal cancer

Studies of short-term (i.e. 2-12 weeks) and chronic (>12 weeks) design were included, as were acute studies that made CVD risk marker assessments between 3 and 8 h after a single intake of flavanones. In acute studies, this postprandial time period (i.e. after 3 to 8 h), coincides with the highest flavanone concentration in plasma [71, 78], and, therefore, is likely to represent the period when flavanones may exert beneficial CV effects.

2.3.2 Search strategy

The Cochrane Library, MEDLINE, EMBASE, CINAHL and AMED were searched by using flavanone terms to January 2013. Indexing and text terms as well as truncation and sensitive RCT and human filters [169] were used in the following format: [human adult] AND ([flavanones OR flavanone-rich food text terms) OR (flavanones or flavanone-rich food indexing terms]) AND human RCT filter. Figure 2.1 illustrates the MEDLINE search strategy that was employed and which was adapted for the other database searches due to differences in search syntax. Furthermore, reference lists of included studies were reviewed to identify any other potential studies that may not have been identified in the searches.

Figure 2.1 – MEDLINE Ovid search strategy for flavanone randomised controlled trials

MEDLINE (Ovid)

#	Searches
1	randomized controlled trial.pt.
2	controlled clinical trial.pt.
3	randomized.ab.
4	control.ab.
5	clinical trials as topic.sh.#
6	randomly.ab.
7	trial.ti.
8	1 or 2 or 3 or 4 or 5 or 6 or 7
9	exp animals/ not humans.sh.
10	8 not 9
11	(flavanone* or hesper* or narin* or narirutin or eriodictyol or citrus or orange* or grapefruit* or lemon* or lime* or pummelo* or tangerine* or satsuma*).ti,ab.
12	exp flavanones/ or exp hesperidin/
13	exp citrus/ or exp citrus aurantiifolia/ or exp citrus paradisi/ or exp citrus sinensis/
14	11 or 12 or 13
15	10 and 14

terms ending with "/" are indexing terms and exploded if proceeding "exp"; "*" is the truncation term, e.g. narin* might stand for naringenin or naringin. Abbreviations: .pt, publication type; .ab, abstract; .ti, title; sh, medical subject heading (MESH)

2.3.3 Study selection

Titles and abstracts were screened for inclusion and those clearly not meeting the inclusion criteria were excluded. In addition to screening by the main author MS (Manuel Schär), a second reviewer, PC (Dr Peter Curtis), independently screened the full search to minimise potential screening error occurrences. Full texts were obtained, and inclusion criteria applied for studies remaining after the initial abstract screen. Again, the two reviewers (MS and PC) independently assessed full texts for inclusion; additionally, a subset of the full texts were checked by Dr Lee Hooper [128]. Any disagreement in study inclusion/exclusion was resolved in a joint meeting. For studies that could not be included because randomisation was not reported, authors were contacted for clarification.

2.3.4 Data extraction and validity of studies

From the studies that met the inclusion criteria, data were extracted using an adapted data extraction form from our group's systematic review [128]. Extracted data included bibliographic data, randomisation, blinding, adverse events reporting, intervention (including duration, design, dose, form and control), participant's characteristics (i.e. age, sex, medication use, lipid status and BP), sample size, dropout rate, study compliance and outcome measures. Authors were contacted when data on outcome measures were missing (e.g. baseline values).

Further data extraction to assess the validity of studies included randomisation procedure, allocation concealment, masking of participants and study scientists to the intervention, industry funding, participant drop outs, intervention compliance and selective outcome reporting.

2.3.5 Data synthesis

For continuous data in parallel studies, the number of participants and the means ± SD of changes between baseline and post intervention values or absolute post intervention values, were used. The data were excluded from analysis if the difference between baseline measures of the experimental and control intervention was greater than the difference between post-intervention and baseline for at least one arm. For continuous data in crossover studies, mean differences (MDs) ± standard error (SE) between the experimental and the control group were used. If these were not provided, absolute post intervention values of the experimental and control group were used separately, despite a consequent reduction in the power of crossover studies to detect effects [170]. For studies examining a time-response relationship, only the data collected at the time-point of highest treatment effect were used. When necessary, SDs were calculated from SE, confidence intervals (CI) or *P*-values [171]; where different units were reported, data were converted to international system units. If non-numerical data were reported, data values were estimated from figures. For studies with multiple experimental interventions, means and SDs were combined in order to achieve a single pair-wise comparison or if only MDs were reported the experimental group showing more effects was used [171].

2.3.6 Data analysis

Due to the small number of studies published, the main analysis was conducted grouping together interventions with flavanone extracts and interventions with flavanone-rich foods. An exception was made for studies which examined bitter orange extract (Nature's Way, Utah, US); these were analysed separately, because as well as being a source of flavanones, a compound in bitter orange (i.e. synephrine) used for weight loss treatment has also been examined for adverse CV effects [172].

Meta-analysis was conducted using the REVMAN software (version 5.1.2; The Cochrane Collaboration, Oxford, United Kingdom) with the DerSimonian and Laird random effect model [173, 174]. MDs ± SEs were obtained from parallel studies and crossover studies that reported means ± SDs for the experimental and control group separately by performing meta-analyses of continuous data. Then using MDs ± SEs from all studies, meta-analyses of generic inverse variance were conducted to receive the pooled effect [173]. Where meta-analyses contained <3 pair-wise comparisons or <50 participants, data were presented in a summary of findings table [175].

Sensitivity analyses were performed to test the quality of trials included in the meta-analysis, and risk of bias was summarised in a table. A Cochrane's test for heterogeneity was performed to verify whether studies included in a meta-analysis examine the same underlying size of effect. P, indicating variation in effect size, and I^2, indicating

inconsistency among studies, were considered as reporting heterogeneity when <0.1 and >50 %, respectively [176].

2.4   Results

Figure 2.2 displays the flow diagram outlining the search results for this systematic review. After roughly deleting duplicates, the search yielded 1766 papers, of which 1715 were excluded in the screening process. Of the 51 papers, which appeared potentially eligible (after abstract scanning), full texts were collected for closer assessment, and 41 papers were excluded which resulted in a final set of 10 papers that met the inclusion criteria.

Figure 2.2 – Systematic review flow diagram

Search results to January 2013:
• AMED   (n = 111)
• CENTRAL   (n = 989)
• CINAHL   (n = 567)
• EMBASE   (n = 1444)
• MEDLINE   (n = 1201)
• **Total roughly de-duplicated   (n = 1766)**

Titles and abstracts excluded because they did not meet inclusion criteria or were duplicate (n = 1715)

Full texts assessed for inclusion (n = 51)

Full texts excluded (n = 41) due to:
• no adequate control (n = 10)
• no randomisation (n = 7)
• multifactorial intervention (n = 8)
• only in Japanese (n = 3)
• acute outcomes at irrelevant postprandial time points (n = 6)
• no CV outcome meeting inclusion criteria (n = 6)
• weight loss included as study objective (n = 1)

Included papers (n = 10)

Primary outcomes:
• endothelial function (n = 2)
• blood pressure (n = 6)

Secondary outcomes:
• blood lipids (n = 7)
• inflammatory markers (n = 2)
• lipoprotein oxidation (n = 2)
• glucose metabolism (n = 3)

The 10 included RCTs examined the effects of citrus fruits (n = 3), flavanone supplements (n = 4), both (n = 1) or bitter orange extract (n = 2) on CVD risk markers (Table 2.1). CVD risk markers that were assessed included primary outcomes; BP (n = 2), endothelial function (n = 2) and biomarkers of vascular function (n = 1) and secondary outcomes; plasma lipids (n = 7), inflammatory biomarkers [i.e. CRP, interleukin-6, von Willebrand Factor, soluble intracellular adhesion molecule (sICAM) and soluble vascular cell adhesion molecule (sVCAM)] (n = 2), metabolic biomarkers (i.e. plasma glucose and insulin sensitivity) (n = 2), and venous insufficiency (n = 1). Across all studies, the flavanone intervention consisted of doses ranging from 292 to 1000 mg and was compared with a control intervention. The studies had a median of n = 19 participants, lasted 3 h to 8 weeks with 5 studies performed in healthy study populations, 4 in patients with elevated CVD risk and one whose study population is not clear.

Table 2.1 – Characteristics of included studies investigating flavanones and CVD risk biomarkers

Study	Study design	n (E/E/C)	Participants	Age (years)[1]	Study duration	Intervention	Outcome
Rizza et al., [60]	crossover	24/24	adults with metabolic syndrome partly medicated[2]	52 ± 10	3 wk	hesperidin supplement (500mg)/d *vs* control supplement/d	BP, FMD, blood lipids, glucose metabolism, inflammation
Morand et al., 2011 [59]	crossover	23/23/23	healthy men	56 ± 5	Acute: (6 h)	500ml orange juice/d (292 mg hesperidin) *vs* 292 mg hesperidin supplement/d *vs* control supplement/d	BP, microvascular reactivity, blood lipids, glucose metabolism, inflammation
Demonty et al., 2010 [177]	parallel	59/64/65	moderately hypercholesterolaemic adults	60 ± 9	4 wk	hesperidin supplement (800mg/d) *vs* naringin supplement (500mg/d) *vs* control supplement	blood lipids
Bui et al., 2006 [178]	crossover	15/15	healthy adults	26 ± 2	6 h	single dose of bitter orange supplements (900mg) (flavanone concentration unknown) *vs* control supplement	BP and HR
Gorinstein et al., 2006 [179]	parallel	19/19/19	hypertriglyceridemic patients with CHD	Range: 39 - 72	4 wk	1 blonde grapefruit/d *vs* 1 red grapefruit/d *vs* usual diet	blood lipids
Min et al., 2005 [180]	crossover	18/18	healthy adults	24.9 ± 4.4	8 h	1 bitter orange supplement (450mg) *vs* 1 control supplement	BP and HR arrhythmia index
Gorinstein et al., 2004 [181]	parallel	22/22/22	hypercholesterolaemic patients with CHD	Range: 48 - 66	4 wk	1 sweetie/d *vs* 2 sweeties/d *vs* usual diet	blood lipids
Vinson et al., 2001 [182]	crossover[2]	16/10	not reported	53 ± 10	8 wk	3 supplements of 990 mg vitamin C + 2700 mg citrus extract/d *vs* 2 supplements of 1000mg vitamin C/d	blood lipids
Harats et al., 1998 [183]	parallel	19/17	healthy men	19.8 range: 18 - 23	8 wk	controlled diet + 1.1l orange juice/d (600mg flavanones) *vs* controlled diet	blood lipids
Cospite et al., 1989 [166]	parallel	43/45	adults with chronic venous insufficiency	Range: 20 - 72	8 wk	hesperidin supplement (900mg diosmin and 100mg hesperidin) *vs* control supplement (900mg diosmin)	venous insufficiency (calf and ankle circumference after 15 min walking)

Abbreviations: n, number of participants per arm; CVD, cardiovascular disease; E, experimental interventions; C, control intervention; d, day; BP, blood pressure; HR, heart rate; CHD, coronary heart disease; FMD, flow mediated dilatation; LDL, low density lipoproteins; [1]mean ± SD unless stated [2]each participant was assigned to two of four intervention supplements; [2] Participants were prescribed to the following therapies: antidiabetic (n = 17), antihypertensive (n = 10), statin (n = 1) and antiplatelet (n = 3)

Table 2.2 – Risk of bias assessment of included studies

Study	Sequence generation (selection bias)	Allocation concealment (selection bias)	Masking of participants and personnel (performance bias)	Masking of outcome assessors (detection bias)	Incomplete outcome data (attrition bias)	Selective reporting (reporting bias)	Carry-over effect (other bias)	Total per study: (low risk/unclear/ high risk)
Rizza et al., [60]	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Unclear	6/1/0
Morand et al., 2011 [59]	Unclear	Unclear	Unclear	Unclear	Low risk	Low risk	NA	2/4/0
Demonty et al., 2010 [177]	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	7/0/0
Bui et al., 2006 [178]	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	7/0/0
Gorinstein et al., 2006 [179]	Unclear	Unclear	High risk	Unclear	Low risk	High risk	NA	1/3/2
Min et al., 2005 [180]	Unclear	Low risk	Unclear	Unclear	Low risk	Low risk	Low risk	4/3/0
Gorinstein et al., 2004 [181]	Unclear	Unclear	High risk	Unclear	Unclear	High risk	NA	0/4/2
Vinson et al., 2001 [182]	Unclear	Low risk	Unclear	Unclear	Unclear	Low risk	Low risk	3/4/0
Harats et al., 1998 [183]	Unclear	Low risk	High risk	Unclear	Unclear	Low risk	NA	2/3/1
Cospite et al., 1989 [166]	Unclear	Unclear	Unclear	Unclear	Low risk	Low risk	NA	2/4/0
Total per domain: (low risk/unclear/high risk)	3/7/0	6/4/0	3/4/3	3/7/0	7/3/0	8/0/2	4/1/0	34/26/5

NA, not applicable due to parallel design

Across the 10 included studies, there was variation in the risk of bias of which half were assessed as "low risk", 44 % as "unclear" and 6 % as "high risk" (Table 2.2). Whilst for most studies the risk of bias assessment was unclear, the studies of Morand et al., [59], Rizza et al., [60] and Bui et al., [178] addressed the risk of bias extensively and were the only RCTs that explained the methods used for randomisation and blinding. In comparison to the 7 day washout period in the majority of studies, with 3 days the study of Rizza et al., [60] might have had some risk of bias. The two studies of [179, 181] were assessed as being at high risk of bias, mainly because baseline values were neither reported nor provided after contacting the authors. Furthermore, Gorinstein et al., [179, 181] together with Harats et al., [183] used a usual diet as control intervention which indicates that participants were not blinded to the intervention.

2.4.1 Primary outcomes: blood pressure, endothelial function and NOx

The data from 2 studies that examined effects of short-term (i.e. 3 - 4 weeks) flavanone intake on BP and endothelial function were mixed [59, 60]. Following a flavanone supplement intervention, Rizza et al., [60] observed an improvement in mean brachial artery flow mediated dilatation (FMD) by 2.5 % (95 % CI: 0.4, 4.6), but no effect on DBP in adults with metabolic syndrome (Table 2.3). In contrast, a study in overweight men by [59] reported that flavanones given as a supplement or within orange juice both reduced mean DBP by -3.2mm Hg (95 % CI: -6.1, -0.3) and -5.5mm Hg (95 % CI:, -9.0, -2.0) respectively, and the orange juice intervention tended to increase mean NOx plasma concentration (12.4µmol/L 95 % CI: 1.4, 23.4; P = 0.08). However, there was no change in NOx plasma concentration following the supplement and no change in microvascular reactivity in either intervention (i.e. orange juice and flavanone supplement) [59].

The same study [59] also explored acute effects of flavanones on endothelial function at 6 h following single/acute intake and reported an improvement in microvascular reactivity in the orange juice intervention [105 % (95 % CI: 55, 155)] and a trend towards an improvement in the flavanone supplement intervention [48 % (95 % CI: -1 %, 98 %)], but no change in NOx (data not shown) (Table 2.4).

Table 2.3 – Summary of findings for short-term effects of flavanones on CVD risk markers

Outcome	Study	Daily experimental intervention	Mean effect[1]	[95 % CI]
Blood pressure				
Systolic (mm Hg)	Morand et al.	orange juice	-3.0	[-8.3, 2.3]
		hesperidin supplement	0.6	[-5.5, 6.7]
	Rizza et al.	hesperidin supplement	2.7	[-1.3, 6.7]
Diastolic (mm Hg)	Morand et al.	orange juice	-5.5**	[-9.0, -2.0]
		hesperidin supplement	-3.2*	[-6.1, -0.3]
	Rizza et al.	hesperidin supplement	0.6	[-2.2, 3.4]
Endothelial function and NOx				
Flow mediated dilatation (%)	Rizza et al.	supplement	2.5*	[0.4, 4.6]
Microvascular reactivity (%)	Morand et al.	orange juice	52	[-8, 113]
		hesperidin supplement	17	[-44, 77]
NOx (µmol/L)	Morand et al.	orange juice	12.4	[1.4, 23.4]
		hesperidin supplement	-3.2	[-15.0, 8.6]
Vascular Inflammation				
CRP (mg/L)	Morand et al.	orange juice	0.3	[-0.1, 0.8]
		hesperidin supplement	-0.1	[-0.4, 0.3]
hsCRP (mg/L)	Rizza et al.	hesperidin supplement	-0.7**	[-0.5, -0.9]
Interleukin-6 (pg/mL)	Morand et al.	orange juice	-0.2	[-0.7, 0.2]
		hesperidin supplement	0.2	[-0.6, 1.0]
Von Willebrand Factor (U/mL)	Morand et al.	orange juice	-0.1	[-0.7, 0.6]
		hesperidin supplement	0.1	[-0.4, 0.6]
sICAM (ng/mL)	Morand et al.	orange juice	28.1	[-17.4, 73.6]
		hesperidin supplement	-4.9	[-62.3, 52.5]
	Rizza et al.	hesperidin supplement	-4.0	[-19.0, 11.0]
sVCAM (ng/mL)	Morand et al.	orange juice	-323.0*	[-617, -29]
		hesperidin supplement	-242.0	[-557, 73]
	Rizza et al.	hesperidin supplement	-28.0	[-71, 15]
Metabolic markers				
Glucose (mmol/L)	Morand et al.	orange juice	-0.1	[-0.5, 0.3]
		hesperidin supplement	-0.2	[-0.6, 0.2]
	Rizza et al.	hesperidin supplement	-0.2	[-0.5, 0.1]
Insulin (pmol/L)	Morand et al.	orange juice	-1.9	[-22.3, 18.5]
		hesperidin supplement	-1.1	[-4.2, 2.0]
	Rizza et al.	hesperidin supplement	-6.0	[-15.9, 3.9]
Venous insufficiency				
Ankle circumference (mm)	Cospite et al.	hesperidin supplement	-5.64**	[-8.7, -2.6]
Calf circumference (mm)	Cospite et al.	hesperidin supplement	-1.69	[-3.8, 0.4]

Abbreviations: CVD, cardiovascular disease; CI, confidence interval; NOx, nitric oxide metabolites; CRP, C-reactive peptide; hsCRP, high-sensitivity CRP; sICAM, soluble intracellular adhesion molecule; sVCAM, soluble vascular cell adhesion molecule; [1] difference between the mean in the experimental intervention and the mean in the control intervention; *P*-values: * < 0.05; ** < 0.01

Table 2.4 – Summary of findings for acute effects of flavanones on CVD risk markers

Outcome	Study	Experimental intervention	Mean effect[1]	[95 % CI]
Endothelial function and NOx				
Microvascular reactivity (%)	Morand et al.	orange juice [2]	105**	[55, 155]
		hesperidin supplement [2]	48	[-1, 98]
NOx (µmol/L)	Morand et al.	orange juice or supplement [2]	No effect (data not reported)	
Bitter orange extract				
SBP (mm Hg)	Min et al.	bitter orange extract	-1.0	[-6.9, 4.9]
	Bui et al.	bitter orange extract	7.3	[-1.7, 16.3]
DBP (mm Hg)	Bui et al.	bitter orange extract	2.6	[-4.8, 10.0]
Heart rate (bpm)	Bui et al.	bitter orange extract	4.2	[-4.6, 13.0]

Abbreviations: CVD, cardiovascular disease; CI, confidence interval; NOx, nitric oxide metabolites; SBP, systolic blood pressure; DBP, diastolic blood pressure; [1] difference between the mean in the experimental intervention and the mean in the control intervention; [2] both containing a matched dose of 292mg hesperidin

2.4.2 Secondary outcomes: plasma lipids

Blood lipids were analysed by pooling together the findings of 6 studies (Figure 2.3). Whilst the overall effects of a short-term (i.e. 3 to 8 weeks) flavanone intervention showed a small increase in plasma high density lipoprotein cholesterol (HDL-C) levels [0.04 mmol/L (95 % CI: 0.00, 0.08)], no effect was observed on plasma levels of low density lipoprotein cholesterol (LDL-C) [-0.30 mmol/L (95 % CI: -0.75, 0.15)], total cholesterol (TC) [-0.32 mmol/L (95 % CI: -0.73, 0.09)] and triglycerides (TG) [-0.19 mmol/L (95 % CI: -0.39, 0.02)].

Within subgroup analyses, in individuals with metabolic syndrome and prescribed to CVD related medication (i.e. antidiabetic, antihypertensive, statin and/or antiplatelet) or with a past history of CVD events, an increase in HDL-C [0.06mmol/L (95 % CI: 0.02, 0.10)] and a decrease in LDL-C [-0.64 mmol/L (95 % CI: -1.07, -0.22)], TC [-0.64 mmol/L (95 % CI: -1.04, -0.24)] and TG [-0.25 mmol/L (95 % CI: -0.48, -0.01)] was found, whereas no changes were observed in studies examining healthy populations (Figure 2.3). Given the differences in study designs, as expected, both meta-analyses on blood lipids showed significant heterogeneity (HDL-C: $P = 0.04$, $I^2 = 56$ % and LDL-C: $P<0.00001$, $I^2 = 97$ %). In the subgroup meta-analyses, while the heterogeneity disappeared in the healthy study population, it remained for the subgroup of studies examining individuals with metabolic syndrome and prescribed CVD related medication or with a past history of CVD events.

Figure 2.3 – Meta-analysis of flavanones and blood lipids

1) HDL-C

Study or Subgroup	Mean Difference	SE	Experimental Total	Control Total	Weight	IV, Random, 95% CI
1.1.1 Population with established CVD or metabolic syndrome and prescribed to medication						
Gorinstein 2004	0.07	0.026	44	22	23.0%	0.07 [0.02, 0.12]
Gorinstein 2006	0.1	0.032	19	19	19.4%	0.10 [0.04, 0.16]
Rizza 2011	0.03	0.017	24	24	29.1%	0.03 [-0.00, 0.06]
Subtotal (95% CI)			87	65	71.6%	0.06 [0.02, 0.10]
Heterogeneity: Tau² = 0.00; Chi² = 4.40, df = 2 (P = 0.11); I² = 55%						
Test for overall effect: Z = 2.88 (P = 0.004)						
1.1.2 Healthy population						
Demonty 2010	-0.02	0.024	123	65	24.4%	-0.02 [-0.07, 0.03]
Harats 1998	0	0.402	19	17	0.3%	0.00 [-0.79, 0.79]
Morand 2011	0	0.1	23	23	3.8%	0.00 [-0.20, 0.20]
Subtotal (95% CI)			165	105	28.4%	-0.02 [-0.06, 0.03]
Heterogeneity: Tau² = 0.00; Chi² = 0.04, df = 2 (P = 0.98); I² = 0%						
Test for overall effect: Z = 0.81 (P = 0.42)						
Total (95% CI)			252	170	100.0%	0.04 [-0.00, 0.08]
Heterogeneity: Tau² = 0.00; Chi² = 11.41, df = 5 (P = 0.04); I² = 56%						
Test for overall effect: Z = 1.90 (P = 0.06)						
Test for subgroup differences: Chi² = 6.37, df = 1 (P = 0.01), I² = 84.3%						

(Scale: -0.5 -0.25 0 0.25 0.5; Favours control — Favours experimental)

2) LDL-C

Study or Subgroup	Mean Difference	SE	Experimental Total	Control Total	Weight	IV, Random, 95% CI
1.2.1 Population with established CVD or metabolic syndrome and prescribed to medication						
Gorinstein 2004	-1.05	0.07	44	22	19.6%	-1.05 [-1.19, -0.91]
Gorinstein 2006	-0.67	0.065	19	19	19.7%	-0.67 [-0.80, -0.54]
Rizza 2011	-0.18	0.119	24	24	19.0%	-0.18 [-0.41, 0.05]
Subtotal (95% CI)			87	65	58.2%	-0.64 [-1.07, -0.22]
Heterogeneity: Tau² = 0.13; Chi² = 42.82, df = 2 (P < 0.00001); I² = 95%						
Test for overall effect: Z = 2.99 (P = 0.003)						
1.2.2 Healthy population						
Demonty 2010	-0.01	0.049	123	65	19.8%	-0.01 [-0.11, 0.09]
Harats 1998	0.48	0.944	19	17	4.6%	0.48 [-1.37, 2.33]
Morand 2011	0.3	0.2	23	23	17.4%	0.30 [-0.09, 0.69]
Subtotal (95% CI)			165	105	41.8%	0.06 [-0.14, 0.26]
Heterogeneity: Tau² = 0.01; Chi² = 2.52, df = 2 (P = 0.28); I² = 21%						
Test for overall effect: Z = 0.57 (P = 0.57)						
Total (95% CI)			252	170	100.0%	-0.30 [-0.75, 0.15]
Heterogeneity: Tau² = 0.27; Chi² = 183.36, df = 5 (P < 0.00001); I² = 97%						
Test for overall effect: Z = 1.29 (P = 0.20)						
Test for subgroup differences: Chi² = 8.70, df = 1 (P = 0.003), I² = 88.5%						

(Scale: -1 -0.5 0 0.5 1; Favours experimental — Favours control)

3) TC

Study or Subgroup	Mean Difference	SE	Experimental Total	Control Total	Weight	IV, Random, 95% CI
1.3.1 Population with established CVD or metabolic syndrome and prescribed to medication						
Gorinstein 2004	-1	0.104	44	22	19.6%	-1.00 [-1.20, -0.80]
Gorinstein 2006	-0.6	0.115	19	19	19.4%	-0.60 [-0.83, -0.37]
Rizza 2011	-0.3	0.135	24	24	19.0%	-0.30 [-0.56, -0.04]
Subtotal (95% CI)			87	65	57.9%	-0.64 [-1.04, -0.24]
Heterogeneity: Tau² = 0.11; Chi² = 17.84, df = 2 (P = 0.0001); I² = 89%						
Test for overall effect: Z = 3.16 (P = 0.002)						
1.3.2 Healthy						
Demonty 2010	-0.03	0.06	123	65	20.2%	-0.03 [-0.15, 0.09]
Harats 1998	0.4	0.87	19	17	4.5%	0.40 [-1.31, 2.11]
Morand 2011	0.2	0.2	23	23	17.3%	0.20 [-0.19, 0.59]
Subtotal (95% CI)			165	105	42.1%	-0.01 [-0.12, 0.10]
Heterogeneity: Tau² = 0.00; Chi² = 1.44, df = 2 (P = 0.49); I² = 0%						
Test for overall effect: Z = 0.16 (P = 0.87)						
Total (95% CI)			252	170	100.0%	-0.32 [-0.73, 0.09]
Heterogeneity: Tau² = 0.21; Chi² = 79.25, df = 5 (P < 0.00001); I² = 94%						
Test for overall effect: Z = 1.53 (P = 0.12)						
Test for subgroup differences: Chi² = 8.99, df = 1 (P = 0.003), I² = 88.9%						

(Scale: -1 -0.5 0 0.5 1; Favours experimental — Favours control)

4) TG

Study or Subgroup	Mean Difference	SE	Experimental Total	Control Total	Weight	IV, Random, 95% CI
1.4.1 Population with established CVD or metabolic syndrome and prescribed to medication						
Gorinstein 2004	-0.41	0.035	44	22	29.5%	-0.41 [-0.48, -0.34]
Gorinstein 2006	-0.13	0.032	19	19	29.7%	-0.13 [-0.19, -0.07]
Rizza 2011	-0.17	0.167	24	24	17.1%	-0.17 [-0.50, 0.16]
Subtotal (95% CI)			87	65	76.2%	-0.25 [-0.48, -0.01]
Heterogeneity: Tau² = 0.04; Chi² = 35.13, df = 2 (P < 0.00001); I² = 94%						
Test for overall effect: Z = 2.08 (P = 0.04)						
1.4.2 Healthy						
Morand 2011	0	0.1	23	23	23.8%	0.00 [-0.20, 0.20]
Subtotal (95% CI)			23	23	23.8%	0.00 [-0.20, 0.20]
Heterogeneity: Not applicable						
Test for overall effect: Z = 0.00 (P = 1.00)						
Total (95% CI)			110	88	100.0%	-0.19 [-0.39, 0.02]
Heterogeneity: Tau² = 0.04; Chi² = 41.33, df = 3 (P < 0.00001); I² = 93%						
Test for overall effect: Z = 1.81 (P = 0.07)						
Test for subgroup differences: Chi² = 2.54, df = 1 (P = 0.11), I² = 60.6%						

(Scale: -0.5 -0.25 0 0.25 0.5; Favours experimental — Favours control)

Meta-analysis of short-term (3 to 8 wk) flavanone interventions and blood lipids in mmol/L: 1) high density lipoprotein cholesterol (HDL-C), 2) low density lipoprotein – cholesterol (LDL-C), 3) total cholesterol (TC) and 4) triglycerides (TG); study populations were sub-grouped as either individuals with established CVD/metabolic syndrome and prescribed medication (i.e. antidiabetic, antihypertensive, statin and/or antiplatelet) or healthy; Meta-analysis used the DerSimonian and Laird random effect model [174].

2.4.3 Secondary outcomes: vascular inflammation, metabolic biomarkers, venous insufficiency and bitter orange extract

In two studies, short-term effects of flavanones on inflammatory and metabolic biomarkers were examined [59, 60] (Table 2.3). Morand et al., [59] found a decrease in sVCAM [−323ng/mL (95 % CI: −617, −29)] following the orange juice intervention, but not the flavanone supplement, and Rizza et al., [60] observed a decrease in high-sensitivity CRP [-0.7mg/L (95 % CI: -0.5, -0.9)]. However, neither of the studies observed a change in any of the other biomarkers measured including Interleukin-6, von Willebrand Factor, sICAM, glucose and insulin.

Furthermore, one study suggested supplementation with hesperidin (100 mg/d) for 8 weeks improved venous insufficiency [166] (Table 2.3). Following a single dose of bitter orange extract (containing an unknown dose of flavanones according to the manufacturer and 6 % synephrine), two studies did not observe a postprandial effect (i.e. 3-8 h after intake) on BP and heart rate in healthy individuals [178, 180] (Table 2.4).

2.5 Discussion

This is the first systematic review that focused on flavanone extracts, flavanone-rich foods and CVD risk markers. The systematic search identified 10 RCTs (n = 2 acute, n = 7 short-term and n = 1 both) that examined potential effects of flavanones on a range of markers of CVD risk. While there were sufficient data (i.e. ≥3 studies and ≥50 participants per intervention group) for meta-analyses on blood lipids and to stratify between study participants, the available data regarding the effects of flavanones on BP, endothelial function and plasma NOx were insufficient to draw definite conclusions. Moreover, data were insufficient to determine how flavanone subclass, intervention duration, intervention dose and food matrix may influence the cardiovascular benefits of flavanones.

Two studies to date, although with inconsistent findings, suggested that short-term (i.e. 3-4 week) intake of hesperidin or orange juice may reduce DBP by -3.2 to -5.5 mm Hg, improve endothelial function and tended to increase plasma NOx concentrations [59, 60] (Table 2.3). Inconsistency in findings may be explained because Rizza et al., [60] studied participants with metabolic syndrome that were prescribed to CVD related medication, had a 3 weeks intervention duration with 500 mg/d hesperidin supplementation and used FMD to assess endothelial function, whereas Morand et al., [59] examined potential effects of 292 mg/d hesperidin ingestion (as a supplement and within 500 ml orange juice) for 4 weeks in overweight healthy men and measured microvascular reactivity to assess endothelial function. In addition, Morand et al., [59] examined the acute effects of a single dose of orange juice or a flavanone supplement (providing 292 mg hesperidin) and observed benefits on endothelial function at 6 h after intake, which were not supported by

increases in vascular NO production (i.e. as assessed by plasma concentration of NOx) (Table 2.4).

Thus, further studies are warranted to provide scientific evidence on potential effects of flavanones on vascular markers of CVD risk. Future directions might include studies in healthy populations at mild to moderate CVD risk, interventions with longer study durations (> 4 weeks), investigations of potential food matrix effects (i.e. additive/synergistic effects with other bioactive compounds in citrus fruits including flavones, vitamin C, β-carotenes), different flavanone doses (i.e. to assess a dose-effect relationship), flavanone forms (e.g. naringin) and assessing unexplored markers of CVD risk such as central arterial stiffness [184], CV autonomic function [18] and vascular-related biomarkers (e.g. nitrite [185], S-nitrosothiols [185], NADPH oxidase [132], endothelin-1 [135] and angiotensin II [5]). Furthermore, to elucidate the potential underlying mechanisms by which flavanones exert cardiovascular effects, acute RCTs are required in which cardiovascular bioactivity can be directly associated with achieved plasma flavanone metabolite concentrations.

A greater number of studies (n = 6) have examined the effects of flavanones and citrus fruit on blood lipids. The conducted meta-analyses suggest that there is a small beneficial effect for individuals with elevated CVD risk (i.e. with metabolic syndrome and prescribed CVD related medication or with past history of CVD event), but not for healthy adults (Figure 2.3). However, these results should be interpreted with caution due to data heterogeneity and high risk of bias of the two studies of Gorinstein et al., [179, 181], specifically because they did not report whether their study population had similar blood lipid concentrations at baseline.

Thus, there is sufficient evidence to conclude that flavanones do not improve the lipid profile in healthy individuals up to doses of 600 mg/d and durations of 8 weeks, yet in individuals with metabolic syndrome and patients with established CVD, further short-term studies (i.e. several weeks to months) with low risk of bias are required to verify reported lipid lowering effects. Whilst animal studies suggest flavanones do lower blood lipids through a decrease of fatty acid metabolism and an increase in hepatic lipid β-oxidation [165, 186], the underlying mechanism in human remains to be established.

Overall, the observed beneficial CV effects of flavanones may be of clinical importance. Although improvements were moderate, if the effects are sustained in the long term, a 5 mm Hg decrease in DBP may account for a 22 % and 41 % reduced risk of stroke and CHD, respectively [187], a 2.5 % increase in FMD has been suggested to decrease CVD risk by 20 % [188] and a 0.64 mmol/L lower plasma TC concentration has been associated with a 32 % reduced risk of CHD [189].

Limitations of the few RCTs (n = 10) that have been performed to date include small numbers of participants, substantial differences in baseline values between the intervention groups. Only three studies adequately reported how risk of bias was minimised, and in two studies it was not clearly stated how data were reported (e.g. post intervention values, changes from baseline or mean differences between the experimental and control interventions). Furthermore, some outcome measures were assessed using different approaches, such as FMD and microvascular reactivity to assess endothelial function making it difficult to pool the effects. Given these various limitations, it might be that reported effects are overestimated.

In summary, this systematic review outlines the findings from RCTs to date on flavanones and CVD risk markers. Having identified only 10 published RCTs, this systematic review illustrates that the area of flavanone research remains to be extended with further RCTs that address current limitations. However, the available limited data suggest that flavanones may decrease diastolic BP, improve endothelial function and blood lipid status, inhibit inflammation and reduce venous insufficiency.

Chapter 3. Acute flavanone randomised controlled trial in men at mild to moderate risk of CVD: study protocol and methods

3.1 Rational for study design

3.1.1 Rational for acute design, study population, intervention materials and dosage

An acute study design was chosen to identify potential physiological and cellular mechanisms by which dietary flavanones exert potential cardioprotective effects, as this offers a number of advantages over a short-term intervention (i.e. several weeks). First, a beneficial change in CVD risk biomarkers can be related to actual presence of flavanone metabolites in plasma, thereby providing more evidence for a causal link between flavanones and improvements in markers of CVD risk. Second, given that each experimental period consists of only one day, potential confounders such as dietary and exercise restrictions can be strictly controlled making the study design more robust. Third, establishing potential acute beneficial effects of flavanones serves as an initial step to inform the design of future longitudinal studies (i.e. study population, flavanone dose and CVD risk biomarkers). Despite these advantages, it is acknowledged that acute studies provide little evidence for a potential CV health benefit of flavanones and short-term and chronic (i.e. weeks to years) flavanone RCTs are of fundamental importance to examine these potential benefits.

Males at mild to moderate CVD risk, but otherwise healthy were chosen as the study population. In the UK, men are at a higher CVD risk than women [1], and early modification of dietary and lifestyle factors has been reported highly effective in primary prevention of CVD [190]. Thus, this population could particularly benefit from bioactive dietary compounds such as flavanones to reduce CVD progression.

Flavanones in orange juice were used in this study as flavanones are a poorly studied subclass in respect to potential vascular effects in humans *in vivo* and orange juice is the main source of flavanones in the human diet [48]. As identified in the conducted systematic review (Chapter 2), there is limited evidence suggesting beneficial CV effects of dietary flavanones in humans [59, 60]. Orange juice from concentrate has been chosen as the dietary intervention product because oranges are a rich source of flavanones (mean ± SD flavanone content: 53 ± 6 mg/100ml of juice (n = 6); in-house analysis Appendix 1) and account for 82 % of total dietary flavanone intake [48]. Furthermore, with hesperidin and narirutin accounting for 80 % and 10 % of the total flavonoid content, respectively [100], orange juice from concentrate has a relatively simple flavanone profile and, therefore, a closely matched flavanone extract for the flavanone supplement

intervention was commercially available. The pharmacokinetics of flavanones from orange juice have been established in a number of studies [71, 72, 78, 107, 108], which provided necessary information for the design of this acute bioefficacy study. In particular, flavanones reach a peak plasma concentration between 4.4 and 6 h after intake of orange juice and flavanone bioavailability strongly correlates with soluble flavanone in orange juice [78], due to which the present study assessed markers of CVD risk at 5 h post intervention and selected an orange juice with a high amount of soluble flavanones.

This study provided 320 mg hesperidin in the orange juice intervention and flavanone supplement intervention which was anticipated to attain a flavanone plasma concentration in the low micromolar range. This is a concentration that has previously been linked with beneficial effects on endothelial function in several acute interventions with flavanones [59], flavan-3-ols [191, 192] and isoflavones [193]. With a pint of orange juice from concentrate providing on average 300 mg hesperidin [100], the tested dose could also be achieved within a general UK diet. As orange juice flavanone content is variable [38], the orange juice required for this trial was bought from a single batch of orange juice, and the flavanone content was assessed in-house to determine the amount of orange juice providing 320 mg of hesperidin.

3.1.2 Rational for selection of clinical measurements

In this acute study, the effects of hesperidin on endothelial function, CV autonomic function and central arterial stiffness were assessed using non-invasive clinical measurements. Impaired endothelial function is considered to occur early in the stage of atherosclerosis and, therefore, endothelial function is a common outcome measure to assess the effects of dietary compounds [17, 194]. Whilst Morand et al., [59] reported an acute improvement in microvasculature endothelial function after intake of flavanones, this acute study examined endothelial function in the digital artery using a validated method [124, 125, 195].

To the author's knowledge, assessment of potential acute effects of dietary flavanones on arterial stiffness and autonomic function were novel in the field of dietary flavanones and CVD risk markers. Both measures have been suggested as independent predictors of CVD risk and development of hypertension [18, 184], and were assessed using validated methods [154, 196].

3.1.3 Rational for selection of CV biomarkers to assess vascular NO production, NADPH oxidase and platelet function

NO has a half-life of a few seconds in blood and, therefore, its more stable metabolites (i.e. nitrate, nitrite and nitroso species) are commonly measured to estimate the amount of

NO produced by the vasculature [197]. In this study, plasma nitrite concentrations were chosen as the biomarker of vascular NO production because it is a more accurate indicator than plasma total NO metabolite concentration [64, 198] and based on the methodology optimised in this laboratory, nitrite concentrations could be quantified with greater reproducibility than nitroso species, another sensitive biomarker of vascular NO production [65].

A few methods are available to assess NADPH oxidase in humans *in vivo*, these include its genetic expression through quantification of serum soluble gp^{91phox} by ELISA [199], and its activity in neutrophils using flow cytometry [200]. In the present study, assessment of serum soluble gp^{91phox} was performed because it allowed time-efficient batch analysis of frozen samples.

Platelet function can be assessed using a range of methodologies including platelet aggregation in platelet rich plasma (the historic gold standard), automated platelet function analysis or quantification of activation-dependent platelet surface proteins (i.e. P-selectin and activated gpIIb/IIIa (i.e. fibrinogen receptor) (Figure 5.1); using whole blood platelet activation flow cytometry) [162, 201]. The latter method was chosen because it is a whole blood based assay (i.e. potentially less artefactual variability than assays in platelet rich plasma) and has been proven as a sensitive method for the monitoring of antiplatelet agents [162] and dietary compounds such as flavan-3-ols [202]. Furthermore, the chosen method allowed simultaneous assessment of distinct platelet activation pathways [162]. As proposed by animal and *in vitro* research [73, 74], collagen and arachidonic acid mediated platelet activation may be most potently inhibited by flavanones followed by ADP and thrombin. For the present study, a method was successfully optimised to assess potential inhibitory effects of flavanones on the ADP and the collagen activation pathways, but not for the arachidonic acid pathway (Section 1.7.4), possibly because it would have required samples to be at 37 °C [203].

3.2   Participants and methods

3.2.1 Study population

16 healthy men aged 50 - 75 years with an estimated absolute 10 year CVD risk of 10 - 20 % (British Hypertension Society (BHS) risk calculator [204]; assessed at screening) were recruited locally through advertisement and collaboration with GP practices. Study exclusion criteria were: smoking (within the last 3 months), past history or existing medical condition likely to affect study outcome measures (i.e. CV, hepatic, renal, digestive, haematological, neurological thyroidal disease, diabetes or cancer), use of lipid lowering (i.e. statins), antihypertensive or vasodilator medication, resting SBP / DBP > 160 / 95 mm Hg, use of antibiotics and flu vaccination ≤ 3 months before and during study participation,

intake of flavonoid containing dietary supplements ≤ 1 month before and during study participation. Prescription to antiplatelet medication was not part of the protocol's exclusion criteria, but medication intake was recorded and, for consistency, participants who had a prescription were asked to maintain their antiplatelet medication intake during study participation. Specifically, participants had no gastrointestinal disease and were not on antibiotics or had vaccinations (for 3 months prior and during study participation), both of which are factors that could affect the gut microflora and, in turn, flavanone metabolism and absorption [205, 206].

The study, which not only consisted of the herein described flavanone trial, but also an isoflavone and anthocyanin trial, was approved by the East of England Research Ethic Committee Norwich (ref 11/EE/0233) and the Norwich & Norfolk University Hospital Research & Development [ref 2011DIET01S (66-05-11)], followed the principles declared in the Declaration of Helsinki Revised Version (2008) and was registered at clinicaltrials.gov (registration no: NCT01530893). Prior to study commencement, written informed consent was obtained from each participant and all subsequent screening visits and experimental periods were performed at the Clinical Research and Trial Unit at the University of East Anglia.

3.2.2 Dietary intervention products

6 orange juices were sourced from 5 local commercial suppliers (i.e. Aldi, Brakes, Cooperative, Tesco and Waitrose) and from the Florida Department of Citrus (FDOC; Lake Alfred, Florida) with the latter being an orange juice previously used in flavanone intervention studies [59]. Flavanone extract was provided by Monteloeder (Alicante, Spain) and carboxymethyl-cellulose (CMC) by Dow Wolff Cellulosics (Bitterfeld, Germany). White opaque gelatine capsules were purchased from Fragon (Minneapolis, Minnesota), and vitamin C (i.e. L-ascorbic acid), fructose, glucose, sucrose and nitrate/nitrite free Buxton bottled water from commercial shops.

Hesperidin, narirutin and vitamin C were quantified in the 6 orange juices and the flavanone extract using an in-house developed HPLC method (Method and Results in Appendix 1). Amongst the 6 orange juices, the FDOC orange juice was selected because it had the highest content of soluble hesperidin (i.e. 23 %), which has been reported an important factor influencing flavanone bioavailability [78]. To provide a hesperidin dose of 320 mg, 767 mL (≈3 servings [207]) of FDOC orange juice were required, and subsequently the hesperidin-matched flavanone extract and the control interventions were matched to the orange juice in terms of vitamin C, sugars and amount of liquid consumed (Table 3.1). Additionally for the flavanone extract intervention, capsules were filled with flavanone extract containing 320 mg hesperidin. All required supplements and sugar

sachets were prepared in an analytical kitchen using calibrated analytical scales, whereby two researchers verified that amounts were accurately weighted out.

A single batch of products was used throughout the study, and potential degradation of the study intervention products was minimised by use of monitored storage conditions. FDOC concentrate was stored at -20 °C, and compound capsules and sugar sachets were held within a temperature monitored pharmacy room.

After study completion, content and solubility of flavanones and vitamin C were reanalysed in the orange juice to verify potential changes during storage at -20 °C over several months (Appendix 1, Table 2). There was no flavanone degradation with even a marginal increase in hesperidin content and in narirutin solubility, which may be due to variability between the different tins of orange juice concentrate. On the other hand, vitamin C levels in the orange juice were reduced from 57.3 mg/100 mL pre-study to 46.7mg/100ml post-study (P<0.01; Appendix 1, Table 2).

Table 3.1 – Composition of the dietary interventions

	Dietary intervention		
	Orange juice	Supplement	Control
Drink volume (mL)	767	767	767
Supplements (#)	CMC (2)	Flavanone (1) vitamin C (1)	vitamin C (1) CMC (1)
Sugar[1] (g)	68	68	68
Hesperidin[2] (mg)	320	320	0
Narirutin[2], (mg)	48	16	0
Vitamin C[2], (mg)	439	439	439
Sinapic acid[3] (mg)	6	ND	ND
Caffeic acid[3] (mg)	3	ND	ND
Ferulic acid[3] (mg)	27	ND	ND
Courmaric acid[3] (mg)	11	ND	ND

[1] Matched sugar composition based on data provided by the Florida department of citrus: 16.6 g glucose, 18.0 g fructose and 33.4 g sucrose. [2] quantified in-house using HPLC. [3] based on reported mean contents in orange juice [208]. Abbreviations: CMC, carboxymethyl-cellulose; ND, not determined.

This study aimed to compare potential beneficial CV effects when 320 mg hesperetin are ingested in orange juice or as a dose matched supplement (Table 3.1). Even though the flavanone extract consisted of 80 % flavanones [as assessed in-house pre- and post-study using HPLC (Appendix 1)], plasma concentrations of flavanone phase II metabolites were not elevated 5 h after the flavanone extract intervention relative to the control intervention [mean ± SD: 60 ± 96 nM and 18 ± 6 nM, respectively; $P = 0.87$; (method described in Chapter 5)]. These achieved plasma flavanone concentrations were considerably lower than anticipated and levels below 100 nM may not be physiologically important. Thus, the CVD risk marker data of the flavanone extract intervention were considered unreliable to assess potential beneficial CV effects of flavanones

3.2.3 Study design

To test potential acute effects of orange juice providing 320 mg hesperidin and 48 mg narirutin on biomarkers of CVD risk, a carefully controlled single-blinded crossover RCT in 16 men was conducted. On three experimental periods ($\geq$ 1 week apart), participants underwent CV measurements and received three dietary interventions (in random order): 1) orange juice, 2) a sugar, energy and vitamin C matched flavanone extract and 3) sugar, energy and vitamin C matched control, whereby independent scientists allocated participants to computer-generated random intervention sequences.

Participants were asked to follow a range of lifestyle restrictions to minimise variability between experimental periods (Figure 3.1). For 4 weeks prior to, and during the entire study, participants abstained from dietary supplements containing flavonoids and maintained their intake of non-flavonoid containing dietary supplements. For 3 days before each experimental period, participants followed a diet low in 4 flavonoid subclasses (i.e. flavanones, flavan-3-ols, anthocyanins and isoflavones) and other bioactive foods (i.e. oily fish, nuts and garlic). Participants received a table with foods that were classified as "to avoid", "to restrict" and "alternatives" based on a consensus made by the study team using information from the phenol-explorer [100, 209] and USDA databases [210] and a previous study in the department [211] (Appendix 2). To note, despite a fair amount of flavan-3-ols in tea, due to the habitual intake in the present study population, participants were only asked to refrain from tea intake for 1 day prior to each experimental period. Prior to each experimental period, participants also refrained from strenuous exercise (for 48 h) and avoided foods containing alcohol, caffeine and nitrate/nitrite (for 24 h). In addition, on the night before each experimental period, participants consumed a low-flavonoid standardised ready meal (i.e. a bread roll with either a beef lasagne or sundried tomato pasta; kept consistent for each participant) providing sufficient volume for the population group studied. The low levels of flavonoids present in these meals originated from tomatoes, estimated to contain less than 1 mg flavonoids/meal [100], and small quantities of onion, red wine and oregano. Thus, no interacting effects on the study outcome were anticipated from consumption of this standardised ready meal after which participants fasted for at least 10 h prior to arrival at the Clinical Research and Trial Unit. Compliance to lifestyle restrictions, occurrence of adverse events and intake of medication were checked at each experimental period using questionnaires. To estimate participants' habitual energy intake, 24 h food recall data were collected on the 3rd or 2nd day before each visit (method described in Section 3.2.9).

As described in further detail hereafter, each experimental period started with anthropometric measurements, collection of baseline CV measurement data and a blood sample, followed by intake of the dietary intervention and collection of the CV

measurement data and a blood sample at 5 h post intervention (Figure 3.1) (5 h being the anticipated timepoint of maximal flavanone absorbance [71]). Between the dietary intervention and the post intervention CV measurements, participants were allowed to leave the research facility.

Factors potentially influencing the study outcome were standardised where possible. Each experimental period started between 0730 and 0830 to minimise circadian variability. CV measurements were performed following in-house developed standard operating procedures (SOPs) and conditions of participants and clinical rooms were controlled. Clinical rooms were kept quiet, lighted-subdued and at ambient temperature (22-24°C), participants were asked to micturate and attend a ≥ 15 min supine rest before start of the CV measurements and remained supine during the measurements. Furthermore, before assessments of CV autonomic function and endothelial function involving finger BP measurements, finger temperature was monitored and where necessary participants' hands were warmed with blankets. Following a standardised sequence, CV measurements consisted of brachial BP, CV autonomic function, central arterial stiffness and digital endothelial function, and were always performed on the same body side of the participant. On the contralateral side and embedded in the sequence of CV measurements, blood samples were collected and immediately processed and stored at -80 °C unless stated otherwise (Figure 3.1). To ensure blinding and allocation concealment of the dietary intervention, study drinks were freshly prepared in opaque bottles by independent scientists, and together with the supplements were administered by the research nurses who were not involved in data collection and analysis (Figure 3.1). Furthermore, the research nurses verified that within 15 min, participants consumed the dietary intervention (i.e. both supplements and the entire study drink which included rinsing the opaque bottle twice with nitrate/nitrite free water).

Throughout the experimental period, participants' diet was controlled. In addition to the dietary intervention, participants ate 2 cheese sandwiches and 1 light yoghurt (containing 660 kcal, 31.1 g protein, 95.6 g carbohydrate and 26.8 g fat) consistently 1.5 h after the dietary intervention. Moreover, participants were allowed to drink nitrate/nitrite free water ad libitum and a light meal was provided at the end of the visit.

Figure 3.1 – Overview of the experimental periods: preparations, schedule, and dietary interventions

Preparations:
- ≥ 1 week wash-out between visits
- 3 days low flavonoid diet
- 2 days exercise restrictions
- 1 day alcohol, caffeine and nitrate/nitrite restrictions
- Standardised evening meal
- Only water and study meals 10 h prior and during the experimental period

24 h food recall
Adverse event and medication check

Anthropometric measurements

Cardiovascular measurements and blood collection

0 h — Orange juice, supplement or control intervention

1.5 h — Standard meal

5 h — Cardiovascular measurements and blood collection

Supine rest

Supine rest (15 min)

Blood pressure (10 min)

Autonomic function (25 min)

Blood collection * (10 min)

Arterial stiffness (15 min)

Endothelial function (25 min)

* Blood processing:
Plasma for nitrite analysis:
- Immediate centrifugation (4500g, 3min) of heparinised blood
- Isolation and preservation of plasma with NEM and DTPA
- Immediate freezing on dry ice
- -80° C storage in light protected tube until analysis

Serum for analysis of NADPH oxidase expression:
- Ensured blood clotting time of 30-60min at room temperature
- Serum isolation and storage at -80° C until analysis

Platelet activation whole blood flow cytometry:
- Collection and processing of sodium citrated blood with careful handling to avoid artefactual activation
- Record of NSAID or aspirin intake within last 10 days
- Incubation of fresh whole blood with platelet agonists, fluorochrome labelled antibodies and formalin fixation
- Flow cytometric analysis on same day

Plasma for flavanone metabolite analysis:
- Heparin vacutainer stored on ice and plasma isolation within 2 h
- Acidification with ascorbate (1mM end concentration)
- Storage at -80° C until analysis

[1]given in random order. [2] consisted of 2 cheese sandwiches and a vanilla yoghurt. Abbreviations: NEM, N-ethylmaleimide, DTPA, diethylenetriaminepentaacetic acid; NADPH, nicotinamide adenine dinucleotide phosphate; NSAID, non-steroidal anti-inflammatory drugs

3.2.4 Anthropometric measurements

Participants (wearing no shoes and light clothing) had their height (in m) and weight (in kg) measured (in duplicate) using calibrated and levelled stadiometer and scale, respectively. During each measurement, participants were standing in an upright position and the head was positioned in the Frankfurt plane for the height measurement. The body mass index (BMI) was calculated from the mean height and weight using the equation: weight/height^2.

3.2.5 Oscillometric blood pressure

Brachial BP measurements were performed using a calibrated and BHS validated Omron 705IT (Omron Healthcare Co., Kyoto, Japan) with appropriate sized cuffs (medium 22-32 cm or large 32-42 cm). The BP cuff was placed 2 cm above the antecubital fossa and snug with only one finger fitting between the cuff and the arm. 3 measurements were taken 3 min apart from each other and the average SBP, DBP and heart rate was calculated from the last two readings. Further readings were taken if the variation was > 10 %.

3.2.6 Assessment of autonomic function using beat-to-beat blood pressure and pulse interval

Equipment. For CV autonomic function testing, a Portapres unit (TNO Biomedical Instrumentation, Amsterdam, Netherlands) was used which was equipped with a height correction unit, finger BP cuffs (sizes: small, medium and large), a USB-serial and port cable laptop connection and Beatscope software. Further equipment included a sphygmomanometer device, 20 mL BD plastic syringes and a hand thermometer.

Data acquisition. A finger BP cuff of appropriate size (i.e. inflatable part not overlapping) was placed snug on the middle finger centred over the proximal phalanges. The height correction unit was nulled and placed with one sensor on the finger cuff and one at heart level. In Beatport of the Beatscope software and in the Portapres control unit, gender, age, anthropometric and BP values of the participant were entered.

To ensure good quality recordings of continuous finger arterial BP data, prior to data acquisition, the participant's finger temperature was monitored and acclimatised if < 28 °C. Furthermore, the Portapres has a built-in calibration system (Physio-Cal) that calibrates the BP recording every 10-70 beats (time between calibrations increases when recording quality improves). Hence, a ≥ 5 min run-in recording period was held to allow stabilisation of the BP signal and ensure a Physio-Cal rate exceeding 30 beats. During the

CV autonomic function measurements, Physio-Cal was turned off to provide uninterrupted recordings, whilst calibration was repeated in-between measurements.

CV autonomic function was assessed through measurements of spontaneous baroreflex sensitivity (BRS) [212] and the Valsalva manoeuvre (i.e. a standard clinical test of autonomic function) [213]. Data for spontaneous BRS were recorded for 12 min while participants were at rest, quiet and with minimal movement. For the Valsalva manoeuvre, participants blew into a 20 mL syringe connected to a sphygmomanometer to achieve a pressure of 40 mm Hg for 15 seconds after which they rested quietly and with minimal movement for 45 sec. The Valsalva manoeuvres were performed three times with 1 min rest between assessments.

Data analysis. From the original finger BP data, sampled at 100 Hz, beat-to-beat SBP (i.e. BP maximum) and pulse interval (PI; i.e. time interval between two BP maxima) data were automatically extracted by the Beatscope software (Appendix 3, Figure 1). Subsequently, markers of autonomic function were derived, including BRS in the frequency and time domain and the Valsalva ratio.

BRS in the frequency domain was calculated through power spectral analysis as described in the literature [214, 215] and using MATLAB with the Signal Processing Toolbox (Release 2013a, The MathWorks, Inc., Massachusetts, USA). 12 min resting SBP and PI signals were visually inspected and spikes such as ectopic beats or movement artefacts were manually removed using linear interpolation, and recordings with more than 2 % ectopic beats were rejected from further analysis (Appendix 3, Figure 2). Linear trends were removed and the signals were subtracted from their mean. To produce a uniform time axis, SBP and PI signals were resampled at a frequency of 2 Hz. The power spectra were then estimated using Welch's method with sections of 1024 samples multiplied by a Hanning window and 75 % overlap between sections. Obtained power spectra were smoothed using a 13 point triangular window, and powers of SBP and PI in the low frequency band (i.e. 0.05 to 0.15 Hz) were calculated through numerical integration. BRS was obtained by calculation of the α-index (αBRS; square root of the ratio of the powers of PI and SBP in the low frequency band) (Appendix 3, Figure 2).

BRS in the time domain was automatically calculated from the 12 min resting beat-to-beat SBP and PI data using a cross-correlation BRS (xBRS) algorithm written by Westerhof et al., [216] and provided within a software package by the manufacturer (TNO Biomedical Instrumentation, Amsterdam, Netherlands). Briefly, whilst artefacts were automatically excluded, signals were resampled at 1 Hz intervals and the cross-correlation between SBP and PI were fitted over 10 seconds sliding windows and with a delay in PI of 0 to 5 seconds (Appendix 3, Figure 3). Using the delay that gives the highest cross-correlation coefficient and if the cross-correlation reaches significance at $P < 0.01$, the corresponding

regression slope was accepted as an xBRS estimate. Because of log-normal distribution, the xBRS data were reported as the geometric average of xBRS estimates collected over the 12 min.

Average HR variability during the 3 Valsalva manoeuvres was analysed by taking the ratio of the minimal HR over the maximal HR (i.e. Valsalva ratio), whilst ectopic beats were manually excluded.

3.2.7 Assessment of endothelial function using peripheral arterial tonometry in the digital artery

Equipment. The EndoPAT equipment (Endo-PAT2000, Itamar Medical, Caesarea, Israel; using software version 3.4.4) comprised a main unit, a pneumatic plethysmograph, connected via pneumo-electric tubes to two finger probes consisting of inflatable latex aircushions surrounded by rigid external cases. Further equipment included a sphygmomanometer with a BP cuff, two arm supports, foam anchors, medical tape and a thermometer probe.

Data acquisition and analysis. Endothelial function was measured in the digital artery as the increase in peripheral arterial tonometry during reactive hyperaemia (PAT-RH). Participants rested both hands comfortably on the arm supports. The EndoPAT finger probes were placed on both index fingers, with the pneumo-electric tubes attached to the participants' index fingers and the finger probes not touching anything (ensured through the use of the arm supports, medical tape and foam anchors). The BP cuff of the sphygmomanometer was placed snug on the upper arm (side of CV measures), while the measurement in the contralateral side served as a control.

To ensure good quality recordings, prior to data acquisition, participants' finger temperature was monitored and acclimatised with blankets if < 28 °C. Throughout the 15 min total recording, participants were asked to keep still and quiet; in particular, not moving the finger probes. Following the first 5 min of baseline recording (Figure 3.2; Baseline period), the BP cuff was inflated for 5 min to 200 mm Hg or systolic BP plus 60mm Hg (whichever was higher) to stop arterial blood flow (Figure 3.2; Occlusion). If the signal did not indicate a complete stop of arterial blood flow, the pressure was further increased step-wise up to 300mm Hg as necessary. Subsequently, recordings continued for an additional 5 min after deflation of the BP cuff to measure occlusion-induced reactive hyperaemia (Figure 3.2; Test period). The PAT-RH index indicating digital artery endothelial function was automatically calculated by the software as the ratio of average finger pulse volume amplitude post occlusion (i.e. 1 min interval starting 1.5 min after cuff deflation) to baseline (i.e. 3.5 min interval before cuff inflation) and corrected for systemic changes recorded in the control arm (Figure 3.2).

Figure 3.2 – Recording peripheral arterial tonometry during reactive hyperaemia

$$PAT - RH = \frac{A}{B} \Big/ \frac{C}{D}$$

Test/baseline ratio of tested arm   Test/baseline ratio of control arm
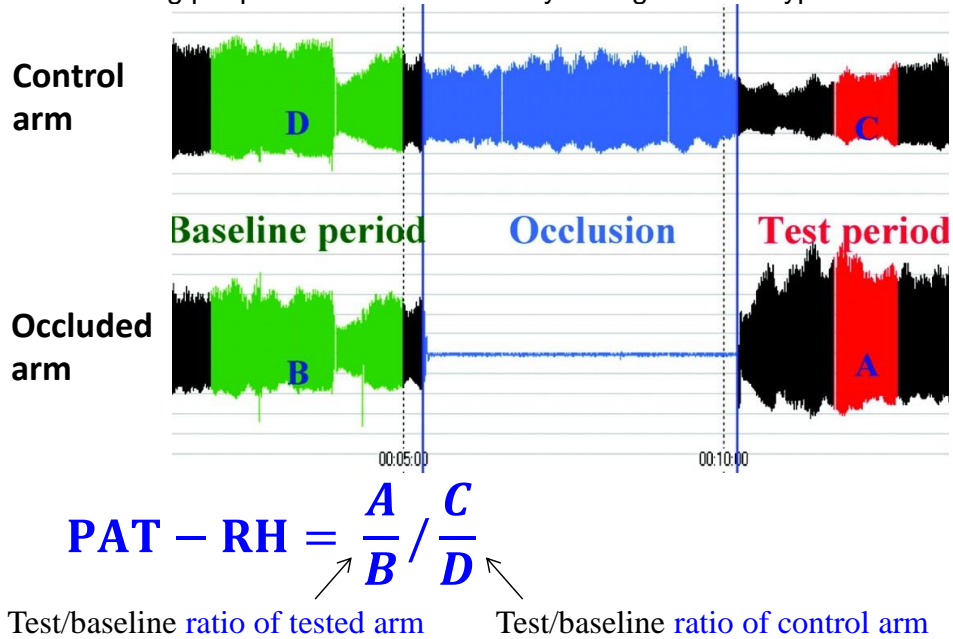
Figure adapted from [217]; PAT-RH, peripheral arterial tonometry-reactive hyperaemia index

3.2.8 Assessment of central arterial stiffness

Equipment. The Vicorder equipment (Skidmore Medical, Bristol, UK) consisted of the main device, two pneumatic tubes, a BP cuff for the neck and a medium-size BP cuff. The main device was connected via a USB cable to a laptop on which Vicorder software version 3.1 was installed.

Data acquisition. Central arterial stiffness, an established independent marker of CVD risk [184, 218], was assessed by carotid to femoral pulse wave velocity (cfPWV) and the central augmentation index (cAIx).

For cfPWV, participants had the upper body inclined by 15° and a pillow placed centred under the thoracic spine so that the head leaned backwards freeing up the carotid artery along the neck. This position ensured good quality recordings of carotid pulse wave traces. The neck cuff was placed snug with its inflatable part over the carotid artery and the medium-sized BP cuff was placed snug on the proximal side of the thigh. The path length was obtained by taking the average of two tape measurements between the carotid cuff and the thigh cuff (mid-points of the cuffs) to the nearest cm. In the Vicorder software, path length and participants' BP were entered and a time-window of 7 seconds was selected, which captures cfPWV variation over an average breath cycle. During the measurements, participants were asked to avoid swallowing, stay still and quiet. Using the Vicorder's digital oscillometric technique, at least three reproducible carotid and femoral pulse wave traces were recorded. The software automatically calculated cfPWV using the transit time between the upstrokes of carotid and femoral pulse wave traces and the path length (Figure 3.3A), whilst operators verified quality and reproducibility of three

recordings in real time [i.e. ensuring < 10 % cfPWV variation and > 6 beat counts (i.e. internal quality control)].

Figure 3.3 – Carotid to femoral pulse wave velocity and pulse wave reflection
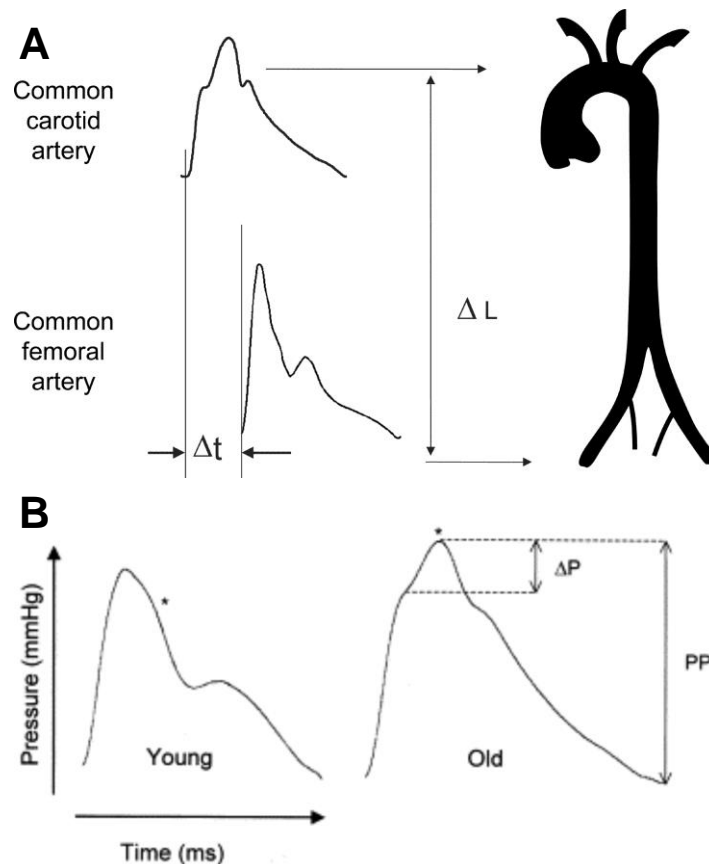
A: Typical carotid and femoral pulse wave traces used for the assessment of carotid to femoral pulse wave velocity (i.e. Δ L / Δ t). (figure adapted from [184])

B: Typical central pulse wave traces in young and old participants used for the assessment of central augmentation index (i.e. ΔP / PP); note the prominent second systolic peak in the old participant (*). (figure adapted from [219])

Abbreviations: Δ t; transition time; Δ L, path length; ΔP, augmentation pressure; PP, pulse pressure

For cAIx, the medium-size BP cuff was placed snug over the upper arm and 2 cm above the antecubital fossa. Participants were asked to stay still and quiet during recordings of brachial pulse wave traces. Central pulse wave traces were transferred from these brachial recordings by the software and the cAIx calculated as the central augmentation pressure relative to the central pulse pressure (Figure 3.3B). Three measurements were taken with good quality and reproducibility (i.e. < 10 % cAIx variation and > 6 beats) as assessed in real time by the operator. Because of strong correlation with heart rate, the cAIx was additionally reported at a normalised heart rate of 75 bpm [220].

3.2.9 Estimation of participant's energy intake using 24 h recall

One or two days before each experimental period, participants' dietary intakes were estimated using a standard 24 h food recall questionnaire. Participants were asked to

estimate portion sizes using household measures and provide details on the brand names of consumed products. Using published food portion size data [207] and the food composition database Weighted Intake Software Package (WISP; version 4; Tinuviel Software, Anglesey, United Kingdom), mean daily energy intakes were estimated.

3.2.10 Analysis of plasma nitrite using reductive gas-phase chemiluminescence

3.2.10.1 Equipment and chemicals

Materials. The chemiluminescence detector 88 (CLD88) was purchased from Eco Physics (Duernten, Switzerland) and included a purge vessel system for liquid sample analysis, a nitrogen gas cylinder, a pressure regulator (operating in the mbar range), a 110 ml/min sampling probe, and Chart software (EDAQ, Dublin, Ireland). Two recirculating heating and chilling water baths were obtained from Grant Instruments (Shepreth, UK), 100 µL Hamilton syringes, human male serum, sodium nitrite, potassium iodide, iodine, N-ethylmaleimide (NEM), sulfanilamide and diethylenetriaminepentaacetic acid (DTPA) from Sigma Aldrich (Dorset, UK). Light protected amber tubes were purchased from Fisher Scientific (Loughborough, UK), lint free wipes from Distinctive Medical (Cheshire, UK) and 2 mL heparin vacutainers and 20 mL plastic syringes from Bunzl Healthcare (London, UK). Acetic acid glacial, hydrochloric acid, sodium hydroxide and acetone were from the communal lab supply. To avoid potential nitrite contamination, fresh MilliQ grade water (18.2 MΩcm^{-1}) was used and all glassware was washed. No nitrite contamination was detectable in plasticware and heparin vacutainers.

3.2.10.2 Method

Blood processing. Due to an 11 minute half-life of nitrite in blood [221], 2 mL freshly collected heparinised blood was immediately processed and nitrite preserved following the procedure described by Nagababu et al., [222]. Briefly, blood was centrifuged for 3 min at 4500 g and room temperature, plasma was isolated and aliquoted into labelled light protected amber tubes containing NEM and DTPA (final concentrations: 6.5 mM and 0.1 mM, respectively; NEM prepared fresh every day). Samples were vortexed for 5 seconds, immediately frozen on dry ice and stored at -80 °C until analysis.

Plasma nitrite analysis. Plasma nitrite was quantified using reductive gas-phase ozone-based chemiluminescence as previously described by Feelisch et al., [223]. Briefly, 100 µL of plasma samples (in triplicate, one freeze thaw cycle and freshly thawed on ice) were injected into a purge vessel containing an iodide/triiodide reaction mixture (kept at 60 °C), which selectively reduces nitrite and nitroso species to gaseous NO. The NO was then transported by nitrogen via a condenser and sodium hydroxide trap (both kept at 0 °C) to the chemiluminescence detector. There, NO undergoes a light-emitting reaction with

ozone which is detected by a photomultiplier and recorded (Figure 3.4). Nitroso species were selectively quantified through pretreatment of plasma with acidified sulfanilamide (i.e. a chemical which prevents reduction of nitrite); however, using the in-house method optimised in this laboratory, sulfanilamide treated samples created a signal below the limit of detection. Hence, the signal obtained from injection of sulfanilamide-free samples was interpreted as originating predominantly from nitrite. To avoid cross-contamination between injections, syringes were carefully cleaned with MilliQ and acetone and dried using lint free wipes. Quantification of plasma nitrite was based on a six point standard curve of freshly prepared standards (concentration range: 0 to 200 nM) and a quality control (i.e. commercially available serum) was injected regularly. Intra-assay and inter-assay %CV were 4.2 % and 3.3 %, respectively.

Figure 3.4 – Raw NO signals recorded following injection of nitrite standards or plasma
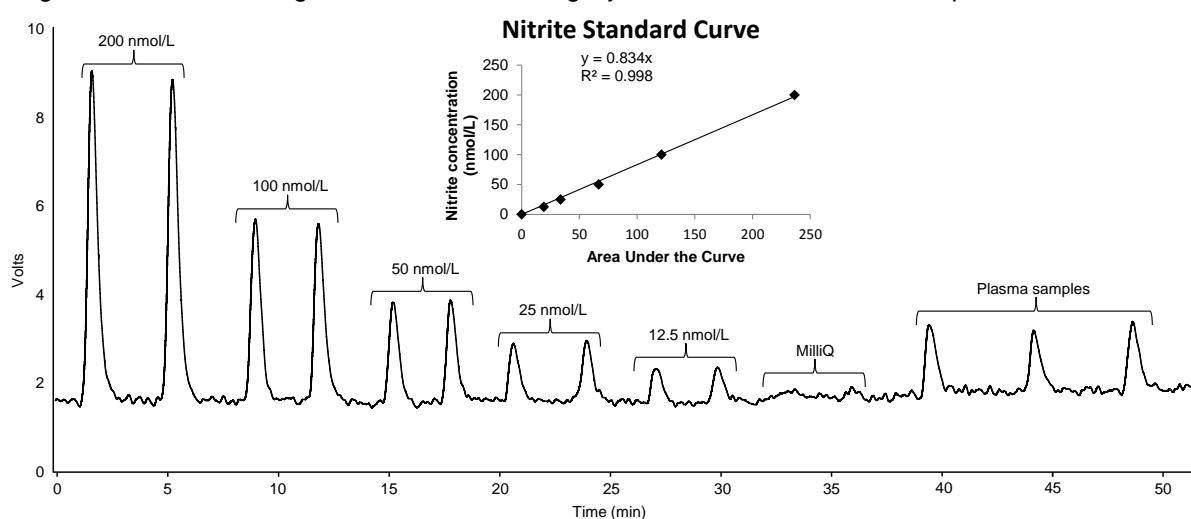
3.2.11 Assessment of NADPH oxidase gene expression

Serum samples were sent to Professor Violi's group (Rome, Italy) for quantification of serum soluble gp91^{phox}, a subunit of NADPH oxidase, using their developed and validated ELISA method [199]. Briefly, samples were analysed in duplicate (1 freeze/thaw cycle), and the assay involved the use of anti-gp91^{phox} (Santa Cruz Biotech, Santa Cruz, US) coated plates and spectrophotometric quantification against standard curves of gp91^{phox} peptide (New England Peptide, Gardner, US). The assay's intra- and inter-day %CV were 5.8 % and 6.7 %, respectively.

3.2.12 Platelet activation whole blood flow cytometry

The following platelet activation whole blood flowcytometry method was based on the protocol published by Krueger et al., [224].

3.2.12.1 Materials

The fluorophore-conjugated monoclonal antibodies anti-human CD61-allophycocyanin (APC), anti-human CD62P-phycoerythrin (PE) and Isotype Control Mouse IgG1 PE were purchased from eBioscience (San Diego, USA), and anti-human PAC-1-fluorescein isothiocyanate (FITC), Isotype Control Mouse IgM, κ FITC, compensation beads, staining buffer and sodium citrate vacutainers from BD bioscience (San Jose, USA). The platelet agonists ADP, crosslinked CRP and phorbol 12-myristate 13-acetate (PMA) were obtained from Alpha Laboratories (Hampshire, UK), the Biochemistry Department of the University of Cambridge (Cambridge, UK) and Sigma Aldrich (Dorset, UK), respectively. Megamix calibration beads were purchased from Biocytex (Marseille, France), paraformaldehyde from Fisher Scientific (Loughborough, UK) and minisart CA 0.2 μm sterile CA syringe filters from Sartorius Stedim (Surrey, UK). Modified HEPES/Tyrode's (HT) buffer solutions and 1 % formalin fixation solution were prepared in-house and syringe filtered.

Fluorophore-conjugated monoclonal antibodies, platelet agonists and buffers were from a single batch, prepared and stored following instructions of the manufacturer and Krueger et al., [224]. Prior to study commencement, individual antibodies were mixed together in the right proportion to a mastermix (Table 3.2), batch aliquoted out into 1.5 ml polypropylene Eppendorf test tubes and stored in the dark at 4 °C. Due to potential photobleaching, antibodies were kept in the dark during storage and incubation periods. To minimise *ex vivo* platelet activation, all reagents were acclimatised to room temperature before usage.

3.2.12.2 Method

Blood collection. To minimise *ex vivo* platelet activation, a 21-G needle was used, the first 18 ml of blood was collected for purposes other than platelet activation, and the tourniquet was removed prior to collection of blood in a sodium citrate vacutainer dedicated for platelet activation analysis. Whole blood was mixed with the sodium citrate anticoagulant by gentle inversion of the vacutainer for 3 times and kept at room temperature until analysis.

Test sample preparation. Within 30 min after the blood collection, working solutions of platelet agonists (i.e. 5 μg/mL CRP, 10 μM ADP or 10 μM PMA) were prepared fresh, and whole blood was diluted 1:10 and mixed by gentle pipetting. 2 control samples were prepared to test proper functioning of the assay and 3 test samples were prepared (i.e. non-activated, ADP activated and CRP activated) for each timepoint (i.e. baseline and 5 h). Platelet agonists, HT buffer and diluted whole blood were added to each test tube and mixed by gentle tapping (Table 3.2). Final concentrations of antibodies and platelet agonists were previously optimised in-house through performance of titration experiments

[[225]; Section 5.1.4] ensuring saturation of CD62p-PE and PAC1-FITC binding, submaximal ADP and CRP induced *ex vivo* platelet activation and maximal PMA induced *ex vivo* platelet activation. Following 20 min incubation at room temperature, samples were fixed with 400 µL filtered 1 % formalin, incubated for 15 min at room temperature and subsequently stored at 4°C until analysis on the flow cytometer.

Table 3.2 – Platelet activation: sample preparation

	Test samples			Control samples	
	Non-activated	ADP activated	CRP activated	Isotype	PMA
Antibody mastermix	1 µL CD61-APC, 2.5 µL CD62p-PE, 10 µL PAC1-FITC	1 µL CD61-APC, 2.5 µL CD62p-PE, 10 µL PAC1-FITC	1 µL CD61-APC, 2.5 µL CD62p-PE, 10 µL PAC1-FITC	1 µL CD61-APC, 0.6 µL iso-PE, 0.5 µL iso-FITC	1 µL CD61-APC, 2.5 µL CD62p-PE, 10 µL PAC1-FITC
Agonist (end concentration)	n/a	2.5 µL ADP WS (1 µM)	2µL CRP WS (0.4µg/mL) [3]	n/a	2.5 µL PMA WS (1 µM)
HT buffer	6.5 µL	4 µL	6.5 µL	17.9 µL	4 µL
dWB	5 µL	5 µL	5 µL	5 µL	5 µL

Abbreviations: CD61-APC, anti-human integrin β3 antibody conjugated to allophycocyanin fluorophore; CD62p-PE, anti-human P-selectin antibody conjugated to phycoerythrin fluorophore; PAC1-FITC, anti-human activated fibrinogen receptor antibody conjugated to fluorescein isothiocyanate fluorophore; WS, working solution; CRP, collagen related peptide (WS: 10µg/mL); ADP, adenosine diphosphate (WS: 10 µM); PMA, 10 µM phorbol 12-myristate 13-acetate (WS: 10 µM); dWB, diluted whole blood; HT buffer, HEPES/Tyrode's buffer;

Flow cytometry data acquisition. Flow cytometry analysis was performed using a BD Accuri C6 flow cytometer and CFlow software (Becton Dickinson, NJ USA). Samples were run on the same day of test sample preparation and the time from sample fixation to data acquisition was kept consistent. 10'000 platelet events were collected in each test sample using a 14 µL/min flow rate and a 20'000 FSC-H threshold. Platelet events were identified based on light scatter (Figure 3.5 A) and presence of integrin β3 (i.e. protein expressed on resting and activated platelets; detected through CD61-APC binding) (Figure 3.5 B). Platelet activation was assessed as % of platelets with positive P-selectin or activated fibrinogen receptor expression (i.e. two proteins expressed on platelets that are activated; detected through CD62p-PE and PAC1-FITC binding, respectively) on platelets under basal conditions (Figure 3.5 D) and upon *ex vivo* stimulation with CRP or ADP (Figure 3.5 C). The assay's intra-day and inter-day coefficient of variation (%CV) were 5.1 % and 9.6 %, respectively.

Before collection of platelet data, cytometer cleanliness was tested by detecting less than 200 events in 50 µL ultrapure water run at a flow rate of 33 µL/min and 80'000 forward scatter threshold (prior to platelet data collection) and flow cytometer performance was verified by running calibration beads (before and after platelet data acquisition). The same gating strategy in logarithmic scale was applied throughout the whole study. Using beads, colour compensation between fluorophores PE and FITC was established and applied to all biological sample data. FL-1 (filter 530/30 nm; green spectrum) was corrected by 4.21 % due to CD62p-PE excitation and FL-2 (filter 585/40 nm; blue spectrum) was corrected by 6.85 % due to PAC1-FITC excitation. No colour compensation was required for the

APC fluorophore which was assessed by FL-4 (filter 675/25nm; red spectrum) and does not excite into FL-2 or FL-1.

Figure 3.5 – Platelet activation whole blood flow cytometry: data acquisition
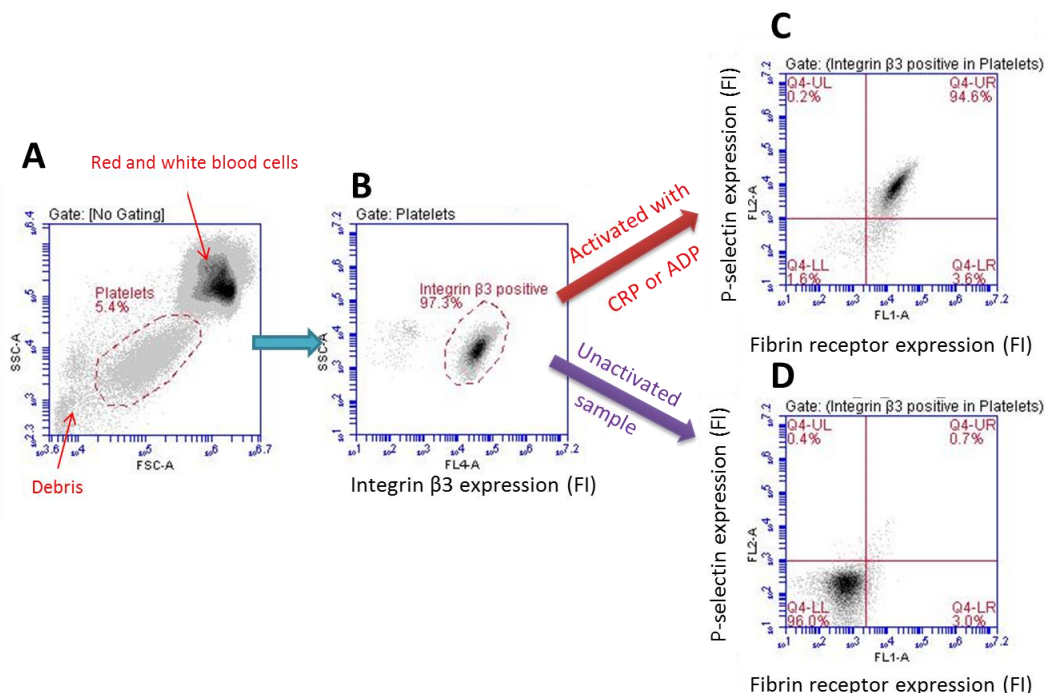
Assessment of platelet activation by whole blood flow cytometry. **A:** log FSC-A/ log SSC-A plot: light-scatter profile of diluted whole blood with platelets broadly identified based on size. **B:** log FL4-A/ log SSC-A plot of events in the "Platelets" gate. Platelets were identified based on expression of integrin β3, a protein expressed on rested and activated platelets. **C** and **D**: Log FL2-A/ log FL1-A plot gated on identified platelets. This plot indicates the amount of activated fibrinogen receptor and P-selectin expression, two proteins that are expressed on activated platelets. Platelet activation is assessed in platelets activated *ex vivo* with collagen related peptide (CRP) or adenosine diphosphate (ADP) (**C**) or in platelets under basal conditions (i.e. non-activated) (**D**). Abbreviations: SSC-A, side scatter area; FSC-A, forward scatter-area; FL4-A, FL2-A and FL1-A, fluorescence bandpass filters (675/25 nm, 585/40 nm and 530/30 nm, respectively); FI, fluorescence intensity

3.2.13 Flavanone metabolite analysis in plasma

3.2.13.1 Materials

Compound standards. Naringenin-7-glucuronide was purchased from Bioquote (York, UK). Sulfate and glucuronide conjugated phenolic acids (protocatechuic acid-3-glucuronide, protocatechuic acid-4-glucuronide, vanillic acid-4-glucuronide, benzoic acid-4-glucuronide, isovanillic acid-3-glucuronide, protocatechuic acid-3-sulfate, protocatechuic acid-4-sulfate, vanillic acid-4-sulfate, isovanillic acid-3-sulfate and benzoic acid-4-sulfate) were synthesised at the University of St Andrews (UK) as previously published [226]. Hesperetin, naringenin, phloridzin, taxifolin and the subsequently listed 53 phenolic acids (CAS number) were purchased from Sigma-Aldrich (Dorset, UK): [2,4-dihydroxybenzaldehyde (95-01-2), 2,4-dihydroxybenzoic acid (89-86-1), 2,4-dihydroxycinnamic acid (614-86-8), 2.3-dihydroxybenzoic acid (303-38-8), 2-hydroxy,4-methoxybenzaldehyde (673-22-3), 2-hydroxy,4-methoxybenzoic acid (2237-36-7), 2-

methoxybenzoic acid (579-75-9), 3,4-dihydroxybenzaldehyde (139-85-5), 3,4-dihydroxyhydrocinnamic acid (1078-61-1), 3,4-dihydroxyphenylacetic acid (102-32-9), 3,4-dimethoxybenzoic acid methyl ester (2150-38-1), 3,5-dihydroxybenzoic acid (99-10-5), 3-hydroxybenzoic acid (99-06-9), 3-hydroxycinnamic acid (14755-02-3), 3-hydroxyphenylacetic acid (621-37-4), 3-methoxybenzoic acid (586-38-9), 3-methoxycinnamic acid (6099-04-3), 3-methyl hippuric acid (27115-49-7), 3-methyl gallic acid (3934-84-7), 4-hydroxybenzaldehyde (123-08-0), 4-hydroxybenzoic acid (99-96-7), 4-hydroxybenzyl alcohol (623-05-2), 4-hydroxyphenylacetic acid (156-38-7), 4-methoxybenzaldehyde (123-11-5), 4-methoxybenzoic acid (100-09-4), 4-methoxycinnamic acid (830-09-1), 4-methyl hippuric acid (27115-50-0), 4-valerolactone (57129-69-8), 5-oxo-5-phenylvaleric acid (1501-05-9), 5-phenylvaleric acid (2270-20-4), 6-methoxysalicyclic acid (3147-64-6), alpha hydroxyhippuric acid (16555-77-4), apocynin (498-02-2), caffeic acid (331-39-5), dihydroferulic acid (1135-23-5), ferulic acid (537-98-4), gallic acid (149-91-7), hippuric acid (495-69-2), homovanillic acid (306-08-1), hydrocinnamic acid (501-52-0), isoferulic acid (537-73-5), isovanillic acid (645-08-9), methyl 3,4,5-trihydroxybenzoate (99-24-1), methyl 3,4-dihydroxybenzoate (2150-43-8), methyl vanillate (3943-74-6), p-coumaric acid (501-98-4), phloretic acid (501-97-3), phloroglucinol aldehyde (487-70-7), protocatechuic acid (99-50-3), salicyclic acid (69-72-7), sinapic acid (530-59-6), syringic acid (530-57-4) and vanillic acid (121-34-6)]. 3-hydroxyhippuric acid (1637-75-8) was purchased from Enamine Ltd (Kiev, Ukraine). Stock solutions of all pure compounds were prepared at 5 mg/mL in dimethyl-sulfoxide, and the stock solutions containing phenolic acid, taxifolin and phloridzin were stored at room temperature, whilst stock solutions containing flavanone or synthesized phenolic acid glucuronides and sulfates were stored at -80 °C.

Solvents. Hydrochloric acid, formic acid, dimethyl-sulfoxide and methanol were obtained from Sigma-Aldrich (Poole, Dorset, UK), and acetonitrile from Fisher Scientific (Loughborough, UK). All solvents and MilliQ water (18.2 MΩ cm-1) were of HPLC grade quality and glassware was washed with HPLC grade solvent prior to use.

Consumables. Strata-X™ solid phase extraction columns (6 mL, 500 mg, 88 Å), Kinetex pentafluorophenyl HPLC column (2.6 μM, 100 x 4.6 mm, 100 Å) and SecurityGuard ULTRA Cartridges (pentafluorophenyl, 2 x 4.6 mm) were purchased from Phenomenex (Macclesfield, UK). Sterile filtered, human male serum, a Hamiltion syringe (1 mL volume) and acrodisc polytetrafluoroethylene syringe filters (13 mm Ø, 0.45 μM pore size) were purchased from Sigma-Aldrich (Dorset, UK).

3.2.13.2 Method

Plasma solid phase extraction. Flavanones and phenolic acids were extracted following previously developed and validated methods [227] with minor modifications. Briefly, 1 mL

of acidified plasma was spiked with an internal standard (i.e. phloridzin at 5 µM) and subsequently extracted using solid phase extraction cartridges. These were washed with 12 mL of 0.1/99.9 v/v hydrochloric acid/water, dried for 30 min under vacuum, soaked in 0.1/99.9 v/v hydrochloric acid/methanol for 10 min and eluted into glass vials with 7 mL 0.1/99.9 v/v hydrochloric acid/methanol. Samples were evaporated to complete dryness under speedvac at room temperature. The dried samples were resuspended in 250 µL of mobile phase (0.1/5/94.9, v/v/v, FA/MeOH/MilliQ) and sonicated in an ultrasonic cold water bath for 15 min at room temperature. Samples were syringe filtered, spiked with an internal standard (i.e. taxifolin at 5 µM) and stored at -80 °C until analysis. The mean ± SD extraction efficiency of phloridzin was 71.8 ± 12.9 %.

HPLC-MS^2 analysis. Samples were analysed following a previously developed and validated method [227] with adaptation for quantification of flavanones metabolites. Briefly, the HPLC-electrospray ionisation-MS^2 system consisted of an Agilent 1200 series HPLC coupled to an AB Sciex 3200 series Q-Trap electrospray ionisation mass spectrometer operated using Analyst software (v. 1.5, Applied Biosystems/MDS Sciex). Compound separation was achieved on a pentafluorophenyl column attached to a Security Guard ULTRA maintained at 37 °C with a flow gradient consisting of 1.5 mL/min at 0 min, 1 mL/min from 7 to 14 min and 1.5 mL/min from 14 to 32 min and a sample injection volume of 5 µL. The mobile phase consisted of 0.1/99.9 v/v water/formic acid (A) and 0.1/99.9 v/v acetonitrile/formic acid (B); and mobile phase gradient consisting of: 1 % B at 0 min, 7.5 % B at 7 min, 7.6 % B at 14 min, 10 % B at 17 min, 12 % B at 18.5 min, 12.5 % B at 20 min, 30 % B at 24 min, 90 % B from 25 to 28 min and 1 % B from 29 to 32 min. Flavanone metabolites and internal standards were detected by scheduled multiple reaction monitoring (sMRM). The following compound dependent parameters were optimised using syringe infusion (in negative and/or positive sMRM mode): declustering potential, entrance potential, collision energy and collision exit potential (Table 3.3). Optimised MS^2 source parameters were 40 psi curtain gas, 700 °C Temperature, 60 psi nebulizer gas, 60 psi auxiliary gas and -4000 V or 5500 V ion spray voltage; the latter depending on negative or positive MRM mode, respectively.

If pure standards were not commercially available, methods to identify and quantify flavanone metabolites were developed using a pooled plasma extract (i.e. plasma collected 5 h after the orange juice intervention and pooled from n = 13 participants) (Figure 3.6). sMRM transitions and parameters were obtained from the literature [71, 228] or derived from the fragmentation pattern of the flavanone aglycones or phenolic acids by adding the m/z of glucuronide (i.e. 176) or sulfate (i.e. 80) to the precursor ion and appropriate MS^2 fragment. 113 m/z was also added as a MS^2 fragment for putative glucuronide conjugated phenolic acids as it is a commonly reported fragment of glucuronic acid [229].

Figure 3.6 – sMRM method development and screening strategy for the identification of 23 flavanones and their phenolic metabolites

Analysis of pure standards

67 tested compounds[1]
- hesperetin, naringenin & eriodictyol
- naringenin-7-glucuronide
- 54 phenolic acids
- 5 phenolic acid sulphates
- 5 phenolic acid glucuronides

10 compounds with poor detection limit

Analysis of pooled plasma[3]

57 tested compounds[1]
- hesperetin, naringenin & eriodictyol
- naringenin-7-glucuronide
- 43 phenolic acids
- 5 phenolic acid sulphates
- 5 phenolic acid glucuronides

91 tested putative compounds[2]
- 2 hesperetin-glucuronides
- 2 hesperetin-diglucuronides
- hesperetin-sulphate
- hesperetin-glucuronide-sulphate
- 2 naringenin-glucuronides
- naringenin-diglucuronide
- naringenin-glucuronide-sulphate
- naringenin-sulphate
- eriodictyol-glucuronide
- eriodictyol-diglucuronide
- eriodictyol-glucuronide-sulphate
- eriodictyol-sulphate
- 38 phenolic acids-glucuronides
- 38 phenolic acid-sulphates

112 compounds not detected in pooled plasma[3]

Compounds detected in pooled plasma[3]

36 compounds
- hesperetin and naringenin
- 2 hesperetin-glucuronides
- 2 naringenin-glucuronides
- 2 hesperetin-diglucuronides
- 15 phenolic acids
- 7 phenolic acid-glucuronides
- 6 phenolic acid-sulphates

13 compounds not identified as flavanone metabolites[4]

Compounds identified as flavanone metabolites

23 compounds
- hesperetin and naringenin
- 2 hesperetin-glucuronides
- 2 naringenin-glucuronides
- 2 hesperetin-diglucuronides
- 9 phenolic acids
- 6 phenolic acid glucuronides

[1] Pure compounds were commercially available or synthesised. [2] Pure compounds were not commercially available and identification was based on known transitions. [3] Collected 5 h after the orange juice intervention and pooled from n = 13 participants. [4] For 9 compounds, concentrations were not significantly different 5 h after the orange juice intervention compared to the control intervention and 4 compounds were below the limit of quantification. Abbreviations: sMRM, scheduled multiple reaction monitoring.

Compounds were identified based on 3-5 sMRM transitions and retention time (RT) of the pure standard (if available). From all 158 compounds originally considered as potential flavanone metabolites, 23 were identified as flavanone metabolites (Figure 3.6) in the final analysis of all clinical samples using optimised sMRM methods (Table 3.3) (i.e. compound concentration was significantly elevated 5 h post orange juice intervention relative to the control).

Table 3.3 – sMRM transitions, parameters and detection limits of identified flavanone metabolites

Metabolite	sMRM mode	RT (min)	LOD (nM)	R^2	Precursor ion (m/z)	MS^2 fragments (m/z)	DP (V)	EP (V)	CE (V)	CXP (V)	Previously identified by (biofluid)[1]
Flavanone aglycones											
Naringenin	-	25.6	0.1	1.000	271	177, 151*, 119, 107	-57	-5	-25, -25, -25, -45	-3	[72] (P, U)
Hesperetin	-	25.7	0.1	0.998	301	286, 242, 164*	-59	-5	-27, -27, -35	-1	[72] (P, U)
Flavanone phase II metabolites:											
Hesperetin-diglucuronide[2]	-	19.5	2.5	N/A	653	477*, 301, 286, 176	-86	-5	-30	-7	[78] (P, U)
Hesperetin-diglucuronide[2]	-	23.0	2.1	N/A	653	477*, 301, 286, 176	-86	-5	-30	-7	[78] (P, U)
Hesperetin-glucuronide[2]	-	24.2	4.1	N/A	477	301*,286, 257, 242	-86	-5	-30	-7	[71, 72, 78] (P, U)
Hesperetin-glucuronide[2]	-	24.8	1.0	N/A	477	301*,286, 257, 242	-86	-5	-30	-7	[71, 72, 78] (P, U)
Naringenin-7-glucuronide	-	24.0	0.2	0.998	447	271*, 176, 151	-41	-5	-27, -22, -42,	0	[72, 78] (P, U)
Naringenin-glucuronide[2]	-	24.3	1.0	N/A	447	271*, 176, 151	-41	-5	-27, -22, -42,	0	[72, 78] (P, U)
Phenolic acid metabolites											
Benzoic acid-4-glucuronide	-	3.1	0.6	0.998	313	175, 137, 113, 93*	-54	-7	-15, -20, -20, -54	-2	
Hydroxyhippuric acid[2, 3]	-	4.5	4.1	N/A	194	150, 93*, 121	-39	-5	-20, -27, -38	-2	
3-hydroxyhippuric acid	-	5	2.4	1.000	194	150*, 93, 121	-39	-5	-20, -27, -38	-2	[113] (U)
Isovanillic acid-3-glucuronide	-	5.2	4.1	0.996	343	152, 113, 167*, 175, 108	-60	-4	-40, -20, -20, -15, -55,	-2	
Vanillic acid-4-glucuronide	-	6.5	5.1	0.990	343	152, 113, 167*, 175, 108	-60	-4	-40, -20, -20, -15, -55,	-2	
Hippuric acid	-	7	778.5	0.997	178	77, 132, 134*	-40	-3	-22, -22, -18	0	[113] (U)
Iso/vanillic acid-glucuronide[2, 3]	-	7	4.6	N/A	343	152, 113, 167*, 175, 108	-60	-4	-40, -20, -20, -15, -55,	-2	
4-hydroxybenzoic acid	-	7.1	18.9	1.000	137	65, 75, 93*	-35	-5	-44, -50, -20	-2	[113] (U)
4-hydroxyphenylacetic acid	-	7.1	148.5	0.994	151	123, 93, 79, 107*	-50	-7	-25, -25, -25, -17	-2	[113] (U)
3-hydroxyphenylacetic acid	-	8.3	7.9	0.994	151	121, 107*, 92.9, 65	-26	-7	-13, -16, -12, -34	0	[113] (U)
Isovanillic acid	+	9.9	13.9	0.997	169	151, 125, 65, 93*	27	6	19, 12, 34, 19	6	
Vanillic acid	+	10.3	9.2	0.998	169	151, 125, 65, 93*	27	6	19, 12, 34, 19	6	
Iso/ferulic acid-glucuronide[2, 3]	-	10.2	2.8	N/A	369	193*, 113, 134, 178	-28	-9	-23, -23, -23, -18	0	
Dihydroferulic acid-3-glucuronide[2, 3]	-	11.5	28.9	N/A	371	151, 195*, 113, 121, 136	-40	-8	-18, -35, -35, -35, -20	0	
Dihydroferulic acid	-	17.1	5.4	1.000	195	151, 136*, 119, 121, 149	-40	-8	-18, -20, -16, -35, -18,	0	[113] (U)

*sMRM transition used for quantification; [1] P, plasma; U, urine; [2] site of conjugation could not be ascertained as identification was based on known transitions where pure standards for isomers were not available or separation of isomers was chromatographically not possible; [3] putatively identified compound which could have different isomeric configuration. Abbreviations: sMRM, scheduled multiple reaction monitoring; RT, Retention time; LOD, limit of detection (Signal/Noise = 3); R^2, linear regression coefficient of standard curve; N/A, putative metabolite, i.e. no analytical standard was available to make standard curve; MS^2, tandem mass spectrometry; DP, declustering potential; EP, entrance potential; CE, collision energy; CXP, collision exit potential.

Quantification was established using the most intense sMRM transition and matrix-matched baseline-adjusted standard curves (concentration range: 0 to 51.2 µM over 11 points) (Table 3.3). Where pure standards were not available, quantification was conducted relative to standard curves of compounds with the most similar structure, such as 3-hydroxyhippuric acid was used to quantify hydroxyhippuric acid and vanillic acid-glucuronide was used to quantify dihydroferulic acid-glucuronide. The limit of detection, which was calculated by Analyst software, was established for each compound as the concentration of a peak with a signal to noise ratio of 3, and used as the threshold for quantification. A quality control consisting of taxifolin spiked into serum extract at 50 µM was run every 12 injections and the between-run coefficient of variation was established as 8.6 %.

3.2.14 Quantification of vitamin C

EDTA plasma was immediately isolated from fresh blood, deproteinised, mixed with diethylenetriaminepentaacetic acid (250 µM) and frozen on dry ice. Vitamin C was analysed using ascorbate assay kits (No. 7004240) from Cayman Chemical (Ann Arbor, Michigan, USA). Intra- and inter-assay coefficients of variation (%CV) were 3.3 % and 13.8 %, respectively.

3.2.15 Statistical analysis

A sample size of 14 participants to complete the study was calculated to be sufficient to detect clinically relevant effect sizes of dietary flavanones for PAT-RH, (i.e. the primary endpoint of the trial), with a power of 80 % and a significance level of 5 %. The clinically relevant effect size and expected variability for PAT-RH [expressed as standard deviations (SD)] was 0.35 ± 0.4 [122]. For secondary endpoints (i.e. BP, Valsalva ratio, BRS, cfPWV, cAIx, platelet activation, plasma nitrite and serum soluble gp[91phox]), the sample size of the present RCT is comparable to previous acute crossover RCTs that tested vascular effects of flavonoids (range: 10 - 22 participants) [158, 159, 192, 193, 202, 230-232].

Following a pre-established statistical analysis plan and whilst investigators remained blinded to the dietary interventions, data were statistically analysed using a per protocol approach [233]. Effects of the dietary interventions on study endpoints were analysed using a linear mixed model specific for crossover studies (described by Jones and Kendward [234]). In this model, subjects nested within intervention sequence were included as a random effect and intervention sequence, experimental period, baseline values and dietary intervention as fixed effects. No carry-over effects were observed as assessed by addition of the period and dietary intervention interaction to the linear mixed

model (i.e. *P*-value of > 0.05). If the mixed model showed a significant difference, pairwise comparisons between interventions were performed on least-square means with Tukey adjustment. Model assumptions were assessed through diagnostic plots (i.e. histograms and boxplots of baseline and 5 h post intervention data, and histograms, Q-Q plots and fitted *versus* residual plots of model residuals) and objective tests (i.e. Shapiro Wilk test to assess normality of the data and model residuals, and Levene test to assess homogeneity of variance of the data).

Exploratory associations between markers of CVD risk and plasma concentrations of flavanone and phenolic acid metabolites (i.e. changes from baseline to 5 h post orange juice intervention which were deemed potentially physiologically important [i.e. defined as ≥ 100 nM mean increases)] were assessed by Pearson's correlation and multivariate regression analysis.

Due to the exploratory nature of this study, statistical tests were not adjusted for multiple comparisons. Data are presented as means ± SD and an effect was considered significant when the *P*-value was < 0.05. The statistical analysis was performed using R 3.0.1 (R Foundation for Statistical Computing).

Chapter 4. Acute vascular and cardiac autonomic response after intake of flavanones in orange juice: in healthy men at mild to moderate CVD risk

4.1 Introduction

Cardiovascular (CV) disease (CVD) is the cause of one in three deaths in the UK with men at a higher risk than women [1]. A change in modifiable risk factors may have the potential to decrease disease burden and death; in particular, encouraging a diet rich in fruit was reported to have the third highest impact after reducing blood pressure (BP) levels and smoking [37]. For example, a diet rich in fruit and vegetables has been linked with a decreased risk of CVD by an odds ratio of 0.7 (99 % CI: 0.64-0.77) [235], and phytochemicals in fruits and vegetables such as flavonoids may partly be responsible for these cardioprotective effects [38].

A growing body of epidemiological evidence suggests that intake of citrus fruit and flavanones, a polyphenol compound mainly consumed in citrus fruits, is inversely associated with both mortality and incidence of coronary heart disease and stroke [43-51, 54, 236]. In two large cohort studies, a high flavanone intake has been linked with a 19 % reduction in risk of ischemic stroke [Relative risk (RR): 0.81 (95 % confidence interval (CI): 0.66–0.99; $P = 0.038$)] [48] and a 22 % reduction in risk of coronary heart disease [RR: 0.78 (95 % CI: 0.65-0.94; $P = 0.010$)] [49] when comparing upper and lower quintile of flavanone intake in cohorts of 70,000 and 35,000 participants followed up for 14 and 16 years, respectively, and using multivariate models that adjusted for known confounding factors including estimated intake of vitamin C. In support of this epidemiological evidence, a number of *in vitro* and animal studies suggest that flavanones exert effects of potential relevance to CV health through an improvement in CVD risk biomarkers including BP, endothelial and autonomic function [60, 62, 74, 93, 94, 148].

Despite these promising epidemiological, *in vitro* and animal data, the systematic review conducted in Chapter 2 clearly demonstrated that there are currently insufficient data from human randomised controlled trials (RCTs) to determine the CV benefits of dietary flavanones. There are also gaps in our understanding of the underlying mechanisms of action of flavanones and their metabolites in humans. Whilst the conducted meta-analysis of 6 short-term RCTs (duration range: 3 to 8 weeks) suggested that flavanones lower mean blood total cholesterol by 0.64 mmol/L (95 % CI: -1.04, -0.24) compared to control in a population at an elevated CVD risk but not at a mild to moderate CVD risk (Chapter 2; Figure 2.3), the effect of dietary flavanones on BP and vascular function is poorly understood. Two short-term RCTs found no effects of flavanones on systolic BP (SBP) but equivocal findings on endothelial function and diastolic BP (DBP) [59, 60]. Following a short-term (i.e. 3 – 4 weeks) flavanone intervention, Morand et al., [59] reported a

decrease in DBP, with no effect on endothelial function, conversely Rizza et al., [60] observed an improvement in endothelial function but no change in DBP (Table 4.1). These inconsistencies in effects on DBP and endothelial function may be explained by inter-study differences such as flavanone dose (i.e. 292 mg versus 500 mg hesperidin), study duration (i.e. 3 versus 4 weeks), study population [healthy overweight men and adults with metabolic syndrome (defined by the [NCEP ATP] III Criteria [237])] and the method used to measure endothelial function [i.e. forearm microvascular reactivity and brachial artery flow mediated dilatation (FMD)].

Table 4.1 – Randomised controlled trials examining the effects of flavanones on blood pressure and endothelial function

Study	Participants/ age (mean ± SD)	duration/ study intervention/ dose	Mean effect[1] [95% CI]
Short-term flavanone interventions:			
Morand et al., 2011 [59]	23 overweight men/ 56 ± 5 y	4 wk/ 0.5 L orange juice/ 292 mg hesperidin + 47 mg narirutin per day	**SBP**: -3.0 mm Hg [-8.3, 2.3] **DBP**: -5.5 mm Hg* [-9.0, -2.0] **Microvascular reactivity[2]**: 52 % [-8, 113] **NOx**: 12.4 µM [1.4, 23.4]
		4 wk/ supplement/ 292 mg hesperidin per day	**SBP**: 0.6 mm Hg [-8.3, 2.3] **DBP**: -3.2 mm Hg* [-9.0, -2.0] **Microvascular reactivity[2]**: 17 % [-44, 77] **NOx**: -3.2 µM [-15.0, 8.6]
Rizza et al., 2011 [60]	24 adults with metabolic syndrome/ 52 ± 2 y	3 wk/ supplement/ 500 mg hesperidin per day	**SBP**: 2.7 mm Hg [-1.3, 6.7] **DBP**: 0.6 mm Hg [-2.2, 3.4] **FMD[2]**: 2.5 %* [0.4, 4.6]
Acute flavanone interventions:			
Morand et al., 2011 [59]	23 overweight men/ 56 ± 5 y	6 h/ 0.5 L orange juice/ 292 mg hesperidin + 47 mg narirutin	**Microvascular reactivity[2]**: 105 %**[55, 155] **NOx:** no mean effect (data not reported)
		6 h/ supplement/ 292 mg hesperidin	**Microvascular reactivity[2]**: 48 % [-1, 98] **NOx:** no mean effect (data not reported)

Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure; NOx, plasma levels of nitric oxide metabolites; FMD, flow mediated dilatation; y, year; *P-value<0.05; **P-value<0.01 [1]Difference between control and experimental intervention; [2]methods to assess endothelial function

Morand et al., [59] not only examined short-term effects of flavanones (i.e. 4 weeks intervention), but also reported acute effects of 292 mg hesperidin on endothelial function in the microvasculature measured 6 h after intake of orange juice or a dose-matched hesperidin supplement (consumed together with a light meal) in healthy overweight men (Table 4.1). Whilst these beneficial effects were associated with a similar hesperetin plasma concentration achieved after intake of orange juice and a flavanone extract (i.e. mean ± SEMs: 860 ± 100 nM and 770 ± 160 nM respectively, P = 0.64), the orange juice dietary intervention resulted in greater effects on endothelial function. This apparent difference may be partly attributable to significantly higher plasma concentrations of vitamin C after the orange juice intervention compared to the flavanone extract and control interventions (i.e. mean ± SEMs: 70.5 ± 4.6 µM, 52.8 ± 4.6 µM and 50.8 ± 4.6 µM respectively, P < 0.001). Given that vitamin C has been suggested to acutely improve endothelial function [238-242], additive or synergistic beneficial effects of flavanones and vitamin C may have occurred.

The aim of the herein reported acute human RCT was to examine the potential physiological mechanisms by which flavanones may exert beneficial CV effects as suggested by the epidemiological and short-term RCT data as described above. Clinical measurements of endothelial function, central arterial stiffness and CV autonomic function were chosen as they are sensitive markers of a range of physiologically important systems in maintaining CV health; i.e. endothelial regulation of vascular homeostasis [17], haemodynamics of central arteries [184] and BP regulation by the autonomic nervous system [18], respectively. Whilst these three clinical measures are established independent markers of CVD risk [18, 122, 149] and CV autonomic function is a marker preceding the development of hypertension [18], to the author's knowledge, there are currently no data available on potential effects of dietary flavanones on CV autonomic function and arterial stiffness. This RCT also aimed to explore the importance of the food matrix on the biological effects of flavanones, by testing acute vascular response following intake of dietary flavanones in orange juice or a hesperidin matched supplement. In addition and unlike previous research [59], the present study accounted for the potential effects of vitamin C by matching the flavanone extract intervention and control intervention with the vitamin C present in the orange juice intervention. This may further our understanding of potential additive/synergistic effects between bioactive dietary compounds such as flavanones and vitamin C. Further study aims, were to explore potential acute effects of flavanones on circulating CVD risk biomarkers (Chapter 4) and to associate changes in markers of CVD risk with plasma concentrations of individual flavanone metabolites including phase II metabolites and phenolic acids (Chapter 5).

4.2   Results

Overall for all three flavonoid intervention trials, 61 eligible participants were identified after obtaining written informed consent from potential volunteers and assessing study inclusion criteria during clinical screening visits. 16 of these eligible participants were allocated to the flavanone trial and randomised to the intervention sequence. Amongst them, n = 2 discontinued trial participation after the first experimental period (n = 1 was excluded due to an infection and n = 1 withdrew due to time commitment) and data from n = 2 participants (i.e. 3 experimental periods) were excluded due to non-compliance with dietary restrictions (as uncovered by plasma nitrite concentrations greater than mean + 3 * SD; described in Chapter 4). Additionally, for cfPWV and cAIx, data from n = 1 participant (i.e. both experimental periods) were excluded due to low quality recordings (i.e. beat counts ≤ 6) and for αBRS, data from n = 4 participants (i.e. 5 experimental periods) were excluded due to > 2 % ectopic beats. The 15 study participants were healthy men (age range 51-69) at an estimated mean absolute 10 year CVD risk of 15.7 % (range: 10 - 20 %; BHS criteria [204]) assessed at screening (Table 4.2). Whilst 6 participants (40 %) had stage 1 hypertension, 10 participants (73 %) were mildly hyperlipidemic (Table 4.2) [204].

Table 4.2 – Characteristics of study participants at screening (n = 15)

	mean ± SD	Range	n (%) outside recommendations	BHS [204] recommendations
n	15			
Age (y)	60.3 ± 6	51-69		
Absolute CVD risk (%)	15.7 ± 0	10-20		
Relative CVD risk	24.6 ± 18.8	1-61		
BMI (kg/m2)	25.7 ± 3.5	22-35	6 (40)	<25
SBP (mm Hg)	137.0 ± 12.2	111-155	6 (40)	<140
DBP (mm Hg)	83.1 ± 6.6	73-94	4 (27)	<90
Glucose (mmol/L)	4.9 ± 0.6	3.9-6	0 (0)	<6.1
Blood lipids: (mmol/L)			11 (73)	
Total cholesterol	5.3 ± 0.6	4.2-6.5	9 (60)	<5
HDL-C	1.31 ± 0.32	0.9-1.9	4 (27)	>1
LDL-C	3.5 ± 0.5	2.6-4.4	11 (73)	<3
Triglyceride	1 ± 0.4	0.6-2.3	1 (7)	<1.7

Abbreviations: SEM; standard error of the mean; BHS, British Hypertension Society; CVD, cardiovascular disease; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL-C and LDL-C, high and low density lipoprotein cholesterol, respectively.

Participants reported good compliance to dietary and exercise restrictions prior to and during the experimental periods (Figure 3.1). In particular, the low mean plasma concentration of flavanone metabolites at baseline further suggests that participants adhered to the 3 day citrus fruit free diet (Table 4.3). Moreover, mean baseline plasma concentrations of vitamin C of fasted participants (i.e. ≈ 32 μM) were relatively low in comparison to levels reported in the literature (e.g. mean ± SD of 47.2 ± 6.5 μM [243]), which further supports good adherence to the dietary restrictions.

5 h after the orange juice intervention, plasma concentration of phase II flavanone metabolites was significantly elevated (P < 0.001) relative to the control intervention (Table 4.3; refer to Table 3.3 for correlation coefficients of standard curves and to Figure 6.1 for a typical spectral trace). At baseline and 5 h after the control intervention, a low plasma concentration of flavanone phase II metabolites was detected. Plasma concentrations of vitamin C were similar at baseline and increased by the same extent 5 h after the dietary interventions (P = 0.27) (Table 4.3), which was expected since dietary interventions provided the same dose of vitamin C.

Food habits and lifestyle did not change between the orange juice and control interventions as suggested by a similar mean energy intake (i.e. 1635 ± 541 kcal; 1749 ± 591 kcal; P = 0.61) and BMI (i.e. 26.4 ± 3.9 kg/m^2; 25.6 ± 3.9 kg/m^2; P-value: 0.53). Likewise, participants' baseline characteristics of CV measurements were similar between the dietary interventions (i.e. P > 0.05) (Table 4.3). No significant acute change in BP, HR, RHI, xBRS, Valsalva ratio and cfPWV was observed 5 h after the orange juice intervention compared to the control intervention (Table 4.3), conversely, cAIx was significantly higher and αBRS was significantly decreased 5 h after the orange juice interventions compared to the control. However, the significant effect on cAIx was attenuated and lost significance when normalised to a HR of 75 bpm (Table 4.3).

Table 4.3 – Markers of cardiovascular risk at baseline and 5 h following interventions in men at moderate cardiovascular disease risk [1]

	Mean ± SD									
	Placebo			Orange Juice			Flavanone extract			
	BL	5h	Δ	BL	5h	Δ	BL	5h	Δ	*P*[3]
SBP (mm Hg)	80.4 ± 6.5	74.8 ± 7.6	-5.6 ± 4.5	78.5 ± 6.7	74 ± 7.2	-4.5 ± 4.5	80.8 ± 7.4	75.6 ± 7.2	-5.2 ± 4.2	0.83
DBP (mm Hg)	128.4 ± 8.9	123.2 ± 7.5	-5.1 ± 10.7	126.9 ± 6.6	124.5 ± 10.6	-2.4 ± 8.5	127.1 ± 7.6	123.9 ± 9.6	-3.2 ± 7.5	0.75
HR (bpm)	55.9 ± 7.9	59.9 ± 8.5	4 ± 5.1	54.2 ± 7.3	59 ± 8.1	4.8 ± 4	54.5 ± 8.2	58.3 ± 7.6	3.7 ± 4.7	0.82
PAT-RH index	2.83 ± 0.72	2.71 ± 0.7	-0.13 ± 0.39	2.82 ± 0.46	2.73 ± 0.69	-0.09 ± 0.44	2.85 ± 0.81	2.69 ± 0.59	-0.16 ± 0.52	0.93
xBRS (ms/mm Hg)	8.4 ± 3.3	8.5 ± 3.4	0.1 ± 2.5	10.8 ± 5.8	9 ± 3.3	-1.8 ± 4.2	11.4 ± 5.4	9.7 ± 3.9	-1.7 ± 3.9	0.83
αBRS (ms/mm Hg)	8.3 ± 3.2	8.7 ± 3	0.3 ± 3.7	9.5 ± 3.9	7.3 ± 3.1	-2.2 ± 2.2*	ND	ND	ND	< 0.05
Valsalva ratio	1.6 ± 0.28	1.62 ± 0.29	0.02 ± 0.16	1.62 ± 0.29	1.67 ± 0.38	0.06 ± 0.16	1.68 ± 0.37	1.72 ± 0.3	0.04 ± 0.24	0.99
cfPWV (m/s)	9.9 ± 1	9.9 ± 0.8	0 ± 0.9	9.9 ± 1.3	9.6 ± 1	-0.3 ± 0.7	9.4 ± 1.9	9.7 ± 1.6	0.2 ± 0.9	0.22
cAIx (%)	25.1 ± 4.5	20.8 ± 4.3	-4.3 ± 2.4	25.2 ± 3.9	22.8 ± 3.3	-2.5 ± 2.1**	25.6 ± 4.4	22.7 ± 4.1	-2.8 ± 3.1	< 0.05
cAIx@HR75 (%)	33.1 ± 7.6	26.9 ± 7.4	-6.2 ± 3.5	34.8 ± 8.6	29.4 ± 5.7	-5.4 ± 4.7	36 ± 8.6	30 ± 7.7	-6.1 ± 6	0.31
Plasma flavanones (nM)	15 ± 11	18 ± 6	2 ± 10	15 ± 7	1601 ± 1331	1586 ± 1326****	13 ± 11	60 ± 96	48 ± 99	P < 0.05
Vitamin C (µM)	32.4 ± 12.2	50.1 ± 14.8	17.7 ± 9.5	32 ± 14	47.8 ± 18.4	15.8 ± 8.6	31.2 ± 9.4	52.6 ± 20.5	21.4 ± 14.5	0.235

[1] Interventions were matched for sugar and vitamin C content, whilst the control intervention was free of flavanones, the orange juice and flavanone extract intervention were matched in hesperidin (dose: 320mg). The sample sizes for the interventions were n = 14 (control), n = 13 (orange juice) and n = 15 (Flavanone extract), except for cfPWV and cAIx [n = 13 (control), n = 12 (orange juice) and n = 14 (flavanone extract)] and αBRS [n = 10 (control) and n = 12 (orange juice)]. [2] Differences between the dietary interventions were established using a linear mixed model for crossover studies with baseline values on each experimental period as the covariate and significant changes were considered at a *P*-value of < 0.05. Differences between the orange juice or supplement intervention and the control intervention were assessed by post-hoc analyses with Tukey adjustment and are indicated as follows; *, P < 0.05; **, P < 0.01; ****, P < 0.0001. Abbreviations: CV, cardiovascular; 5H – BL; 5 h post intervention changes from baseline; SBP and DBP, systolic and diastolic blood pressure; HR, heart rate; PAT-RH, peripheral arterial tonometry-reactive during reactive hyperaemia; cfPWV, carotid to femoral pulse wave velocity; cAIx, central augmentation index; cAIx@HR75, cAIx normalised at a 75bpm HR.

4.3   Discussion

Our data suggest that flavanones were highly bioavailable 5 h after intake of 3 servings of orange juice (providing 320 mg hesperidin and 48 mg narirutin) (Table 4.3), and that these absorbed flavanone metabolites from orange juice did not exert acute beneficial effects on BP, heart rate, arterial stiffness, endothelial and CV autonomic function in healthy men at a mild to moderate CVD risk compared with an energy and vitamin C matched control (Table 4.3).

Amongst flavanone bioavailability studies, Mullen et al., [71] have previously reported that the mean ± SEM time of maximal flavanone phase II metabolite plasma concentration is at 4.4 ± 0.5 h after intake of orange juice when unaccompanied by a meal and in fasted participants. Thus, the herein measured mean flavanone phase II metabolite plasma concentration of 1.60 ± 1.33 µM at 5 h after intake of 368 mg flavanones in orange juice is likely to reflect maximal plasma concentration of flavanone phase II metabolites and is towards the upper end of previously reported concentrations (i.e. range: 0.2 to 1.6 µM) [71, 72, 78, 108], which may be due to the relatively high administered dose and solubility of hesperidin in the FDOC orange juice.

Inconsistent with the orange juice intervention, plasma concentration of flavanone phase II metabolites were not elevated 5 h after intake of the dose-matched flavanone extract intervention. In turn, CVD risk marker data of the flavanone extract intervention were considered unreliable to assess potential beneficial CV effects of flavanones and were, therefore, omitted from analysis. In contrast to the present findings, another study reported a similar plasma flavanone concentrations after intake of the same dose of flavanones administered in a supplement or from orange juice [59]. A significant discrepancy might be that whilst participants of the conducted RCT were fasted and consumed the dietary intervention (i.e. a test drink and two supplements) without any accompanying meal, participants of Morand et al., [59] consumed their dietary interventions together with a meal, which may increase absorption of the water-insoluble hesperidin powder in the supplement. Indeed, animal studies have shown that the bioavailability of water-insoluble flavonoids was enhanced 7 to 14 fold when isolated flavonoids were consumed together with fish oils or dissolved in a polar aprotic liquid than when consumed with water [244, 245]. Thus, these findings may indicate that flavanone bioavailability depends on the food matrix in which flavanones are consumed and that supplements may be poorly bioavailable when consumed on an empty stomach. Alternatively, it could also be speculated that the timepoint of maximal flavanone metabolite concentration following flavanone extract intake may considerably differ from 5 h post consumption.

Despite increased circulating levels of flavanones 5 h after the orange juice intervention, no beneficial acute effect of absorbed flavanones on BP, endothelial function, CV autonomic function and arterial stiffness was found compared with the energy and vitamin C matched control.

Endothelial function was measured in the present study in the digital artery indicated by the PAT-RH index. Although no flavanone studies were included, a meta-analysis of 18 studies (n = 255 participants) considering all six flavonoid subclasses provides strong evidence that flavonoids acutely improve endothelial function with an overall mean increase of 2.33 % (95 % CI: -1.58, -3.08) in FMD of the brachial artery [246]. In contrast with this and the limited evidence from another acute flavanone intervention [59] suggesting beneficial effects on endothelial function, the present study did not observe an acute beneficial effect of flavanones on endothelial function. Whilst the present acute flavanone intervention and the one by Morand et al., [59] were both in healthy men at a similar CVD risk, discrepant findings between the two studies may be explained by differences in the arterial sites and methods used to assess endothelial function (i.e. in the digital artery using PAT and in the forearm microvasculature using laser Doppler flow imaging and acetylcholine iontophoresis, respectively), dissimilarities in the control intervention (i.e. matched or not matched for vitamin C, respectively), the timepoint of measurement (i.e. 5 h and 6 h after flavanone intake, respectively) and the administered flavanone dose (i.e. 368 mg and 292 mg, respectively) which led to unequal mean plasma concentrations of absorbed flavanones (i.e. 1.60 and 0.86 µM, respectively). Although a number of studies validated digital artery endothelial function against invasive methods and FMD [124, 125, 195] and reported correlations with CVD risk [122, 123] and NO bioavailability [129], other studies found no correlation of digital artery endothelial function with the Framingham CVD risk score, microvascular reactivity and FMD [126, 127]. Moreover, whilst intake of 75 g glucose or smoking are known interventions to acutely decrease FMD by 30 and 60 %, respectively, these two established acute interventions did not induce a change in PAT-RH [247]. In summary, digital artery endothelial function assessed by the PAT-RH index may not have been the most sensitive method to detect a potential beneficial effect of flavanones on endothelial function.

To the author's knowledge, the present study is the first to examine potential acute effects of dietary flavanones on arterial stiffness and CV autonomic function. However, neither arterial stiffness nor CV autonomic function were acutely improved by flavanones as assessed by cfPWV and cAIx or BRS and the Valsalva ratio, respectively (Table 4.3) with the cAIx being significantly increased and the αBRS being significantly decreased 5 h after the orange juice intervention compared to the control intervention.

In contrast to xBRS, which was similar between the two dietary interventions, the αBRS was significantly lower in the orange juice intervention compared to the control

intervention (Table 4.3). A possible explanation may be that unlike xBRS, αBRS is not a commercially available method and, therefore, less robust and more subjective in dealing with noise in the raw data. In contrast to the present findings on CV autonomic function, Takumi et al., [62] reported 500 mg non-dietary glucosyl hesperidin (reported to reach a C_{max} of >10 μM [70]) to acutely improve autonomic function assessed using heart rate variability in young healthy women. This limited evidence from two studies may suggest that supraphysiological plasma concentrations of flavanone metabolites may be required to beneficially affect CV autonomic function.

The cAIx was significantly higher 5 h after the orange juice intervention relative to the control intervention (Table 4.3). This could, at least in part, be explained by the participants' HR since the significant difference was lost when the cAIx was normalised for HR of 75 bpm. Previous studies reported mixed findings regarding acute effects of flavonoids on arterial stiffness. Whilst three acute flavan-3-ol interventions reported a decrease in arterial stiffness [158, 159, 248], an acute anthocyanin intervention reported no improvement [232]. Together with the present data, this limited evidence may suggest that only certain flavonoid subclasses may decrease arterial stiffness.

No acute effect of flavanones on BP was observed (Table 4.3). This is consistent with evidence from flavonoid meta-analyses [128, 249] which reported flavonoids to decrease DBP after daily intake over several weeks but not after acute intake.

Despite a growing body of epidemiological evidence suggesting habitual intake of dietary flavanones and citrus fruits to reduce CVD risk [43-51, 54, 236] and supporting *in vitro*, animal and human studies [60, 62, 74, 93, 94, 148] reporting beneficial effects of isolated flavanones on CVD risk biomarkers including BP, endothelial function and CV autonomic function, data from this acute human RCT did not suggest an acute beneficial CV effect 5 h after intake of a dietary flavanone intervention relative to a vitamin C matched dietary control intervention. In both the control and orange juice interventions, a similar increase in plasma vitamin C concentration from baseline to 5h post intervention was observed which may have prevented the detection of a potential flavanone-induced beneficial CV effect (Table 4.3). Indeed, a comparable increase in circulating levels of vitamin C of 20 μM has been associated with a 30 % reduction in CVD mortality when sustained in the long term [243] and there are acute RCTs that report vitamin C to beneficially affect endothelial function [238-242] and autonomic function [250]. However, whilst these acute improvements were observed in regular smokers and patients with hypertension, hypercholesterolemia, CVD or diabetes, within the same studies, no effects were reported for apparently healthy participants [240-242]. Furthermore, vitamin C was administered at high oral doses ranging from 1 to 2 g or even through venous infusion reaching plasma concentrations unachievable through dietary intake but only through supplements. In summary, little is known regarding potential CV bioactivity of a physiological dose of

vitamin C on CVD risk biomarkers in a healthy population and, thus, vitamin C alone may have had beneficial CV effects in the present study.

Whilst as a long-term goal, evidence for a potential health benefit of flavanone intake needs to be examined through the conduct of long-term RCTs, as a preliminary step, further acute RCTs are required to establish; a) populations that may benefit most from flavanone intake, b) optimal doses of flavanone intake consumed within a food matrix supporting flavanone absorption, and c) timepoints of flavanone CV bioefficacy linked to the presence of circulating flavanone metabolites. The present RCT and the other previous acute study by Morand et al., [59] examined the potential acute effects of flavanones in healthy men at a moderate CVD risk; however, potential effects in individuals at a higher CVD risk (e.g. smokers and patients with hypertension, hypercholesterolemia, metabolic disorder or past CVD event) remain to be examined. Whilst only 6 % of ingested flavanones have been recovered as phase II metabolites in 24 h urine [71], 37 % of ingested flavanones have been recovered as phenolic acid metabolites predominantly in urine collected 5 - 24 h after orange juice intake [113]. Thus, potential acute cardioprotective effects of flavanones might occur later than 5 h after their intake. Although little is known regarding the dose-response of flavanones and CVD risk biomarkers, a meta-analysis including RCTs in all flavonoid subclasses reported an inverted U shape relationship between ingested flavonoid dose and FMD [246]. Morand et al., [59] linked their observed acute beneficial effects of flavanones on endothelial function to a mean plasma flavanone phase II metabolite concentration of 0.82 µM which was considerably lower than mean concentration of 1.48 µM reached in the present study that was not linked with an acute beneficial effect. Hence, an acute dose-response study is required to establish optimal flavanone intakes that elicit CV benefits.

There were some limitations to the present study worth noting. Although the control intervention was matched for energy, vitamin C content and liquid volume against the orange juice intervention, orange juice also contains other potentially bioactive compounds which were not control for; including flavones, flavonols, carotenoids, vitamin B9 and folate [100, 210]. However, in comparison to the high content of hesperidin and vitamin C, the sum of these additional potentially bioactive compounds in orange juice is several magnitudes lower and, therefore, their contribution to potential beneficial CV effects of orange juice is potentially negligible. Although the flavanone dose administered in this study (i.e. 368 mg) can be achieved in a diet rich in citrus fruit, this dose was relatively high and does not reflect average flavanone intakes, such as estimated in several European countries (range: 24 to 51 mg/day) [99]. Even though the target of 14 participants completing the study was achieved, quality assessment resulted in exclusion of data, in particular for arterial stiffness and αBRS endpoints, due to which statistical power to detect potential beneficial CV effects of flavanones may have been reduced. The

CVD risk estimation based on a one-off SBP and blood cholesterol assessment at screening might have been overestimated. In comparison to mean SBP assessed at baseline of the experimental periods, participants' mean SBP at screening was 8 mm Hg higher which might have been caused by the white coat syndrome or participants were not adequately rested at screening before BP monitoring. Hence, some participants might have been at < 10 % CVD risk and may not have shown an acute CV response to flavanone intake.

In conclusion, although plasma flavanones concentration was highly elevated 5 h after intake of orange juice but not after intake of a dose-matched flavanone extract, the present data suggest that flavanones from orange juice do not have acute beneficial effects on clinical markers of CVD risk in healthy men at a mild to moderate CVD risk 5 h post intervention compared with a vitamin C matched placebo intervention. Further acute flavanone RCTs that test importance of the food matrix, a dose-response and a longer time-course in various study populations are required to inform the design of short and long term RCTs that are required to examine a potential health benefit of dietary flavanones.

Chapter 5. Acute response of nitric oxide production, NADPH oxidase gene expression and platelet activation to flavanone intake in men at mild to moderate cardiovascular disease risk

5.1   Introduction

Epidemiological evidence has shown an inverse association between the intake of dietary flavanones and cardiovascular disease (CVD) mortality [48, 49] and limited evidence from two recent randomised controlled trials (RCTs) suggests that these cardiovascular (CV) beneficial effects of flavanones may partly be attributed to a reduction in diastolic blood pressure (BP) and an improvement in endothelial function [59, 60]. However, as clearly identified in the systematic review of human randomised controlled trials (RCT) (Chapter 2), the underlying mechanisms by which flavanones mediate beneficial CV effects remain to be established and to the author's knowledge, to date, no human RCTs have examined the potential inhibitory effects of flavanones on platelet function.

Nitric oxide (NO), produced by endothelial NO synthase (eNOS), plays a key role in CV homeostasis [17]; in fact, reduced vascular NO production contributes to a range of CV disorders including endothelial dysfunction and atherosclerosis [17], hypertension [251], arterial stiffness [252], autonomic dysfunction [145] and increased platelet reactivity [253, 254]. Treatment of cultured endothelial cells (from bovine arteries or human veins) with 10 to 100μM hesperetin induced a significant increase in NO production [60, 68-70], accepting that these studies used supraphysiological concentrations and often flavanone forms different to circulating flavanone metabolites (i.e. predominantly glucuronide-conjugates) [71]. Similarly, 4 weeks daily administration of 500 to 2000 mg flavanones per kg diet significantly elevated urinary nitrate excretion in stroke-prone spontaneously hypertensive rats [94]. Proposed subcellular mechanisms of action by which flavanones may increase vascular NO production include elevation of NO through increased eNOS activity [60, 68-70] and gene expression [69] and decreased inactivation of NO through inhibition of superoxide-generating nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity [70] and gene expression [93]. Despite these promising findings, a human RCT did not relate their observed improvement in endothelial function and reduction in diastolic BP after acute or 4 weeks flavanone intake, respectively, with an increase in plasma concentrations of total NO metabolites (NOx; a marker of endothelial NO production) in overweight men [59]. Given the limited evidence to date, further human RCTs are required to establish whether the mechanisms by which flavanones mediate potential beneficial CV effects may be through an increase in NO production and/or inhibition of NADPH oxidase.

Platelets contribute in the early stages to atherosclerosis progression (by stimulation of monocyte and lymphocyte recruitment, macrophage activation, low density lipoprotein oxidation and cell proliferation via platelet derived growth factor; in particular at atherosclerotic sites) and play an important role in the development of arterial thrombosis which may result in an acute CV event [255, 256]. Indeed, increased susceptibility of platelets to activate and aggregate has been linked with a reduced risk of reoccurring coronary artery disease [10] and a large meta-analysis established that antiplatelet therapy (most commonly with aspirin) reduces CVD risk in secondary prevention by 25 % [257]. In primary prevention, however, a meta-analysis found only insufficient data to conclude that there is a net benefit of antiplatelet therapy on CV health because the therapy also increases risk of extracranial bleeding and haemorrhagic stroke [258]. Similarly to antiplatelet therapy, an inhibitory effect of dietary flavanones on platelet activation/aggregation might be a potential mechanism by which dietary flavanones mediate beneficial CV effects. Preclinical studies have observed potent inhibition of platelet aggregation when animal or human platelets were treated *in vitro* with 10 to 200 µM flavanone aglycones or glycosides [73-75]. Moreover, a significantly decreased risk of cerebral thrombosis was reported in stroke-prone spontaneously hypertensive rats that were daily fed with 500 to 2000 mg flavanones per kg diet over 4 weeks [94], and acute inhibition of platelet aggregation and increased tail bleeding time were observed in rats 2 h after oral administration of 10 mg hesperidin per kg body weight or 100 mg citrus extract per kg body weight in an extent comparable to 50 mg aspirin per kg body weight [74]. The *in vitro* studies observed flavanone induced inhibition of platelet aggregation following stimulation with several platelet activating/aggregating factors and most potently after collagen or arachidonic acid stimulated platelet aggregation [73, 74] (Figure 5.1). Jin et al., [73] reported that the subcellular mechanisms of action by which flavanones inhibit platelet activation/aggregation include inhibition of phospholipase C-γ2 phosphorylation, cyclooxygenase1 activity (i.e. two enzymes specific to the collagen and arachidonic acid activation pathways, respectively) and a decrease in intracellular Ca^{2+} (Figure 5.1). However, it is unclear if these effects translate to humans *in vivo*, in particular given that the animal studies fed relatively high flavanone doses [74, 94] and all *in vitro* studies used supraphysiological concentrations of flavonoid forms that do not occur in the circulation [73-75]. Limited evidence from one non-controlled human study did not observe inhibited *ex vivo* ADP, collagen or thrombin stimulated platelet aggregation after daily intake of 250ml orange juice or grapefruit juice for 7 days in a healthy population, whereas 250ml grape juice per day, a rich source of anthocyanins containing 3 times more flavonoids than the citrus juices, inhibited *ex vivo* collagen stimulated platelet aggregation relative to baseline [259]. Given the promising *in vitro* and animal evidence and to clarify the null findings from one non-controlled human study, human RCTs are warranted that test potential effects of flavanones on platelet function.

Figure 5.1 – Schematic presentation of the known pathways leading to platelet activation[1, 2]

[1]The main known platelet agonists include collagen, thrombin, ADP and thromboxane A_2 (TxA$_2$). [2]Platelet activation results in surface expression of the activated fibrinogen receptor (i.e. gpIIb/gpIIIa) and P-selectin (i.e. as a result of dense granule release). Figure adapted from [76]

In this chapter, data from the acute human RCT aimed to examine whether dietary flavanones from orange juice mediate potential beneficial CV effects, at least in part, by increasing NO production, as assessed by plasma nitrite concentrations, and by inhibition of NADPH oxidase expression, as assessed by serum soluble gp^{91phox} concentration. Furthermore, the study aimed to explore potential acute inhibitory effects of dietary flavanones from orange juice on platelet activation under basal conditions and upon *ex vivo* collagen related peptide (CRP) or ADP activation.

5.2   Results

As previously described in Chapter 4, data from n = 15 participants were included in the analysis (n = 12 for both interventions, n = 2 for the control intervention and n = 1 for the orange juice intervention). These data were incomplete because n = 2 participants did not attend the second experimental period and n = 2 participants did not adhere to dietary restrictions (as uncovered by plasma nitrite concentrations greater than mean + 3 * SD). Additionally, for platelet activation, data from 3 experimental periods had to be excluded due to haemolysis of the sodium citrated blood. Furthermore, one participant was prescribed naproxen which is a non-steroidal anti-inflammatory drug; nevertheless, his platelet data were included in the analysis because he served as his own control in this crossover study. The study participants were healthy men (age range: 51 to 69 years) at an estimated mean absolute 10 year CVD risk of 15.8 % and 13.6 %, respectively (BHS

criteria [204]) (Table 5.1). Participants reported good compliance to dietary and exercise restrictions prior to and during the experimental periods.

Table 5.1 – Characteristics of study participants at screening

	Mean ± SD	Range
n	15	
Age (y)	60.3 ± 6	51-69
Absolute CVD risk (%)	15.7 ± 0	10-20
BMI (kg/m2)	25.7 ± 3.5	22-35
Systolic BP (mm Hg)	137 ± 12.2	111-155
Diastolic BP (mm Hg)	83.1 ± 6.6	73-94
Blood lipids: (mmol/L)		
TC/ HDL-C ratio	4.2 ± 1.0	2.7 - 5.8
LDL-C	3.5 ± 0.5	2.6-4.4
Triglyceride	1.05 ± 0.4	0.6-2.3
Haematology:		
White cell blood count (10^9/L)	5.4 ± 1.4	3.5-8.4
Red cell blood count (10^12/L)	4.9 ± 0.3	4.3-5.6
Platelet blood count (10^9/L)	33.7 ± 49.5	176-389
Haemoglobin (g/dL)	43.4 ± 2.4	13.3-163
Haematocrit (%)	236.6 ± 54.8	38.4-47.6

Abbreviations: CVD, cardiovascular disease; BMI, body mass index; BP, blood pressure; TC, total cholesterol; HDL-C and LDL-C, high and low density lipoprotein cholesterol

At baseline, CVD risk biomarkers and vitamin C plasma concentrations (method and data reported in Chapter 3) were similar between the dietary interventions. Plasma concentration of total flavanone metabolites was significantly elevated 5 h after the orange juice intervention relative to the control intervention (mean ± SD: 1.60 ± 1.33 µM; $P < 0.0001$). Negligible amounts of flavanone metabolites were present at baseline and 5 h after the control intervention (i.e. ≤ 0.02 µM). 5 h post intervention, the orange juice intervention did not mediate an acute significant change in plasma nitrite, serum soluble gp91[phox], platelet activation (assessed as P-selectin and fibrinogen receptor expression on platelets under basal conditions or after *ex vivo* activation with ADP or CRP) compared to the control intervention (Table 5.2).

Table 5.2 – Biomarkers of cardiovascular risk at baseline and 5 h following interventions in men at moderate cardiovascular disease risk [1]

| | Mean ± SD | | | | | | | | | |
| | Placebo | | | Orange Juice | | | Flavanone extract | | | |
	BL	5h	Δ	BL	5h	Δ	BL	5h	Δ	*P* [2]
Plasma nitrite (nmol/L)	54.8 ± 16.8	54.7 ± 21.9	-0.1 ± 16	58.9 ± 14.4	55.7 ± 18.2	-3.2 ± 15.6	ND	ND	ND	0.388
Serum soluble gp91^{phox} (pg/mL)	49.5 ± 22.2	48.6 ± 13	-0.8 ± 22.1	50.3 ± 22.1	54.9 ± 22.4	4.6 ± 18.7	ND	ND	ND	0.828
P-selectin expression: (%) [3]										
Platelets under basal conditions	3.9 ± 4.4	1.9 ± 2.4	-1.9 ± 2.2	4.8 ± 3.7	2.9 ± 2.9	-1.8 ± 1.3	3.6 ± 3.5	2.9 ± 2.5	-0.7 ± 2.3	0.112
ADP activated platelets	95.7 ± 3.1	93.7 ± 4.8	-2 ± 2.6	96.4 ± 2.9	94.5 ± 4.7	-1.9 ± 4.3	94.8 ± 4.4	94.8 ± 3.1	0 ± 2.5	0.269
CRP activated platelets	86.8 ± 14.1	87.7 ± 12.2	0.9 ± 7.1	88.7 ± 12.7	87.8 ± 14.6	-0.9 ± 5.7	87.6 ± 14.2	86 ± 15.1	-1.6 ± 3.6	0.411
Fibrinogen receptor expression: (%) [3]										
Platelets under basal conditions	1.4 ± 1.7	1.6 ± 2	0.3 ± 1.3	1.5 ± 1	1.1 ± 0.8	-0.4 ± 0.5	1.3 ± 1.3	1.6 ± 1.5	0.2 ± 1.6	0.454
ADP activated platelets	76.9 ± 11	73.6 ± 11.9	-3.2 ± 5.2	79.4 ± 10.1	75.5 ± 10.2	-4 ± 8.4	75.6 ± 12.8	75.6 ± 11.1	0 ± 4.9	0.202
CRP activated platelets	79.7 ± 16.7	84.2 ± 10.2	4.5 ± 11.3	82.3 ± 14.1	82.1 ± 14.6	-0.2 ± 5.2	81.3 ± 16.6	80.9 ± 15.7	-0.4 ± 4.9	0.151

[1] The sample sizes for the dietary interventions were n = 14 (control), n = 13 (orange juice) and n = 15 (flavanone extract), except for platelet activation (n = 12 for control and orange juice interventions). [2] differences between the dietary interventions were established using a linear mixed model for crossover studies with baseline values on each experimental period as the covariate. [3] expressed as % positive platelet events; ND, not determined

5.3 Discussion

CV biomarker and platelet data from this RCT suggest that at 5 h post intervention, the orange juice intervention (767 ml; providing 320 mg hesperidin and 48 mg narirutin) did not acutely increase nitrite plasma concentrations, decrease serum concentrations of soluble gp^{91phox} or inhibit platelet activation in men at a mild to moderate risk of CVD relative to a sugar and vitamin C matched control intervention (Table 5.2), even though plasma concentrations of flavanone metabolites were significantly elevated 5 h after the orange juice intervention (Chapter 6).

Although a number of preclinical studies suggested that flavanones may enhance vascular NO production [60, 68-70, 94], a previous RCT did not observe an increase in total NO metabolite plasma concentrations after acute (i.e. at 6 h) or short-term (i.e. after 4 weeks) intake of flavanones in overweight men [59]. To better understand these conflicting data, the present study assessed potential acute effects of flavanones on nitrite plasma concentrations which was reported to be a more accurate indicator of vascular NO production than total NO metabolite plasma concentrations [64, 198]. Furthermore, animal data suggested that flavanones may enhance vascular NO production through inhibition of NADPH oxidase gene expression [95] which led us to assess the potential acute effects of flavanones on serum concentration of soluble gp^{91phox}, an *in vivo* marker of NADPH oxidase gene expression [199]. However, and consistent with total NO metabolite data of Morand et al., [59], no acute increase in plasma nitrite concentration or decrease in serum gp^{91phox} was observed 5 h after the orange juice intervention relative to the control intervention in healthy men at a mild to moderate CVD risk. This is consistent with the present clinical data (Chapter 3) suggesting no acute beneficial effects of the orange juice intervention on endothelial function, arterial stiffness and CV autonomic function, three markers of CVD risk that are, at least in part, regulated by vascular NO [17, 145, 252]. In contrast, most other flavonoid subclasses have been reported to increase vascular NO production as reported by a number of acute human RCTs using a range of assessment methods (i.e. plasma concentrations of total NO metabolites, nitrite or nitroso species or urinary excretion of total NO metabolites), various study populations (i.e. healthy and smokers) and flavonoid doses (range: 80 to 200 mg) [191, 192, 230, 260, 261]. Mixed findings from two acute human RCTs are available regarding effects of flavonoids on NADPH oxidase gene expression, with one suggesting flavan-3-ols [260] to decrease serum concentration of soluble gp^{91phox} in young smokers and one observing no change following an anthocyanin intervention in healthy men [232]. In summary, unlike these very similar acute RCTs intervening with other flavonoid subclasses, flavanones might not mediate potential beneficial CV effects in humans *in vivo* through enhancement of NO production or inhibition of NADPH oxidase gene expression, and further human RCTs are

required to establish the mechanisms by which dietary flavanones mediate potential beneficial CV effects.

In a critical review of 25 human RCTs examining the effects of flavonoids on platelet function, sufficient evidence was found to suggest that intake of flavan-3-ols and cocoa containing foods inhibit platelet function with consumption of 100 g dark chocolate having an effect comparable to 81 mg aspirin, but only inconclusive data were available for other flavonoid subclasses and no data were published for flavanones [164]. Hence, to the author's knowledge, the present acute human RCT was the first to examine potential inhibitory effects of dietary flavanones on platelet activation which have been suggested by a number of *in vitro* and animal studies [73-75, 94]. However, 5 h after intake of an orange juice intervention, no acute inhibition of platelet activation (under basal conditions or upon submaximal *ex vivo* stimulation with ADP or CRP) was observed in healthy men at a mild to moderate risk of CVD relative to a control intervention (Table 5.2). These data are consistent with a previous non-controlled human study that did not observe an inhibition of collagen, ADP or thrombin stimulated *ex vivo* platelet aggregation after daily intake of 250 ml orange juice or grapefruit juice for 7 days relative to baseline [259]. Overall, data from these two human studies suggest that inhibition of platelet activation/aggregation may not be a mechanism by which flavanones exert potential beneficial CV effects. However, further human studies are required to fully establish whether or not flavanones may inhibit platelet activation/aggregation. In particular, potential inhibitory effects of dietary flavanones on arachidonic acid stimulated *ex vivo* platelet activation/aggregation remain to be examined, because this particular pathway was reported to be potently inhibited by flavanones in *in vitro* and animal studies [73, 74]. Furthermore, whilst platelets were activated on average by 78 up to 96 % (Table 5.2), the acute human RCT of Rein et al., [202] observed inhibitory effects of flavan-3-ols at a much lower activation of platelets (i.e. 10 % or 60 % when stimulated with 20 μM epinephrine or 20 μM ADP, respectively). Moreover, a number of human studies found attenuation of inhibitory effects up to no inhibition induced by flavan-3-ols or anthocyanins on platelet aggregation, the more platelet agonist was used [259, 262, 263]. Thus, in contrast to the herein used submaximal stimulation of platelet activation, further human RCTs need to examine potential inhibitory effects of flavanones on platelet activation/aggregation upon minimal to medium *ex vivo* stimulation by platelet agonists. Ideally, these studies include both platelet activation and aggregation assessments and use a range of platelet agonists.

The following limitations in this study are worth noting. The present study had an acute study design with a single assessment at 5 h post intervention; however, flavanone derived metabolites were detected in urine up to 24 h after intake [113]. Therefore, an acute investigation over a longer time period may have been more adequate in fully

establishing potential mechanisms by which flavanones mediate beneficial CV effects and examine potential inhibitory effects on platelets activation. Furthermore, a human RCT with a short-term or chronic (rather than an acute) flavanone intervention would have provided more relevant information with regards to potential health benefits of a diet rich in flavanones and citrus fruits. Although to the author's knowledge, this is the first human RCT that examined potential acute effects of flavanones on plasma nitrite concentrations, serum concentrations of soluble gp^{91phox} and platelet activation and they were not acutely affected by intake of flavanones, inclusion of a wider range of assays may have provided a more complete understanding of the potential mechanism of action by which flavanones mediate beneficial CV effects. These assays include the assessment of plasma concentrations of nitroso species [192], urinary excretion of total nitric oxide metabolites [191], serum concentrations of asymmetric dimethylarginine (i.e. an endogenous eNOS inhibitor) [133], NADPH oxidase activity in neutrophils [232], plasma endothelin-1 [191] plasma prostacyclin/leukotriene ratio [264], platelet microparticles [202], plasma soluble P-selectin or CD40L [265] and, as explained above, measuring both platelet activation and aggregation following only minimal to medium *ex vivo* stimulation with platelet agonists (i.e. similar to Rein et al., [202]), ideally using collagen and/or arachidonic acid *ex vivo* stimulation (as proposed by Jin et al., [73] and Yu et al., [74]).

In conclusion, the orange juice intervention did not acutely increase nitrite plasma concentrations, decrease serum concentration of soluble gp^{91phox} or inhibit platelet activation in healthy men at a mild to moderate risk of CVD at 5 h post intervention relative to the sugar and vitamin C matched control intervention. Further studies are required to determine the mechanisms by which flavanones exert beneficial CV effects and more platelet data are warranted to fully establish whether or not flavanones inhibit platelet function.

Chapter 6. Plasma concentrations of flavanone and phenolic acid metabolites 5 hours after a single oral dose of flavanones

6.1 Introduction

Evidence from epidemiological studies [48, 49] and human intervention trials [59, 60] suggests that consumption of flavanones and citrus fruits may have cardiovascular (CV) health benefits. Examination of the absorption, distribution, metabolism and excretion (ADME) of dietary flavanones is important to help us understand what forms of flavanone metabolites may act on which target tissues (e.g. the vascular endothelium and platelets) and, in turn, mediate potential beneficial CV effects. ADME of flavanone phase II metabolites has previously been examined, and relative urinary recovery of these metabolites ranged from only 1.5 to 14 % [71, 72, 78, 108]. Thus, it is understandable that dietary flavanones may also be subject to microbial degradation and bioavailable as phenolic acid metabolites.

However, relatively little is known regarding ADME of flavanone-derived phenolic acid metabolites. Limited evidence from one orange juice intervention trial identified 5 phenolic acids as flavanone metabolites in 24 h urine at levels reaching 37 % of ingested flavanones relative to a flavanone-free control intervention [113]. Furthermore, Vallejo et al., [78] identified 7 circulating glucuronide conjugated phenolic acid metabolites and suggested that they were derived from oral intake of orange juice [78]. However, they did not include a flavanone-free control intervention and did not quantify plasma concentrations, and, therefore, it is unclear whether these putative phenolic acid metabolites originate from ingested flavanones or other sources such as the background diet [101, 266].

Moreover, little is known regarding the potential CV bioactivity of these absorbed flavanone and phenolic acid metabolites. In an acute flavanone RCT, a mean ± SEM plasma concentration of hesperetin of 0.86 ± 0.16 µM correlated with improved microvascular endothelial function suggesting that hesperetin metabolites may be partly mediating the observed beneficial CV effects [59]. However, this study enzymatically hydrolysed the glucuronides and sulfates from phase II metabolites of hesperetin and did, therefore, not quantify the unique circulating phenolic acid metabolites.

The aim of the present study was to identify and quantify concentrations of a broad range of flavanone phase II metabolites and of previously reported and novel phenolic acid metabolites in 5 h post-intervention plasma samples from an acute crossover placebo-controlled orange juice intervention in men at a mild to moderate risk of CVD. Relationships between plasma levels of flavanone metabolites and markers of CVD risk

(described in Chapter 3 and Chapter 4) were also explored to indicate which metabolites may be potential mediators of beneficial CV effects.

6.2 Results

Plasma samples collected at baseline and 5 h after participants received the control intervention and the orange juice intervention were analysed by HPLC-MS^2 in sMRM analysis mode. Hesperetin, naringenin, 6 phase II metabolites of flavanones and 15 phenolic acid metabolites were identified in plasma collected 5 h after participants received the orange juice intervention (Figure 6.1).

Hesperetin, naringenin, naringenin-7-glucuronide and 11 phenolic acid metabolites were identified based on RT and having ≥ 3 sMRM transitions relative to a pure standard, whilst 5 phase II metabolites of flavanones were putatively identified based on having ≥ 3 sMRM transitions (Table 3.3 & 5.3; Figure 6.1), which were previously reported and specific to phase II metabolites of flavanones [71, 72]. Moreover, 4 phenolic acids were putatively identified based on 3 to 5 sMRM transitions (i.e. 2 isomer phenolic acids and 2 phenolic acid glucuronides for which analytical standards were not available) (Figure 6.1; Table 3.3).
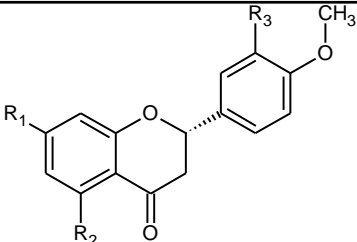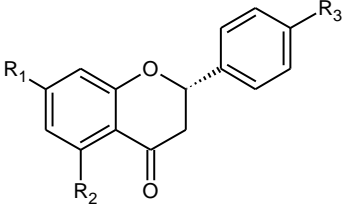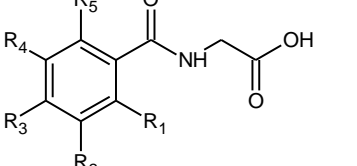
There were 13 further phenolic acids included in the original analysis (Appendix 4). However, 4 of these phenolic acids were below the limit of detection and plasma concentrations of the other 9 phenolic acids did not significantly differ between the orange juice and control interventions indicating they are not specific flavanone metabolites.

Figure 6.1 – Typical HPLC-ESI-MS^2 trace of flavanone metabolites in plasma extracts collected 5 h after participants consumed the orange juice intervention
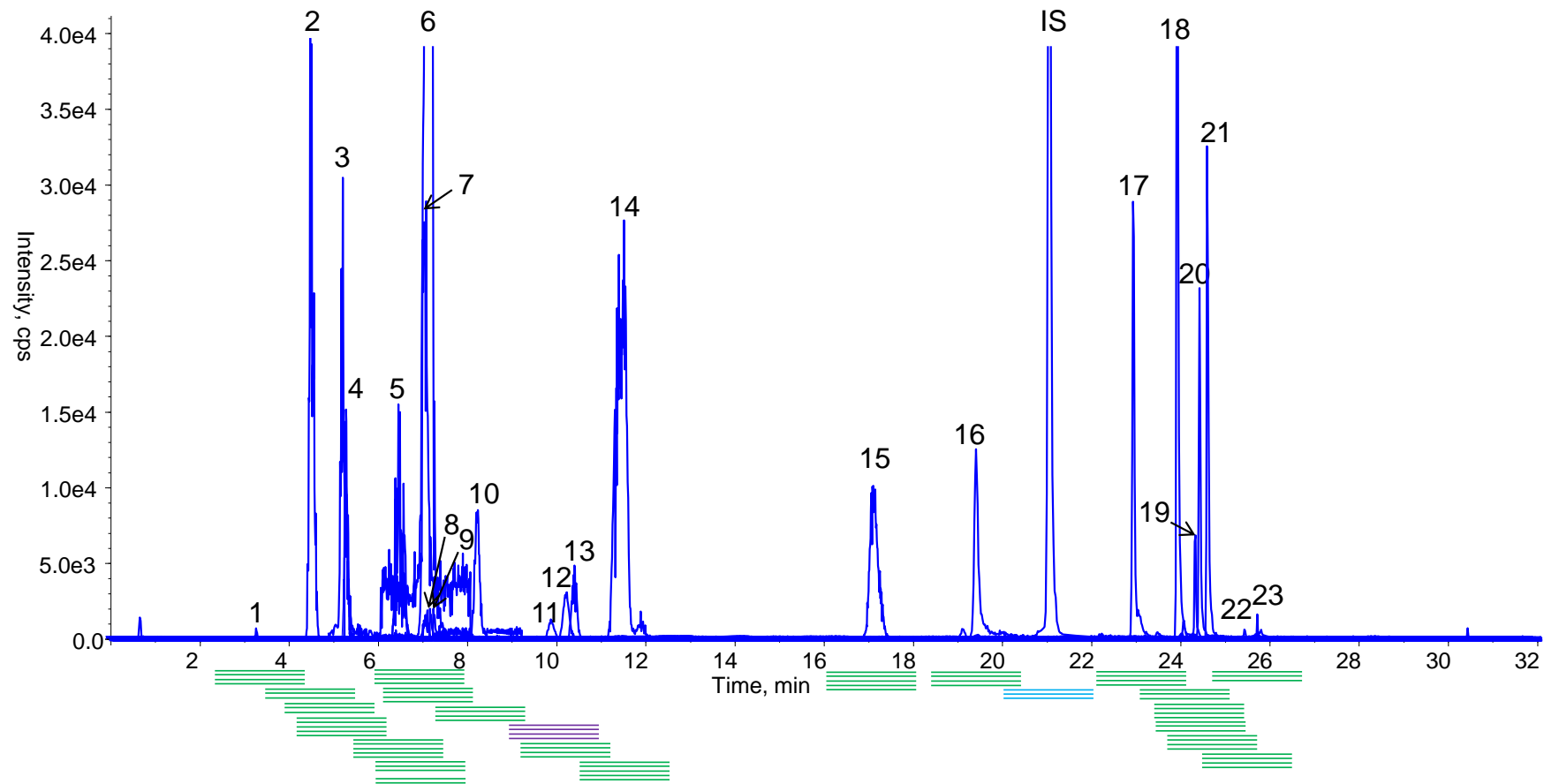
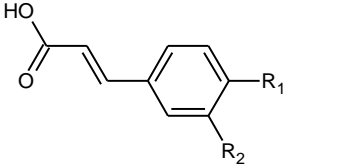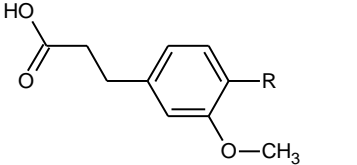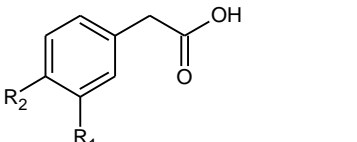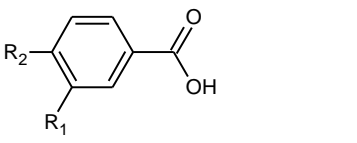Only highest sMRM transitions are displayed for each peak. Benzoic acid-4-glucuronide[1], hydroxyhippuric acid[2], 3-hydroxyhippuric acid[3], isovanillic acid-glucuronide[4], vanillic acid-glucuronide[5], hippuric acid[6] (reached 3.2e5 cps), vanillic or isovanillic acid-glucuronide[7], 4-hydroxyphenylacetic acid[8], 4-hydroxy-benzoic acid[9], 3-hydroxyphenylacetic acid[10], isovanillic acid[11], isoferulic or ferulic acid-glucuronide[12], vanillic acid[13], dihydroferulic acid-3-glucuronide[14], dihydroferulic acid[15], hesperetin-diglucuronide[16], taxifolin[IS] (reached 7.0e4 cps), hesperetin-diglucuronide[17], naringenin-7-glucuronide[18] (reached 7.6e4 cps), hesperetin-glucuronide[19], naringenin-glucuronide[20], hesperetin-glucuronide[21], naringenin[22], hesperetin[23]. Bars below the time axis represent number of sMRM transitions per compound with a window length of 2 min (green, negative sMRM mode; purple, positive sMRM mode; light blue, internal standard) Abbreviations: ESI, electrospreay ionisation; sMRM, scheduled multiple reaction monitoring; IS, internal standard; cps,counts per sec

99 / 150

Table 6.1 – Chemical structure of identified flavanone and phenolic acid metabolites

Chemical structure	#[1]	Analyte name	Configuration[2]
	23	hesperetin	R = H
	19 & 21	hesperetin-glucuronide[3]	R_1, R_2 or R_3 = O-GlcA
	16 & 17	hesperetin-diglucuronide[3]	$R_{1\&2}$, $R_{1\&3}$ or $R_{2\&3}$ = O-GlcA
	22	naringenin	R = H
	18	naringenin-7-glucuronide	R_1 = O-GlcA
	20	naringenin-glucuronide[3]	R_2 or R_3 = O-GlcA
	6	hippuric acid	R = H
	3	3-hydroxyhippuric acid	R_3 = OH
	2	hydroxyhippuric acid[3]	R_1, R_2, R_4 or R_5 = OH
	12	ferulic acid-glucuronide[3]	R_1 = O-GlcA, R_2 = CH_3
		isoferulic acid-glucuronide[3]	R_1 = CH_3, R_2 = O-GlcA
	15	dihydroferulic acid	R = OH
	14	dihydroferulic acid-3-glucuronide	R = O-GlcA
	8	4-hydroxyphenylacetic acid	R_2 = OH
	10	3-hydroxyphenylacetic acid	R_1 = OH
	13	vanillic acid	R_1 = OCH_3, R^2 = OH
	5	vanillic acid-3-glucuronide	R_1 = OCH_3, R_2 = O-GlcA
	11	isovanillic acid	R_1 = OH, R^2 = OCH_3
	4	isovanillic acid-3-glucuronide	R_1 = O-GlcA, R_2 = OCH_3
	9	4-hydroxy-benzoic acid	R_2 = OH
	1	benzoic acid-4-glucuronide	R_2 = O-GlcA

[1] Number refers to peak in Figure 5.3; [2] R = H, unless stated otherwise; [3] site of conjugation could not be ascertained as identification was based on known transitions where pure standards for isomers were not available or separation of isomers was chromatographically not possible

5 h after consumption of the orange juice intervention, aglycones and phase II metabolites of flavanones were detected at mean plasma concentrations of 1.60 ± 1.33 µM, which was significantly increased relative to the control intervention and baseline (P < 0.01) (Figure 6.2 A1 and Table 6.2). A negligible amount of flavanone aglycones and their phase II metabolites were detected in baseline and 5 h samples following the control intervention (Figure 6.2 A1). The plasma concentration of hesperetin-glucuronide was significantly elevated 5 h post control intervention relative to baseline, but only to a negligible extent (Figure 6.2 A1 and Table 6.2). Of the flavanone aglycones and their phase II metabolites,

hesperetin-glucuronides represented 64.4 % of the flavanones identified, followed by naringenin glucuronides (19.3 %), hesperetin-diglucuronides (18.9 %) and flavanone aglycones (0.8 %) (Figure 6.2 A2).

Figure 6.2 – Plasma concentrations and relative proportions of flavanones and phenolic acid metabolites
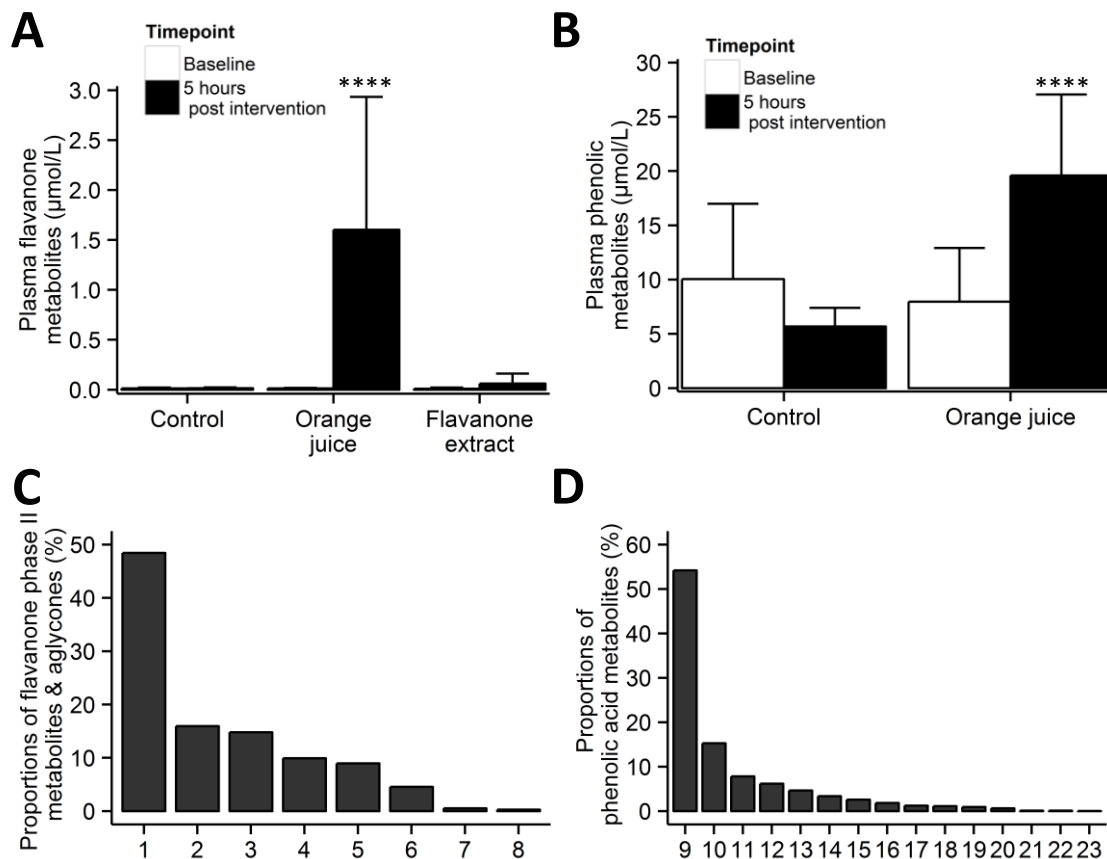
Mean (± SD) plasma concentrations of flavanone aglycones and their phase II metabolites (**A**) and total phenolic acid metabolites (**B**) quantified at baseline and 5 h after the control intervention (n = 14), orange juice intervention (n = 13) and flavanone extract intervention (n = 15; phenolic analysis was not performed). Individual metabolites and their relative proportions 5 h after the orange juice intervention (n = 13): (**C**): 1, hesperetin-glucuronide; 2, hesperetin-glucuronide; 2, hesperetin-glucuronide; 3, hesperetin-diglucuronide; 4, hesperetin-diglucuronide; 5, naringenin-7-glucuronide; 6, naringenin-glucuronide; 7, hesperetin; 8, naringenin; (**D**): 9, hippuric acid; 10, dihydroferulic acid; 11, dihydroferulic acid-3-glucuronide; 12, 4-hydroxyphenylacetic acid; 13, vanillic acid; 14, hydroxyhippuric acid; 15, iso/ferulic acid-glucuronide; 16, 3-hydroxyhippuric acid; 17, isovanillic acid; 18, 3-hydroxyphenylacetic acid; 19, vanillic acid-glucuronide; 20, isovanillic acid-glucuronide; 21, iso/vanillic acid-glucuronide; 22, 4-hydroxy-benzoic acid; 23, benzoic acid-4-glucuronide; 5 h post intervention differences between the dietary interventions were established using a linear mixed model for crossover studies with baseline values on each experimental period as the covariate. Differences between the orange juice or supplement intervention and the control intervention were assessed by post-hoc analyses with Tukey adjustment and are indicated as follows; ****, P < 0.0001.

There was a high mean background concentration of phenolic acids present in baseline plasma, yet mean plasma concentrations of total phenolic acid metabolites were significantly elevated 5 h after the orange juice intervention compared to the control intervention (19.59 ± 7.46 µM and 5.69 ± 1.70 µM, respectively; $P < 0.0001$) and baseline ($P < 0.001$) (Figure 6.2 B1 and Table 6.3). Plasma concentrations of total phenolic acid metabolites was significantly decreased 5 h post control intervention relative to baseline ($P < 0.05$); in particular, hippuric acid and 3-hydroxyhippuric acid contributed significantly to this decrease, whereas some compounds such as isovanillic acid and 3-hydroxyphenylacetic acid significantly increased (Table 6.3). Of the 15 identified phenolic acid metabolites, the ones found in the highest concentrations were hippuric acid contributing 54.2 % to total phenolic acid plasma concentration, followed by dihydroferulic acid (15.3 %), dihydroferulic acid-glucuronide (7.8 %) and 4-hydroxyphenylacetic acid (6.2 %) (Figure 6.2 B2 and Table 6.3).

Table 6.2 – Plasma concentrations of flavanone aglycones and their phase II metabolites at baseline and 5 h after the dietary interventions [1]

	Mean ± SD (nM)						
	Placebo		Orange Juice		Flavanone extract		
	BL	5h	BL	5h	BL	5h	P^2
Total	12 ± 9	15 ± 7	12 ± 6	1601 ± 1331 ****	10 ± 11	61 ± 100	< 0.0001
Hesperetin-glucuronide	13[a]	6 ± 4[c]	5[a]	756 ± 605 ****	ND	51 ± 77[h]	< 0.0001
Hesperetin-glucuronide	ND	6 ± 2[h]	5[a]	245 ± 223 ****	4[a]	25 ± 33[h]	< 0.0001
Hesperetin-diglucuronide	ND	ND	ND	146 ± 121 ****	3[a]	11 ± 10[d]	< 0.0001
Hesperetin-diglucuronide	ND	ND	ND	133 ± 137 ****	3[a]	4 ± 1[b]	< 0.0001
Naringenin-7-glucuronide	4 ± 5	2 ± 2	4 ± 4	222 ± 247 ****	4 ± 6	4 ± 3	< 0.0001
Naringenin-glucuronide	3 ± 2	2 ± 1	3 ± 1	82 ± 90 ****	5 ± 4	3 ± 2	< 0.0001
Hesperetin	1 ± 0	1 ± 0	1 ± 0	9 ± 8 ****	NM	NM	< 0.01
Naringenin	4 ± 2	4 ± 2	4 ± 2	9 ± 4 ****	NM	NM	< 0.05

[1] The sample sizes for the dietary interventions were n = 14 (control), n = 13 (orange juice) and n = 15 (Flavanone extract). Some metabolites were detected only in a subset of the participants: a, n = 1; b, n = 3; c, n = 4; d, n = 5; e, n = 6; f, n = 7; g, n = 8; h, n = 9; i, n = 10; j, n = 12; k, n = 13)]. [2] Differences between the dietary interventions were established using a linear mixed model for crossover studies with baseline values on each experimental period as the covariate and considered significant at a *P*-value < 0.05. Differences between the orange juice or supplement intervention and the control intervention were assessed by post-hoc analyses with Tukey-Kramer adjustment and are indicated as follows; ****, P < 0.0001. If plasma concentration was ND, the concentration was replaced with the limit of detection for statistical analysis. ND; not detected.

Table 6.3 – Plasma concentrations of phenolics at baseline and 5 h after the dietary interventions [1]

| | Mean ± SD (nM) | | | | |
| | Placebo | | Orange Juice | | |
	BL	5h	BL	5h	*P* [2]
Total	10041 ± 6944	5689 ± 1702 *	7964 ± 4945	19590 ± 7464 ***	< 0.0001
Hippuric acid	7917 ± 6832	3127 ± 1298 *	6245 ± 4763	10100 ± 4097 **	< 0.0001
Dihydroferulic acid	8 ± 4[g]	6[a]	14 ± 12[c]	2432 ± 2651 **	< 0.01
Dihydroferulic acid-3-glucuronide	ND	ND	ND	1250 ± 1742 *	< 0.05
4-hydroxyphenylacetic acid	850 ± 594	853 ± 613	577 ± 299	1567 ± 812 ***	< 0.01
Vanillic acid	19 ± 6[k]	19 ± 6[k]	19 ± 8[j]	759 ± 777 **	< 0.01
Iso/ferulic acid-glucuronide	27 ± 24[k]	114 ± 54 ***	27 ± 17	521 ± 163[i] ****	< 0.0001
Hydroxyhippuric acid	129 ± 103	93 ± 78 **	110 ± 108	611 ± 382 ***	< 0.001
Isovanillic acid	48 ± 54[j]	205 ± 201*	43 ± 23	393 ± 218[i] ****	< 0.05
3-hydroxyphenylacetic acid	686 ± 268[k]	848 ± 209[k] *	639 ± 213	978 ± 206 ****	< 0.05
Isovanillic acid-glucuronide	12 ± 13[i]	165 ± 286[k] **	13 ± 9[f]	264 ± 256 **	< 0.05
Vanillic acid-glucuronide	19 ± 24[e]	73 ± 53[i] *	6 ± 1[g]	199 ± 85 **	< 0.001
3-hydroxyhippuric acid	224 ± 360	117 ± 215 *	179 ± 321	362 ± 337 **	< 0.001
Iso/vanillic acid-glucuronide	ND	ND	ND	17 ± 16 **	< 0.01
Benzoic acid-4-glucuronide	3 ± 4	2 ± 2	3 ± 3	7 ± 4 ****	< 0.001
4-hydroxy-benzoic acid	141 ± 109	121 ± 56	161 ± 91	159 ± 81	< 0.05

[1] The sample sizes for the dietary interventions were n = 14 (control) and n = 13 (orange juice). The analysis was not performed for the flavanone extract intervention. Some metabolites were detected only in a subset of the participants: a, n = 1; b, n = 3; c, n = 4; d, n = 5; e, n = 6; f, n = 7; g, n = 8; h, n = 9; i, n = 10; j, n = 12; k, n = 13)]. [2] 5 h post intervention differences between the two dietary interventions were established using a linear mixed model for crossover studies with baseline values on each experimental period as the covariate and changes between baseline and 5 h post intervention were established using paired t-test. Significant changes of the statistical tests are indicated with * for P < 0.05, ** for P < 0.01, *** for P < 0.001 and **** for P < 0.0001. If plasma concentration was ND, the concentration was replaced with the limit of detection for statistical analysis. ND; not detected.

Cumulatively, phenolic acid metabolites reached a 10 fold higher plasma concentration than the flavanone aglycones and their phase II metabolites (15.96 μM *versus* 1.47 μM, respectively) 5 h after the orange juice intervention relative to baseline and the control intervention. There was a large inter-individual variability for the plasma concentration of flavanone and phenolic acid metabolites (5 h changes from baseline ranging from: 0.46 to 5.29 μM and -1.2 to 30.8 μM, respectively) (Figure 6.3).

Figure 6.3 – Inter-individual variability in plasma concentrations of flavanone and phenolic acid metabolites 5 h after the orange juice intervention relative to baseline[1]
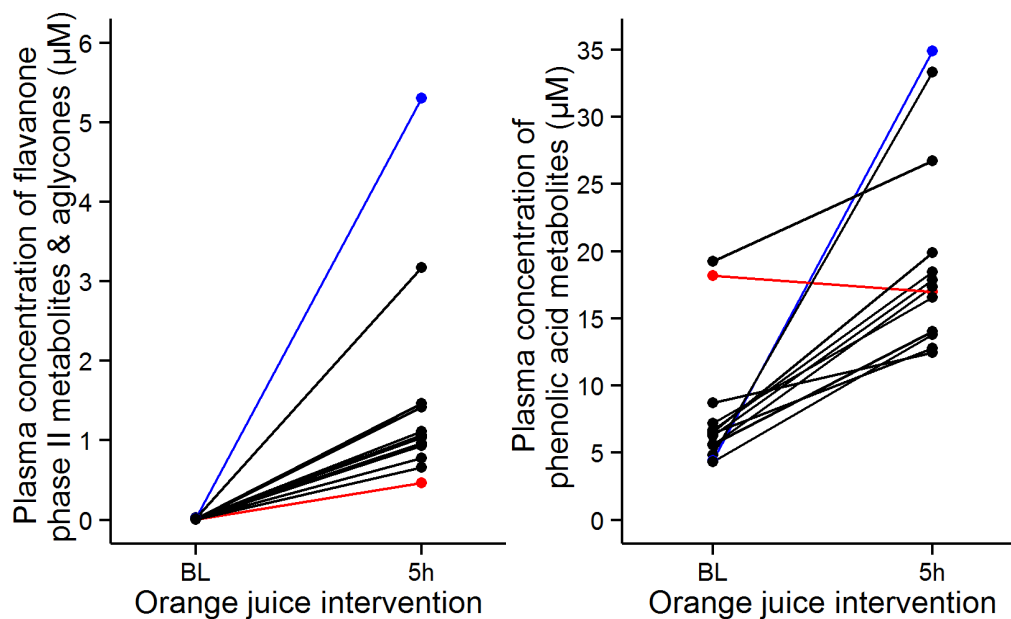
[1] Increase in plasma concentrations of total flavanone aglycones and their phase II metabolites (**A**) and phenolic acid metabolites (**B**) for each participant. The blue and red lines highlight the participant with the highest and lowest change in plasma concentration of the metabolite, respectively.

Plasma concentrations of 5 flavanone metabolites, 12 phenolic acid metabolites and their individual sums increased by ≥ 100 nM from baseline to 5 h post orange juice intervention (Table 6.2 & Table 6.3) and were, therefore, deemed as potentially physiologically important changes and included in correlation and regression analyses. Increased plasma concentrations of several metabolites were correlated (using Pearson's correlations) with changes in specific markers of CVD risk (Appendix 5). For instance, increased total phenolic acid metabolites or 4-hydroxyphenylacetic acid levels from baseline to 5 h post orange juice intervention were negatively correlated with systolic BP or pulse wave velocity, respectively (where a decrease represents a biologically favourable change); conversely, increased levels of isovanillic acid-glucuronides were also positively correlated with heart rate (where an increase represents an unfavourable biological change). Yet, these relationships lost significance when using a multivariate regression model that included all 19 plasma metabolites.

6.3 Discussion

The present study identified 23 plasma flavanone metabolites which were significantly elevated 5 h after an orange juice intervention (providing 320 mg hesperidin and 48 mg narirutin) compared to a flavanone-free control intervention, and these metabolites included 2 flavanone aglycones, 6 flavanone phase II metabolites, 9 additional previously unidentified phenolic acid metabolites and 6 phenolic acid metabolites that were previously reported in urine [113]. To the author's knowledge, this was the first time phenolic acid metabolites have been quantified in plasma following consumption of a single dose of flavanones.

The findings regarding flavanone aglycones and their phase II metabolites were similar to those reported in previous pharmacokinetic studies feeding orange juice [71, 72, 78, 107, 108]. The mean plasma concentrations of total flavanone aglycones and their phase II metabolites 5 h after the orange juice intervention was in accordance with reported mean maximal plasma concentrations (i.e. 0.2 to 2.2 µM) [71, 72, 78, 107, 108], whilst only negligible amounts were detected at baseline and after the flavanone-free control intervention. These negligible amounts possibly originated from other dietary sources (other than citrus fruits) in the habitual and intervention diets, such as tomatoes or herbs containing small amounts of flavanones [100]. Consistent with previous studies [71, 78, 108], the vast majority of the identified metabolites were glucuronide conjugates of the parent flavanones, whereas only trace amounts of flavanone aglycones were detected in plasma. The present study did not detect hesperetin-sulfate and hesperetin-sulfate-glucuronide, two metabolites that were previously detected in urine [71, 72, 78] and only inconsistently in plasma by some studies [78, 108] but not by others [71]. Genetic polymorphisms in sulfotransferase enzymes between study populations could be a possible explanation for these discrepant findings [267]. Hesperetin was also observed to undergo phase I metabolism to the flavanone eriodictyol in an *ex vivo* pig caecum model [268]. However, neither eriodictyol aglycones nor its putative phase II metabolites (Figure 3.6) were detected. Possible reasons may be that eriodictyol is a transient metabolite not reaching the human circulation but undergoing degradation to smaller phenolic acids or that the human microflora does not metabolise hesperetin to eriodictyol unlike the colonic microflora of pigs.

Phenolic acids were present in the baseline diets and biological samples of individuals because they are ubiquitously present in plant based foods including fruit, vegetables, cereals, coffee and tea [266, 269] for which the present study could only control for to a limited extent. Moreover, certain phenolic acids such as hippuric acid may originate from endogenous metabolism [270]. Nevertheless, by using the control intervention to account for these influencing factors, plasma concentrations of 15 phenolic acids were significantly

elevated from baseline to 5 h after the orange juice intervention relative to the control intervention (Figure 6.2B), suggesting that these metabolites were derived from flavanones or phenolic acids in the orange juice intervention. These phenolic acids included 9 novel metabolites and 6 metabolites that were previously quantified in 24h urine by a controlled orange juice pharmacokinetic study of Roowi et al., [113] (Table 3.3, Table 6.3). Their study quantified 9 phenolic acids in 24 h urine samples and consistent to the data presented here, 24 h urinary excretion of dihydroferulic acid, 3-hydroxyhippuric acid and 3-hydroxyphenylacetic acid was significantly elevated after intake of orange juice compared to a control [113]. Furthermore, they identified 3-methoxy-4-hydroxyphenylhydracrylic acid and 3-hydroxyphenylhydracrylic acid as flavanone metabolites [113]; however, the present study did not include these two metabolites because standards were not available for method development and, therefore, their presence and concentration in plasma remains unknown. Roowi et al., [113] did not observe increased 24 h urinary excretion of 4-hydroxy benzoic acid, hippuric acid and 4-hydroxyphenyl acetic acid after intake of orange juice relative to control, whereas the present study observed significantly increased plasma concentrations of these 3 phenolic acids. Differences between analysed biofluids (i.e. urine *versus* plasma), assessment of a single timepoint *versus* a 24 h period and differences in the dietary restrictions before and during the experimental period may potentially explain these discrepant findings. Additionally, Vallejo et al., [78] identified a number of glucuronidated phenolic acid metabolites in plasma after intake of orange juice (i.e. phloretic acid-glucuronide, p-courmaric acid-glucuronide, cinnamic acid-glucuronide, homovanillic acid-glucuronide, hydroxyl-benzoic acid-glucuronide and hippuric acid-glucuronide and hydroxyphenyl acetic acid-glucuronide). However, the data presented here suggest that p-courmaric acid-glucuronide may not be a flavanone metabolite (Appendix 4) and the other phenolic acids were not detected in plasma. Possible explanations for identification of different phenolic acid glucuronide metabolites could result of small differences in flavanone metabolism between the study participants or that the study of Vallejo et al., [78] did not have a comparative control intervention and therefore their reported metabolites may be attributed to a different dietary source than orange juice. Nevertheless, the present study identified similar flavanone metabolites to those that they identified such as isoferulic acid-glucuronide, dihydroferulic acid-glucuronide and vanillic acid-glucuronide, or the same metabolites but not conjugated to a glucuronide, such as hippuric acid, 4-hydroxy-benzoic acid and hydroxyphenylacetic acid. Overall, in addition to these previous studies [78, 113], 9 previously unidentified phenolic acid metabolites were identified and, to the author's knowledge, phenolic acid metabolites (i.e. a total of 15) were quantified in plasma for the first time.

In comparison with plasma concentrations of flavanone aglycones and their phase II metabolites, phenolic acid metabolites reached 10 fold higher plasma concentrations. These findings suggest that phenolic acid metabolites contribute substantially to the circulating pool of flavanone metabolites and together with phase II metabolites of flavanones, dietary flavanones may be more bioavailable than previously thought [105, 109]. In fact, the cumulative plasma concentrations of the 23 identified flavanone metabolites increased to a similar extent as did vitamin C (i.e. $P = 0.70$) 5 h after the orange juice intervention that provided 368 mg flavanones and 439 mg vitamin C relative to baseline and the control intervention. A large inter-individual variability in plasma concentrations changes from baseline of both flavanone phase II metabolites (i.e. range: 0.46 to 5.29 µM) and phenolic acid metabolites (i.e. range: -1.2 to 30.8 µM) was observed 5 h after the orange juice intervention relative to baseline and the control intervention. This is consistent with Vallejo et al., [78] who reported large differences in pharmacokinetics and urinary excretion of flavanone phase II metabolites between participants and at the same time they observed low within-participant variability and found no correlation with gender or BMI. These inter-individual differences in pharmacokinetics were observed not only for flavanones but for a wide range of dietary phytochemicals, and factors including the composition and activity of gut microflora, genetic variation in phase II enzymes (e.g. expression, stability and activity of glucuronsyltransferases and/or sulfotransferases) and intestinal transporters of each individual may determine to which extent they metabolise and absorb phytochemicals, including dietary flavanones [109, 267]. In turn, this may also translate to large inter-individual variability in how individuals might benefit from intake of dietary flavanones in relation to potential beneficial CV effects.

Short-term (i.e. 3-4 week) intake of flavanones may reduce diastolic BP [59] and improve endothelial function [60] as recently reported in two human RCTs. However, little is known regarding which phase II metabolites of flavanones and phenolic acid metabolites are the responsible mediators of these beneficial CV effects. The present study observed no beneficial acute effects of the orange juice intervention on the assessed markers of CVD risk relative to the vitamin C matched control intervention (Chapter 3 and 4). Consistently, none of the multivariate regression analyses suggested a significant association between plasma concentrations of flavanone / phenolic metabolites and markers of CVD risk and only some significant correlations of an exploratory nature were found (Appendix 5) that would require further investigations. However, there is supportive evidence associating flavanones and phenolic acids with beneficial CV effects. In an acute flavanone RCT, improved endothelial function strongly correlated with plasma hesperetin concentrations (albeit they were using a method which enzymatically hydrolysed glucuronide and sulfate conjugates) [59], and multivariate regression analysis of an acute flavonoid intervention (with blueberry anthocyanins) suggested that differences in plasma concentrations of a

range of phenolic acid metabolites predict beneficial responses in endothelial function and NADPH oxidase activity [232]. Moreover, urinary excretion of hippuric acid has been inversely associated with BP in a large population-based study [271].

Despite the identification and quantification of some novel flavanone-derived circulating metabolites, this study has limitations. Even though a pooled (n = 13) plasma extract collected 5 h after the orange juice intervention was screened for presence of 158 potential metabolites and identified 23 of them as flavanone metabolites (Figure 3.6), some flavanone metabolites might have been missed. Underlying reasons may include methods for two known flavanone metabolites (i.e. 3-methoxy-4-hydroxyphenyl hydracrylic acid and 3-hydroxyphenylhydracrylic [113]) were unavailable, and the used HPLC-MS^2 method, albeit sensitive for quantification of flavanone phase II metabolites, has a relatively high detection limit for certain phenolic acids due to their lower molecular weight or poor ionisation, and more metabolites might have been identified if gas chromatography-MS^2 had been used. To the author's knowledge, this is the first study that quantified plasma concentrations of 15 flavanone-derived phenolic acid metabolites; yet, concentrations were only assessed 5 h after an orange juice intervention and, therefore, a more complete time course (e.g. up to 24 h) and inclusion of urine and faecal sample analysis would be required to establish absorption, clearance kinetics and elimination routes of the identified circulating metabolites. 5 flavanone phase II metabolites and 4 phenolic acid metabolites were only tentatively identified based on ≥3 sMRM transitions due to lack of availability of analytical standards, which would have been required to confirm a RT match. As a result, the site of glucuronide conjugation or the isomeric configuration could not be determined (Table 3.3). Whilst the used sMRM transitions of the phase II metabolites of flavanones match with those previously reported [71, 228], the use of synthesised standards or methods such as nuclear magnetic resonance spectroscopy would be necessary to confirm the exact structural identities of the putative phenolic acids. Although phenolic acid metabolites derived from flavanones of an orange juice intervention were identified relative to a flavanone-free control intervention, an intervention with isotopically labelled flavanones would provide more robust evidence and complete insights in regards to the metabolic fate of flavanones. Lastly, the phenolic acid content of the orange juice was not measured in-house but only estimated based on previously reported mean levels [208]. However, this estimate may not reflect the exact dose provided in the present orange juice intervention because phytochemical content of orange juice varies depending on geographic cultivation region, seasonal changes and processing [38].

In conclusion, the present study established that 5 h after an orange juice intervention 9 novel and 14 previously identified flavanone and phenolic acid metabolites were significantly elevated in plasma relative to the control intervention. Future human studies

are required to establish the pharmacokinetics of these metabolites and their potential beneficial CV effects.
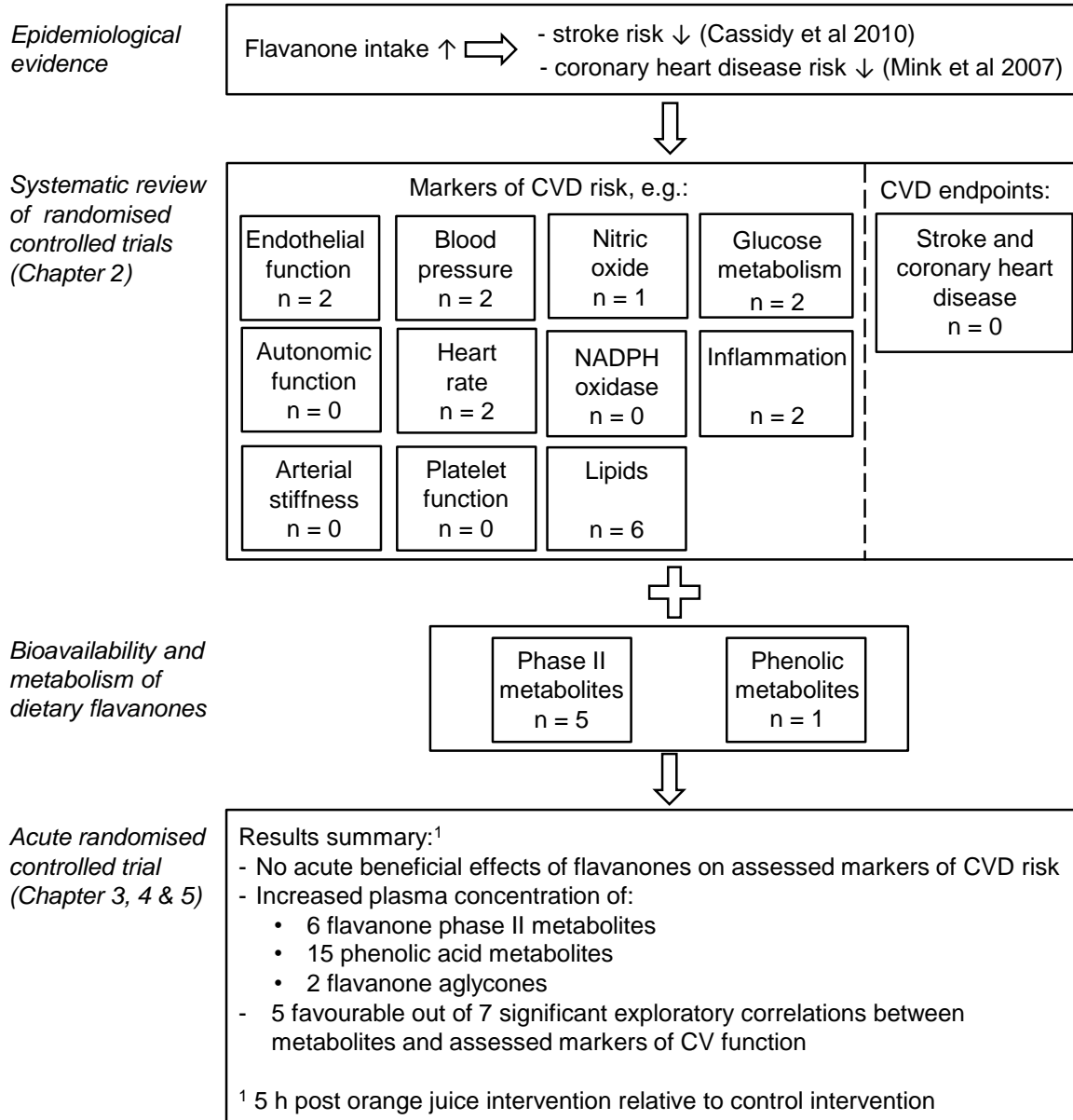
Chapter 7. General discussion and future research perspectives

7.1 Overview

A third of the UK's mortality is attributed to cardiovascular disease (CVD) [1] and a diet rich in fruit and vegetables is a key factor in potentially reducing CVD risk [37]. Citrus fruits and flavanones may substantially contribute to these benefits given the large body of epidemiological evidence that suggests a high intake of either is inversely associated with CVD morbidity and mortality [43-51, 54, 236]. For example, in two large cohort studies using multi-variable adjusted models (including estimated vitamin C intakes), a diet rich in flavanones was associated with a significantly reduced risk of stroke [relative risk (RR): 0.81 (95 % confidence interval (CI): 0.66 - 0.99)] [48] and coronary heart disease [RR: 0.78 (95 % CI: 0.65 - 0.94)] [49].Furthermore, a number of supportive studies using animal models proposed that flavanones mediate beneficial cardiovascular (CV) effects by favourably altering lipid profile [165], vascular function [principally via enhanced bioavailability of nitric oxide (NO)] [60] and CV autonomic function [148] as well as through a reduction in blood pressure (BP) [93] and platelet reactivity [73]. However, scientific evidence that intake of dietary flavanones reduces CVD risk in humans has not yet been established. Thus, the aim of this work was to systematically review published human randomised controlled trials (RCT) that examined the effects of flavanones and citrus fruit on CVD risk markers, to identify the current state-of-the-art and the key research gaps in the literature (Chapter 2) and to address some of them by conducting an acute flavanone intervention RCT (Chapter 3-5) (Figure 7.1).

In the systematic review (Chapter 2), ten human RCTs were identified that met the inclusion criteria (adopted from the methodology our group employed in a previous systematic review [128]) and examined the effects of flavanones and/or citrus fruits on a range of markers of CVD risk (Figure 7.1). A meta-analysis of six RCTs suggested that short-term (i.e. 3-8 weeks) daily intake of flavanones may reduce plasma total cholesterol (for individuals with elevated CVD risk (i.e. with metabolic syndrome or past history of CVD event) (-0.64 mM, 95 % CI: -1.04, -0.24; n = 87; 3 studies), but not in healthy adults (-0.01 mM, 95 % CI: -0.12, 0.10; n = 165; 3 studies). However, these findings have to be interpreted with caution because two studies in individuals with elevated CVD risk that found a highly significant reduction in blood lipids also had a high risk of bias.

Figure 7.1 – Overview of existing data regarding cardiovascular beneficial effects of dietary flavanones and summary of conducted acute trial

Epidemiological evidence

Flavanone intake ↑ ⟹
- stroke risk ↓ (Cassidy et al 2010)
- coronary heart disease risk ↓ (Mink et al 2007)

Systematic review of randomised controlled trials (Chapter 2)

Markers of CVD risk, e.g.:

Endothelial function n = 2	Blood pressure n = 2	Nitric oxide n = 1	Glucose metabolism n = 2
Autonomic function n = 0	Heart rate n = 2	NADPH oxidase n = 0	Inflammation n = 2
Arterial stiffness n = 0	Platelet function n = 0	Lipids n = 6	

CVD endpoints:

Stroke and coronary heart disease n = 0

Bioavailability and metabolism of dietary flavanones

Phase II metabolites n = 5

Phenolic metabolites n = 1

Acute randomised controlled trial (Chapter 3, 4 & 5)

Results summary:[1]
- No acute beneficial effects of flavanones on assessed markers of CVD risk
- Increased plasma concentration of:
  • 6 flavanone phase II metabolites
  • 15 phenolic acid metabolites
  • 2 flavanone aglycones
- 5 favourable out of 7 significant exploratory correlations between metabolites and assessed markers of CV function

[1] 5 h post orange juice intervention relative to control intervention

Abbreviations: n, number of existing studies in the literature; CVD, cardiovascular disease; NADPH, nicotinamide adenine dinucleotide phosphate; n refers to numbers of studies that have been published in the scientific literature

Data on all other examined markers of CVD risk were insufficient to conduct meta-analyses, yet limited data from individual studies suggest that short-term (i.e. 3 – 4 weeks) flavanone intake may reduce diastolic BP (DBP) and inflammation through decreasing soluble vascular cellular adhesion molecules in overweight men [59] or improve brachial artery endothelial function and reduce inflammation through decreasing highly soluble C reactive peptide in individuals with metabolic syndrome [60]. No effects were observed on glucose metabolism (i.e. plasma insulin or glucose concentration) [59, 60] or plasma NO metabolite concentration [59]. Acutely, flavanones were reported to improve microvascular endothelial function (i.e. 6 h after intake; assessed using microvascular reactivity) and this change strongly correlated with hesperetin plasma concentrations, but the underlying mechanism could not be explained through an increase in plasma NO metabolite

concentration [59]. In summary, this systematic review suggests that flavanones may beneficially affect a range of markers of CVD risk; however, existing human RCT data are insufficient to provide evidence for a beneficial relationship between intake of flavanones and a reduction in CVD risk. In particular, optimal daily flavanone intakes, their potential health implications in various study populations and the potential importance of the food matrix in which flavanones are consumed (e.g. whole citrus fruit, citrus juices or purified supplements) remain to be established. Furthermore, potential effects of flavanones on other established markers of CVD risk markers including arterial stiffness [184], CV autonomic function [18] or platelet function [256] have not been examined and at present the mechanism by which flavonoids mediate beneficial CV effects are incompletely understood. Similarly, no acute human RCT to date has simultaneously assessed plasma concentrations of individual flavanone metabolites and looked at associations between these metabolites and changes in CVD risk markers. Even though previous studies have examined the bioavailability and pharmacokinetics of phase II metabolites of flavanones [71, 72, 78, 107, 108] and a few studies identified phenolic acid metabolites [78, 113] and quantified levels in urine but not in plasma [113].

Hence, an acute flavanone RCT was conducted in men at a mild to moderate CVD risk to address some of these existing research gaps (Chapter 3-5). An acute study design was chosen to identify which potential markers of CVD risk may be beneficially modulated by the intake of dietary flavanones. The aim was also to explore potential mechanisms of action underlying these modulations, and to correlate these bioactivity data with plasma concentrations of previously reported and novel flavanone metabolites. Even though it is acknowledged that a human RCT with a short-term or chronic flavanone intervention may have been more informative with regards to potential long-term health effects, results from this acute study may help inform the design of these longer term studies (e.g. selection of study population, flavanone dose and CVD risk maker endpoints).

In regards to potential markers of CVD risk, data reported in Chapter 3 suggested that despite high concentrations of flavanone / phenolic metabolites in plasma 5 h after intake of orange juice (767 ml; providing 320 mg hesperidin and 48 mg narirutin), no parallel beneficial effects on BP, heart rate, central arterial stiffness, digital endothelial function and CV autonomic function were observed in healthy men at a mild to moderate risk of CVD relative to a sugar and vitamin C matched control intervention. Whilst no previous studies were found that examined potential effects of flavanones on arterial stiffness and CV autonomic function (Figure 7.1), the present null findings on digital endothelial function are inconsistent with the acute human RCT by Morand et al., [59] which suggested a beneficial effect of flavanones on microvascular endothelial function. Dissimilarities in the design of the present study and the study by Morand et al., [59] may explain these inconsistent findings; in particular that different methods and arterial sites were used to

measure endothelial function (i.e. in the digital artery using peripheral arterial tonometry and in the forearm microvascular using laser Doppler flow imaging and acetylcholine iontophoresis, respectively), that achieved flavanone plasma concentrations were unequal (i.e. 1.46 and 0.86 µM, respectively) and that the control interventions differed (i.e. matched or not matched for vitamin C, respectively).

Similarly, data reported in Chapter 4 suggested that 5 h after intake of the orange juice intervention, no acute increase in plasma nitrite concentrations, reduction in serum concentration of soluble gp^{91phox} or inhibition in platelet activation was observed in men at a mild to moderate risk of CVD relative to a sugar and vitamin C matched control intervention. The nitrite and platelet activation data, respectively, are consistent with the acute and short-term flavanone intervention human RCT by Morand et al., [59] that observed no increase in plasma total NO metabolites, and the uncontrolled human trial by Keevil et al., [259] that observed no effect of a one week orange juice or grapefruit juice intervention on platelet aggregation relative to baseline. Hence, current data indicate that an increase in NO bioavailability or inhibition of NADPH oxidase may not be the key mechanisms by which flavanones exert beneficial CV effects and also that platelet activation/aggregation may not be affected by intake of dietary flavanones.

The aims addressed in Chapter 5 were to identify and quantify a range of novel and previously identified flavanone and phenolic acid metabolites in plasma samples of the acute human RCT and to examine, on an exploratory basis, whether their concentrations correlate with acute changes of assessed markers of CVD risk (Chapter 3 & 4). Out of 158 considered potential metabolites, a total of 2 flavanone aglycones (albeit at low concentrations), 6 flavanone phase II metabolites and 15 phenolic acid metabolites were identified on the basis that their concentration was significantly elevated 5 h after the orange juice intervention compared to the flavanone-free control intervention. Comparing mean increases in plasma concentrations from baseline, phenolic acid metabolites reached 10 fold higher levels than the flavanone phase II metabolites (i.e. 15.96 µM and 1.47 µM, respectively). Whilst the identified flavanone phase II metabolites have previously been detected at similar concentrations in plasma post orange juice consumption [71, 72, 78, 108], to the author's knowledge, none of the 15 phenolic acid metabolites have previously been identified or quantified in plasma and 6 of them only in urine [113]. These novel data suggest that flavanones might reach higher plasma concentrations than previously thought [105, 109] and a great proportion are present as phenolic acid metabolites for which potential beneficial CV effects are currently poorly understood. Despite the overall null findings of the present acute human RCT, plasma concentrations of 7 certain flavanone and phenolic acid metabolites significantly correlated (using Pearson's correlations) with changes in specific markers of CVD risk, and 5 of them were biologically favourable (Appendix 5). However, these correlations are

exploratory and may help in devising studies and hypothesis to test in future human RCTs.

7.2 Future research perspectives for flavanone human randomised controlled trials
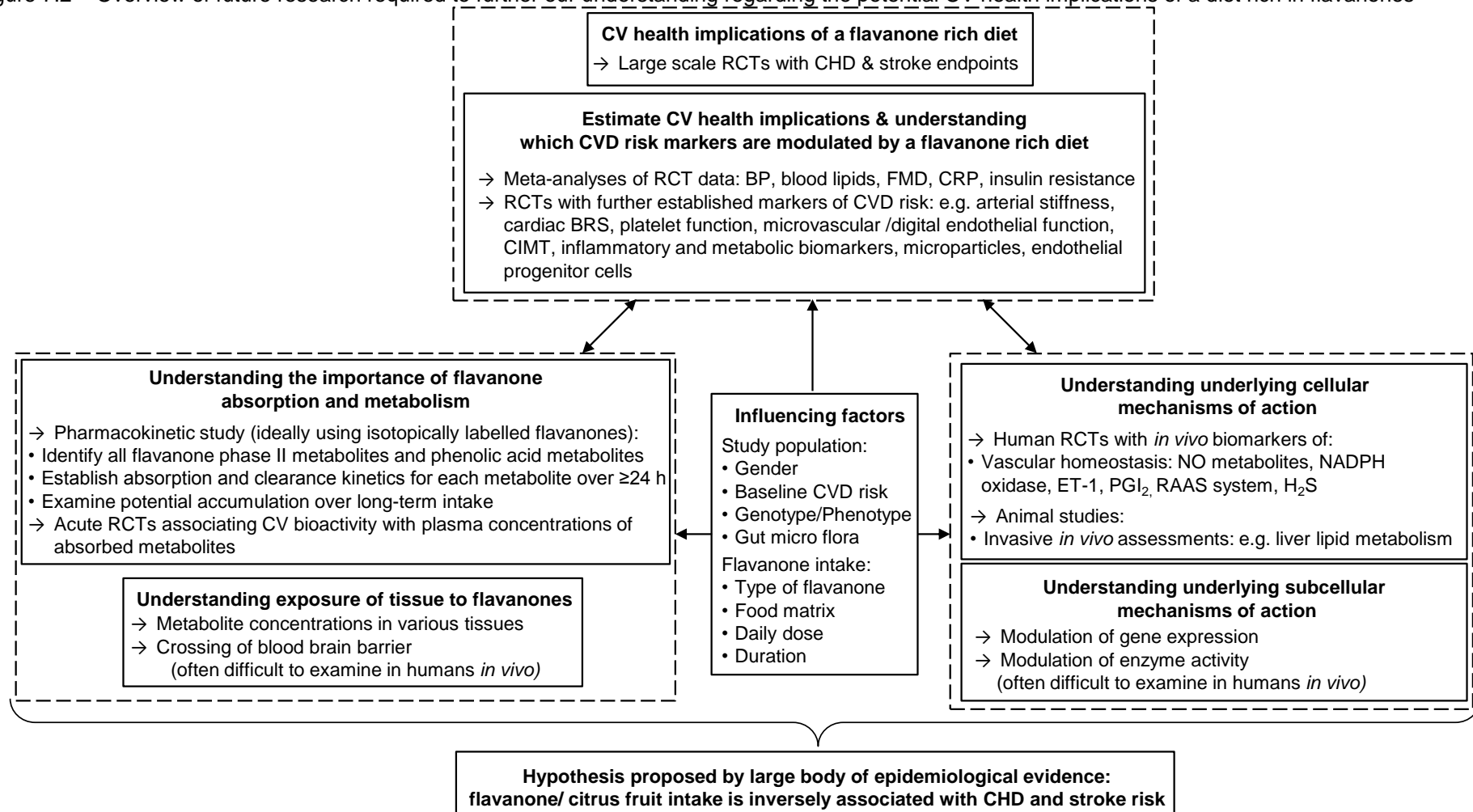
As outlined in previous sections of this thesis, current research has not yet fully established the CV health implications of a diet rich in flavanones (Figure 7.2). Furthermore, only little is known about which CVD risk factors might be beneficially modulated, what the underlying cellular mechanisms of action are and how this potential bioactivity is affected by flavanone metabolism and absorption (Figure 7.2).

In contrast to the flavanone subclass, the CV health implications of a diet rich in flavan-3-ols and isoflavones have been more extensively examined. A large meta-analysis of human RCTs (n = 1297; 42 RCTs) provides scientific evidence that intake of flavan-3-ols and cocoa containing foods improves markers of CVD risk including a clinically significant improvement in brachial artery endothelial function, reductions in insulin resistance and DBP, but only marginal improvements in blood lipids and no inhibition of inflammation (as assessed by C reactive protein) [249]. Sensitivity analysis further suggested that effects on endothelial function were independent of the dose of flavan-3-ols ingested with doses below 50 mg/d still having a significant effect, whilst a dose above 50 mg/d is required to induce a reduction in DBP [249]. Furthermore, evidence from 10 human RCTs suggested that intake of flavan-3-ols and cocoa containing foods inhibit platelet function with consumption of 100 g dark chocolate having an effect comparable to 81 mg aspirin [164, 272]. Similarly, three meta-analyses (n = 789-1281; 11-17 RCTs) provided data to suggest that intake of dietary isoflavones beneficially affects markers of CVD risk through clinically significant improvements in brachial artery endothelial function [273], reductions in total and low density lipoprotein cholesterol [274] and systolic BP (SBP) [275], but no change in triglycerides, high density lipoprotein cholesterol [274] or DBP [275]. Sensitivity analysis further revealed that there might be a synergistic or additive effect of isoflavones with soy protein (i.e. food matrix effect), that SBP lowering effects were more pronounced in studies intervening more than 3 months and cholesterol lowering effects were more pronounced in individuals with hypercholesterolemia. Thus, for dietary flavanones, further human RCTs with short-term to chronic interventions are required in addition to the 10 existing RCTs previously identified in the conducted systematic review (Chapter 2). To enable future meta-analyses, RCTs should measure (consistently and in a standardised way) established independent markers of CVD risk, including the traditional modifiable CV risk markers (i.e. DBP and SBP and blood total, high and low density lipoprotein cholesterols) [276], brachial artery flow mediated dilatation as a marker of endothelial function [277], fasting circulating insulin as a marker of insulin resistance [278, 279], C

reactive protein as a marker of inflammation [280], and platelet aggregation as a marker of platelet function [201]. Collectively, these RCTs should aim to determine effects of flavanone types (e.g. naringenin or hesperetin), flavanone dose, food matrix of intake (e.g. supplement, whole citrus fruits or citrus juice), intake duration (i.e. acute to several months), baseline CVD risk of study populations (i.e. low up to high CVD risk and individuals who suffered CV event) and other characteristics of study populations (e.g. gender, gut microflora or genotype/phenotype) on markers of CVD risk. Importantly, RCTs should compare effects of a flavanone intervention against a closely matched control intervention and employ a design with minimal risk of bias [168]. In addition to RCTs examining established CVD risk markers that may provide data for future meta-analyses, potential effects of dietary intake of flavanones on other established CVD risk markers should also be explored. These may not only include digital endothelial function, BRS, central arterial stiffness and platelet activation tested in the present acute RCT (Chapter 3 & 4) and microvascular endothelial function, inflammatory and metabolic biomarkers tested in previous RCTs [59, 60], but also previously unexplored or novel CVD risk markers such as carotid intima media thickness [281], endothelial microparticles [282] and endothelial progenitor cells [283].

Ultimately, long-term RCTs with a flavanone supplemented dietary intervention, an adequate control intervention and primary CV events as endpoints are required to fully determine the CV health implications of a diet rich in flavanones (Figure 7.2). These studies could be similar to a recently published large-scale human RCT (n = 7447; 4.8 y median follow up) that causally linked Mediterranean diets supplemented with olive oil or nuts with a significantly reduced risk of experiencing a CV event of 30 % or 28 %, respectively, relative to a control diet [284]. However, the conduct of such studies demands immense financial resources and multicentre involvement and, possibly therefore, no such data are currently available for flavonoid subclasses including flavanones.

Figure 7.2 – Overview of future research required to further our understanding regarding the potential CV health implications of a diet rich in flavanones

CV health implications of a flavanone rich diet

→ Large scale RCTs with CHD & stroke endpoints

Estimate CV health implications & understanding which CVD risk markers are modulated by a flavanone rich diet

→ Meta-analyses of RCT data: BP, blood lipids, FMD, CRP, insulin resistance
→ RCTs with further established markers of CVD risk: e.g. arterial stiffness, cardiac BRS, platelet function, microvascular /digital endothelial function, CIMT, inflammatory and metabolic biomarkers, microparticles, endothelial progenitor cells

Understanding the importance of flavanone absorption and metabolism

→ Pharmacokinetic study (ideally using isotopically labelled flavanones):
• Identify all flavanone phase II metabolites and phenolic acid metabolites
• Establish absorption and clearance kinetics for each metabolite over ≥24 h
• Examine potential accumulation over long-term intake
→ Acute RCTs associating CV bioactivity with plasma concentrations of absorbed metabolites

Understanding exposure of tissue to flavanones
→ Metabolite concentrations in various tissues
→ Crossing of blood brain barrier
   (often difficult to examine in humans *in vivo*)

Influencing factors

Study population:
• Gender
• Baseline CVD risk
• Genotype/Phenotype
• Gut micro flora

Flavanone intake:
• Type of flavanone
• Food matrix
• Daily dose
• Duration

Understanding underlying cellular mechanisms of action

→ Human RCTs with *in vivo* biomarkers of:
• Vascular homeostasis: NO metabolites, NADPH oxidase, ET-1, PGI_2, RAAS system, H_2S
→ Animal studies:
• Invasive *in vivo* assessments: e.g. liver lipid metabolism

Understanding underlying subcellular mechanisms of action

→ Modulation of gene expression
→ Modulation of enzyme activity
   (often difficult to examine in humans *in vivo*)

Hypothesis proposed by large body of epidemiological evidence: flavanone/ citrus fruit intake is inversely associated with CHD and stroke risk

Abbreviations: CV, cardiovascular; RCT, randomised controlled trial; CHD, coronary heart disease; BP, blood pressure; FMD, flow mediated dilatation; CRP, C-reactive peptide; BRS, baroreflex sensitivity; CIMT, carotid intima media thickness; NO, nitric oxide; NADPH, nicotinamide adenine dinucleotide phosphate; ET-1, endothelin-1; RAAS, renin, angiotensin and aldosterone system

For those CVD risk markers that previous human RCTs suggested a beneficial change in response to flavanone intake (i.e. blood lipids (Chapter 2), DBP [59] and endothelial function [59, 60]), the potential underlying cellular mechanisms need to be examined (Figure 7.2). Modulations of hepatic lipid β-oxidation and fatty acid metabolism have been proposed by animal studies [165, 186] as the key cellular mechanisms underlying potential flavanone-induced blood lipid lowering effects. However, examining these proposed mechanisms in humans *in vivo* would possibly require unethical invasive methods and has, for example, not been addressed in any of the 11 RCTs of the isoflavone meta-analysis with blood lipid endpoints [274]. In contrast, a range of human *in vivo* biomarkers are available to examine possible mechanisms by which flavanones may induce DBP lowering and endothelial function improving effects. NO and superoxide-generating NADPH oxidase are reported as the two key factors in the regulation of vascular homeostasis [17, 132]. However, the two existing RCTs to date (i.e. the present acute RCT (Chapter 4) and the RCT of Morand et al., [59]) suggested that flavanones do not increase NO production (as assessed by plasma nitrite and total NO metabolite concentrations, respectively) and the present acute RCT also found no decrease in NADPH oxidase gene expression (as assessed by serum concentration of gp^{91phox}). Further human RCTs are thus required that confirm these findings; ideally, in study populations with low baseline NO production (e.g. smokers [285]) and assessing several *in vivo* biomarkers of vascular NO production and NADPH oxidase. For example, in addition to plasma nitrite concentrations and NADPH oxidase gene expression that were assessed (Chapter 4), urinary excretion of NO metabolites and plasma concentration of nitroso species have also been proven sensitive biomarkers to detect flavonoid-induced increases in NO production in previous RCTs [191], as has NAPDH oxidase activity in neutrophils to detect flavonoid-induced inhibition of NADPH oxidase [232]. Furthermore, other mechanisms, such as inhibitory effects on endothelin-1 [191] or the renin-angiotensin-aldosterone system [286], modulation of eicosanoid synthesis (e.g. an increase in prostacyclin) [287] or an increase in hydrogen sulfides [134] could explain potential DBP lowering and endothelial function improving effects of flavanones and require further investigation through human RCTs.

A more complete understanding of the absorption, distribution, metabolism and excretion (ADME) of dietary flavanones is also required to fully determine the potential CV health implications of a diet rich in flavanones (Figure 7.2). At present, the metabolic fate of 49 % of ingested flavanones is unknown as only 51 % have been recovered in urine as flavanone phase II metabolites and phenolic acid metabolites (i.e. 14.3 % [78] and 37 % [113], respectively). Furthermore, whilst the absorption and clearance kinetics of flavanone phase II metabolites have been well established and although 15 phenolic acid metabolites were detected in plasma collected 5 h after flavanone consumption (Chapter

5), the absorption and clearance kinetics of these phenolic acid metabolites remain to be established. Data presented in this thesis also showed that the plasma concentration of flavanone phase II metabolites was significantly elevated 5 h after intake of flavanones in orange juice, but only a negligible plasma concentration was detected when the same dose of flavanones was given in a supplement (Chapter 3). However, the underlying reason for this discrepancy remains unknown and potential effects of food matrix on ADME of flavanones require further examination. To address these research gaps, well-controlled pharmacokinetic studies are warranted; that use comprehensive methods to detect a large number of flavanone phase II metabolites and phenolic acid metabolites; that quantify metabolite concentrations in plasma, urine and faecal samples over an adequate duration post flavanone consumption (i.e. at least 24 h); and that examine impact of food matrix on bioavailability of flavanones, in particular, when flavanones are ingested as a purified supplement. It is also critical that a well-controlled study uses isotopically labelled flavanones to verify with greater certainty that detected metabolites originate from flavanone intake and to determine the metabolic pathway of each metabolite; however, synthesis of these labelled compounds is labour- and costs-intensive [106]. Human studies are also limited in terms of knowing whether metabolites detected in plasma are also distributed into body tissue such as endothelial cells, blood cells/platelets or organs where they might potentially mediate beneficial CV effects. Although animal studies detected flavanones in tissues and organs [114] and suggested they can cross the blood brain barrier [288], these findings are difficult to be replicated in humans *in vivo* because they require the use of potentially C14 labelled flavanones which are expensive and challenging to synthesise. Given the bioavailability of phenolic acid metabolites over 24 h [113], short- and long-term placebo-controlled human studies with a standardised low phenolic and flavonoid diet are also warranted to examine whether some of the metabolites may accumulate in the body. Ultimately, a complete understanding of which forms, to what extent and over which duration body tissues are exposed to metabolites originating from acute or regular intake of flavanones will be critical for the design and interpretation of human flavanone intervention RCTs with CVD risk marker endpoints [106].

Morand et al., [59] reported a significant association (using Pearson's correlation) between plasma concentrations of hesperetin and improvements in microvascular endothelial function assessed 6 h after intake of flavanones, and the present data indicate exploratory associations between certain flavanone phase II metabolites or phenolic acid metabolites and changes in specific markers of CVD risk assessed 5 h after intake of flavanones (Chapter 5; Appendix 5). Although these data suggest that flavanone and phenolic acid metabolites may, at least in part, be mediators of beneficial CV effects; the overall picture is far from understood and data from the two existing acute RCTs comprise

some obvious limitations. Whilst Morand et al., [59] enzymatically removed glucuronides or sulfates from hesperetin metabolites before analysis and did not quantify phenolic acid metabolites, the present acute RCT found no overall effect in assessed markers of CVD risk relative to the control intervention and, therefore, the significant correlations were only of exploratory nature proposing hypothesis that require confirmation by future human RCTs (Chapter 5). Furthermore, both acute RCTs assessed plasma concentrations of flavanone metabolites and markers of CV assessments at a single timepoint (i.e. 6h and 5h post flavanone consumption); however, flavanone-derived phenolic acid metabolites have been detected in urine up to 24h [113] suggesting that potential effects might also occur at later timepoints. Thus, acute RCTs are warranted that examine a time-course effect of acute flavanone intake (e.g. up to 24h) on markers of CVD risk and include a comprehensive quantification of flavanone phase II and phenolic acid metabolites to enable analysis of potential associations through simple correlations and/or multivariate regression analyses. These data may contribute critical information to improve our understanding of which metabolites are the key mediators of beneficial CV effects and at which timepoints or over which duration these effects may occur.

Further studies using preclinical models are required to complement data from human RCTs. These studies should treat cultured endothelial cells *in vitro* or platelets *ex vivo* with physiologically relevant concentrations of flavanone phase II metabolites and/or phenolic acid metabolites and explore bioactivity of metabolites on a cellular to subcellular level to determine which metabolites may elicit beneficial CV effects and which signalling pathways might be modulated (via changes in gene expression or post-translational modifications of enzymes and signalling molecules).

In conclusion, on the basis of the large body of epidemiological evidence suggesting that intake of flavanones is associated with improved heart health, the work conducted in this thesis contributed to the current literature by systematically reviewing existing human RCTs with a flavanone intervention and CVD risk marker endpoints and by conducting an acute RCT. Although this acute RCT established that 5 h after consumption of orange juice a number of flavanone / phenolic metabolites (including some previously identified as well as novel metabolites) were present in the circulation, assessed markers of CVD risk were not beneficially modulated in men at a mild to moderate risk of CVD. Thus, from this work it is clear that a number of further human RCTs are warranted to fully establish the health implications of a diet rich in flavanones and to comprehensively understand the CVD risk factors that are modulated, the cellular mechanisms underlying these modulations and the responsible key mediating circulating metabolites.

Appendix 1. Analysis of flavanones and vitamin C in orange juice and flavanone extract

Appendix 2.1 Materials

Orange juice from concentrate was provided by the Florida Department of Citrus (FDOC; Lake Alfred, Florida) and further 5 orange juices were purchased from 5 local supermarkets (i.e. Waitrose, Brakes, Aldi, Tesco and Cooperative). Flavanone extract was provided by Monteloeder (Alicante, Spain). HPLC grade methanol and acetonitrile were purchased from Fisher Scientific (Loughborough, UK), while hesperidin, narirutin, quercetin-3-rutinoside (rutin), taxifolin, L-ascorbic acid, hydrochloric acid and formic acid were purchased from Sigma-Aldrich (Poole, Dorset, UK).

Appendix 2.2 Method

In triplicate, 400 µL aliquots of orange juice were spiked with taxifolin (extraction efficiency standard; 50 µM end concentration) and subsequently extracted. Each aliquot was centrifuged (2500 rpm, 5 min, room temperature) to separate the soluble flavanones found in the supernatant from the insoluble flavanones found in the pellet. Whilst the supernatant was diluted in 400 µL acidified methanol (0.1 % hydrochloric acid), syringe filtered and spiked with rutin (calibration standard; 1 µM end concentration), flavanones from the pellet were extracted twice through reconstitution with 700 µL acidified methanol, sonication at 55C for 10 min and centrifugation (5000 rpm, 5 min, room temperature). Both pellet extracts were combined, syringe filtered and spiked with rutin (calibration standard; 1 µM end concentration). In triplicate, flavanone extract was reconstituted in methanol/MilliQ/formic acid (5:94.5:0.5 v/v/v) and ready for analysis.

Samples were analysed on an Agilent 1200 series high performance liquid chromatography (HPLC) with a HPLC pump, a 250 to 600 nm diode array detector and an autosampler cooled to 4 °C. Separation was achieved on an Eclipse XDB-C18 (5 µM, 150 x 4.6 mm) column kept at 37 °C, a flow rate of 1 ml/min and a sample injection volume of 5µl. Whilst mobile phase A consisted of MilliQ/FA (99.5:0.5 v/v) and mobile phase B of methanol/FA (99.5:0.5 v/v), the course of the gradient indicated by mobile phase B was 5 % B at 0 min, 35 % B at 6 min, 38 % B at 10 min, 100 % B at 11 min, 100 % B at 13 min, 5 % B at 14 min and 5 % B at 16 min. The compounds of interest (i.e. hesperidin, narirutin and vitamin C) and calibration and extraction efficiency standards (i.e. rutin and taxifolin, respectively) were quantified in the samples based on a six point standard curve. Every 10 injections, a sample consisting of diluted orange juice spiked with the compounds of interest was run as a quality control.

In the orange juice and flavanone extract used for the study, potential flavanone and vitamin C degradation was verified through re-analysis after study completion. Statistical

differences were determined using pairwise two tailed T-test and considered significant at a *P*-value of less than 0.05.

Appendix 2.3　　Results

Based on chromatography of analytical standards, the retention times (RT) and absorbance spectra (λ) for each compound have been identified. Whilst hesperidin, narirutin, taxifolin had a common λ_{max} (i.e. 284 nm) and a RT of 10.2 min, 9.1 min and 7.6 min, respectively, vitamin C had a λ_{max} at 250 nm and RT of 1.5 min and rutin a λ_{max} at 350 nm and RT of 9.2 min (Appendix 4, Figure 1). The method had extraction efficiencies for the orange juice samples ranging from 97.3 to 109.1 % (Appendix 4 Table 1) and a mean intra-day variability of 3.4 % based on the calibration standard and the quality control samples.

The 6 analysed orange juices from concentrate had a hesperidin content ranging from 41.8 to 51.8 mg/100ml, a narirutin content ranging from 6.3 to 8.7 mg/100ml and a vitamin C content ranging from 39.5 to 57.6 mg/100ml (Appendix 4, Table 1). The soluble fraction was higher for narirutin (range: 55.8-70.7 %) compared to hesperidin (range 13.3-23.2 %). The flavanone extract consisted of 76.5 % hesperidin and 3.7 % narirutin, whereas no vitamin C was detected.

Appendix 2 Table 1 – Flavanone and vitamin C content in orange juices from concentrate

	Narirutin		Hesperidin		vitamin C		EE [1]
	mg/100ml	% soluble[2]	mg/100ml	% soluble[2]	mg/100ml	% soluble[2]	%
FDOC (pre-study)	6.3	55.8 %	41.8	23.2 %	57.3	67.2 %	107.7
FDOC (post-study)	6.6	60.7 %*	45.74*	23.9 %	46.7**	65.9 %	n/a
Waitrose	7.4	64.0 %	42.3	13.3 %	39.5	42.4 %	109.1
Aldi	7.4	67.2 %	46.6	17.2 %	57.5	29.5 %	98.1
Tesco	6.4	70.7 %	42.2	18.6 %	48.0	33.8 %	97.3
Cooperative	8.7	69.3 %	51.8	16.3 %	50.1	34.0 %	99.7
Brakes	8.6	69.2 %	51.1	15.4 %	50.6	32.7 %	103.6

[1] based on recovery of 50uM spiked taxifolin; [2] % of flavanones detected in the supernatant extract compared to the total flavanones detected in supernatant and pellet extracts; Abbreviations: EE, extraction efficiency; FDOC, Florida Department of Citrus; n/a, extraction efficiency was not established post-study; Statistical analysis: differences between pre- and post-study flavanone and vitamin C contents and solubility were determined using pairwise t-test (two-tailed) whereby significant differences are indicated with * for $P<0.05$, ** for $P<0.01$.

Based on the post-study analysis of the FDOC orange juice and the flavanone extract, there was no flavanone degradation with even a marginal increase in hesperidin content and in narirutin solubility, which may be due to variability between the different tins of orange juice concentrate (Appendix 4, Table 1). On the other hand, vitamin C levels degraded in the FDOC orange juice.

Appendix 2 Figure 1 – Chromatograms of orange juice and flavanone extract analysis
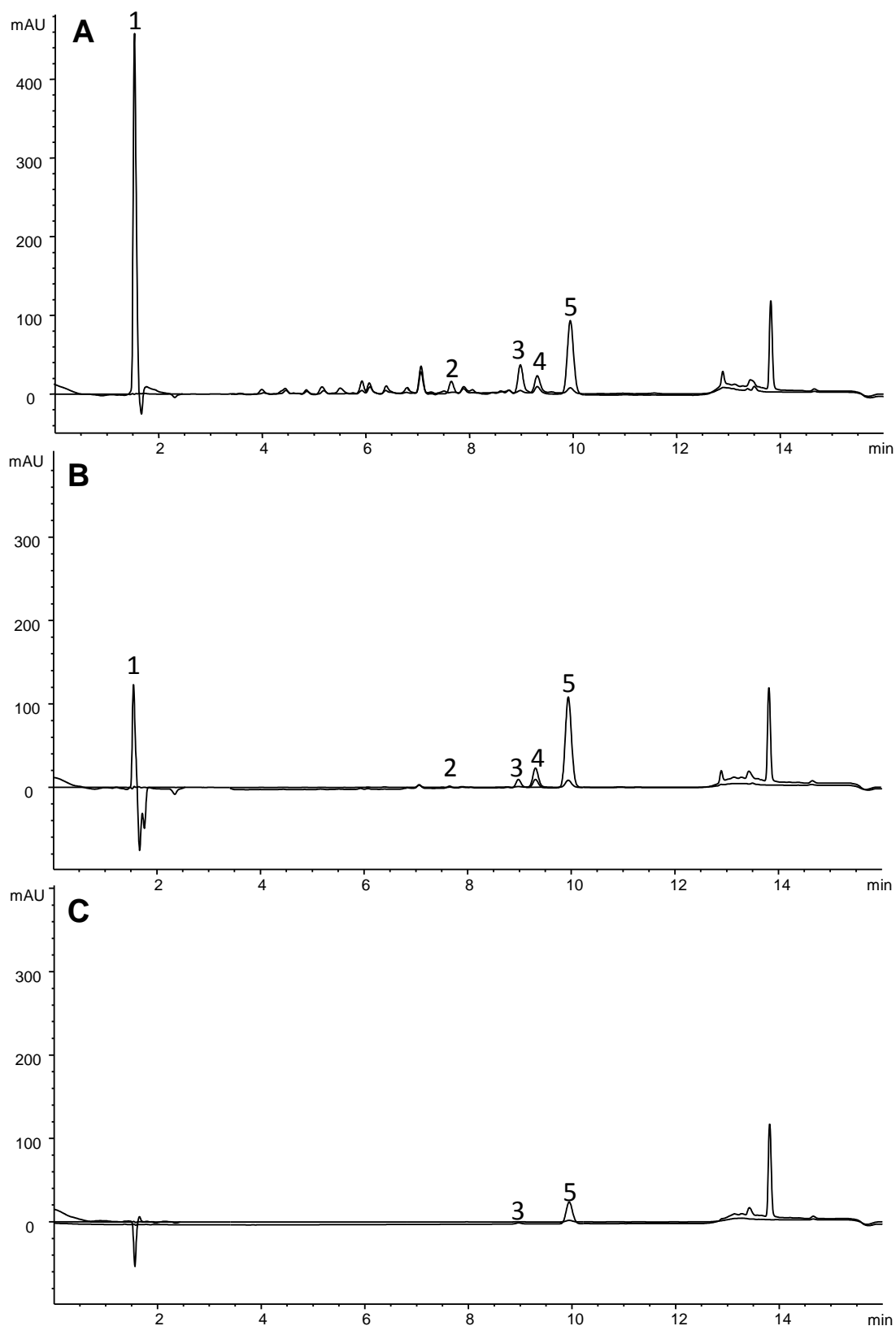
Chromatogramms of A) florida department of citrus (FDOC) supernatant extract, B) FDOC pellet extract and C) Monteloeder flavanone extract. Compounds were identified based on retention time (RT) and absorbance spectrum (λ): vitamin C (RT 1.6 min; λ_{max} 250 nm)[1], taxifolin (RT 7.7 min; λ_{max} 284 nm)[2], narirutin (RT 9.1 min; λ_{max} 284 nm)[3], rutin (RT 9.2 min; λ_{max} 350)[4] and hesperidin (RT 10.2 min; λ_{max} 284 nm)[5]

Appendix 2. Food restrictions prior to experimental period

Table 1: Food restriction for 3 days prior to each assessment visit

Foods to avoid	Restrict		Alternatives (*see table 2, for foods to be avoided 24hr before assessment)
Fruit: including fruit juices and products (e.g. desserts, jams, yoghurts) containing:			
Citrus fruit – for example: oranges and tangerine / satsuma, grapefruit, lemon, lime and mixed tropical fruit items *Berry fruit* – for example blackberry, blueberry, strawberry, raspberry, cranberry, blackcurrant, redcurrant *Other fruit* - Black / red grapes, raisins, plum, prune, cherry, pomegranate	Apple Nectarine Nuts (e.g. almond, hazelnut, peanut, macadamia nut, walnut, cashew nut, chestnut, pecan nut)	1 per 3 days 2 per 3 days 2 handful per 3 days	Mango, banana, melon, pineapple, kiwi, pear, peach apricot, green grapes, fig, date gooseberry, avocado
Vegetables, salad and products (e.g. quiche, pizza, casserole) containing:			
Broad bean, red onion, aubergine, red cabbage, red skinned potatoes, purple carrot	Black bean and black olives Kidney beans Garlic cloves	2 handful per 3 days 1 handful per 3 days 4 per 3 days	Onion (excluding red), shallot, green bean, tomato, broccoli*, carrot*, cauliflower*, celery*, cucumber*, peas, leek, green/white cabbage*, potato (excluding red skinned)*, pumpkin, radish*, parsnip, pepper, beetroot, lentil, sweetcorn, lettuce*, brussel sprouts*, green olive
Other foods: including products (e.g. drinks, desserts, vegetarian meat alternatives) containing the following:			
Dark chocolate, baking chocolate and cocoa products Soy, soy milk and soy containing products (e.g. quorn, tofu) Cider, red wine	White or milk chocolate Oily fish (e.g. tuna, mackerel, kippers, salmon, sardines, herring)	2 chunks per 3 days 1 portion per 3 days	Sugar based and/or dairy desserts and snacks, egg based desserts (e.g. plain biscuits, cereal bar, ice cream, custard) Milk and milk containing products (e.g. non berry fruit yoghurt, cheese) Water, carbonated drinks (e.g. coca-cola, sprite), beer, white wine, spirits

Table 2: Further food restriction 24 hrs prior to each assessment visit

Foods to avoid for 24hr (*In addition to Table 1)	Alternatives
Vegetables, salad and products (e.g. quiche, pizza, casserole) containing:	
Beetroot, broccoli, cabbage, carrot, cauliflower, celery, cucumber, lettuce, brussel sprouts, parsley, potato, radish, spinach	Onion (excluding red), shallot, green bean, tomato, peas, leek, green/white cabbage, pumpkin, parsnip, pepper, lentil, sweetcorn, green olive
Other foods: including products (e.g. roll, sandwich) containing the following:	
Drinks containing caffeine e.g. tea, coffee, hot chocolate, coke, energy drinks (such as RedBull) Drinks containing berry fruit and citrus fruit (including cordials) Bottled water (excluding Buxton mineral water) Drinks / foods containing alcohol Cured and canned meat (e.g. bacon, ham, sausages, corned beef) Smoked fish	Milk (excluding soya milk), Buxton water (provided by the researchers for the 24hr before you attend an assessment visit) Fresh meat (e.g. chicken, turkey, beef, pork, lamb) Fresh fish (excluding oily fish)

Appendix 3. Analysis of baroreflex sensitivity using finger arterial pressure data

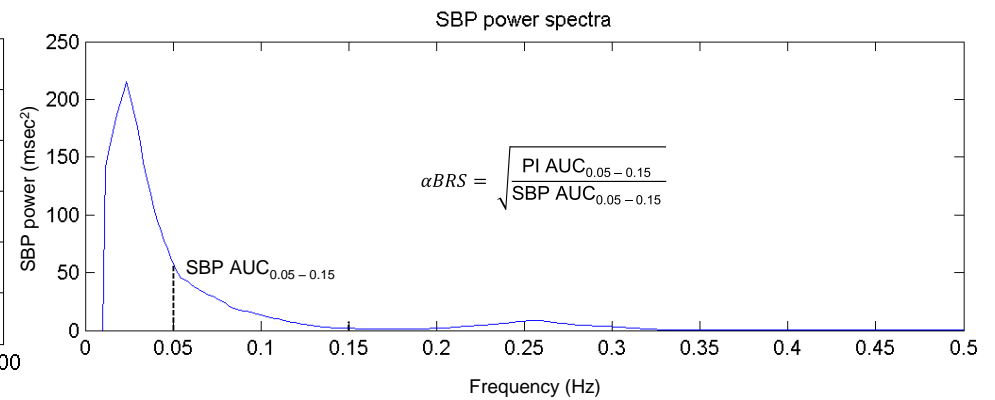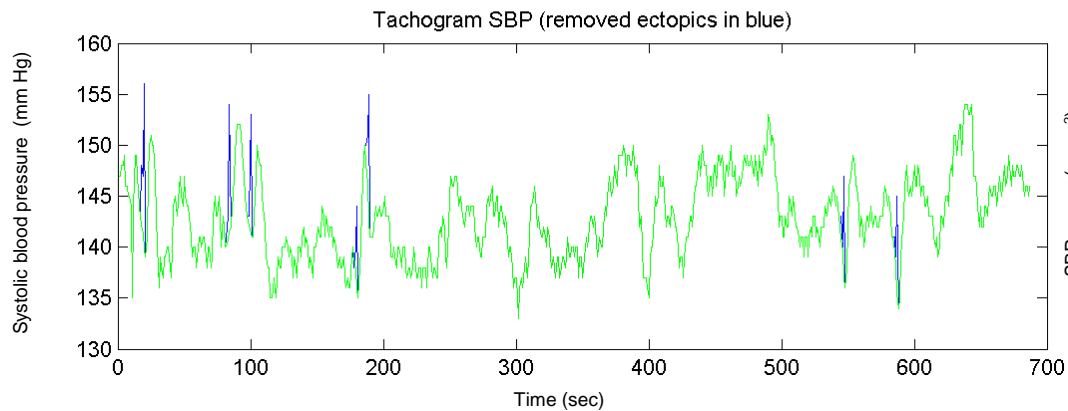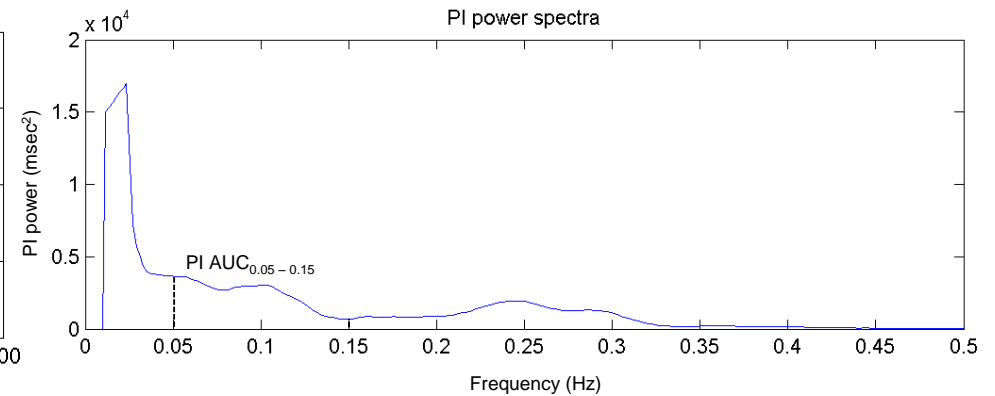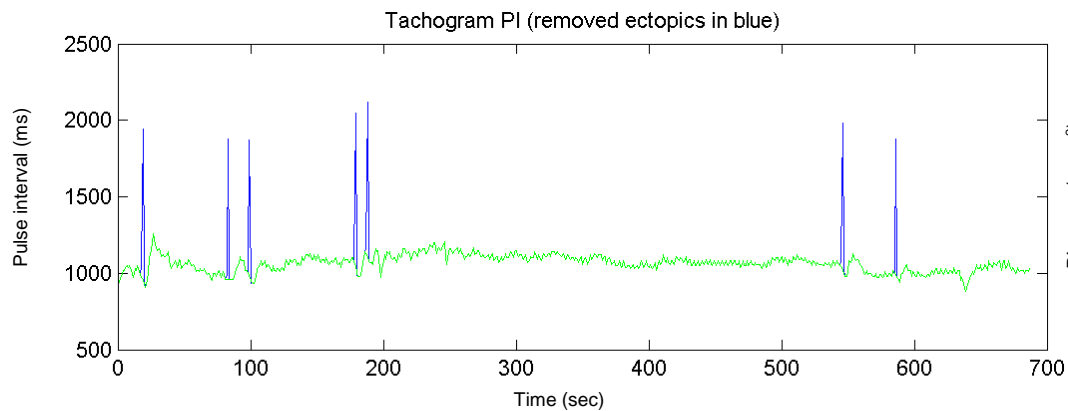**Appendix 4 Figure 1 –** Raw signal of finger arterial pressure
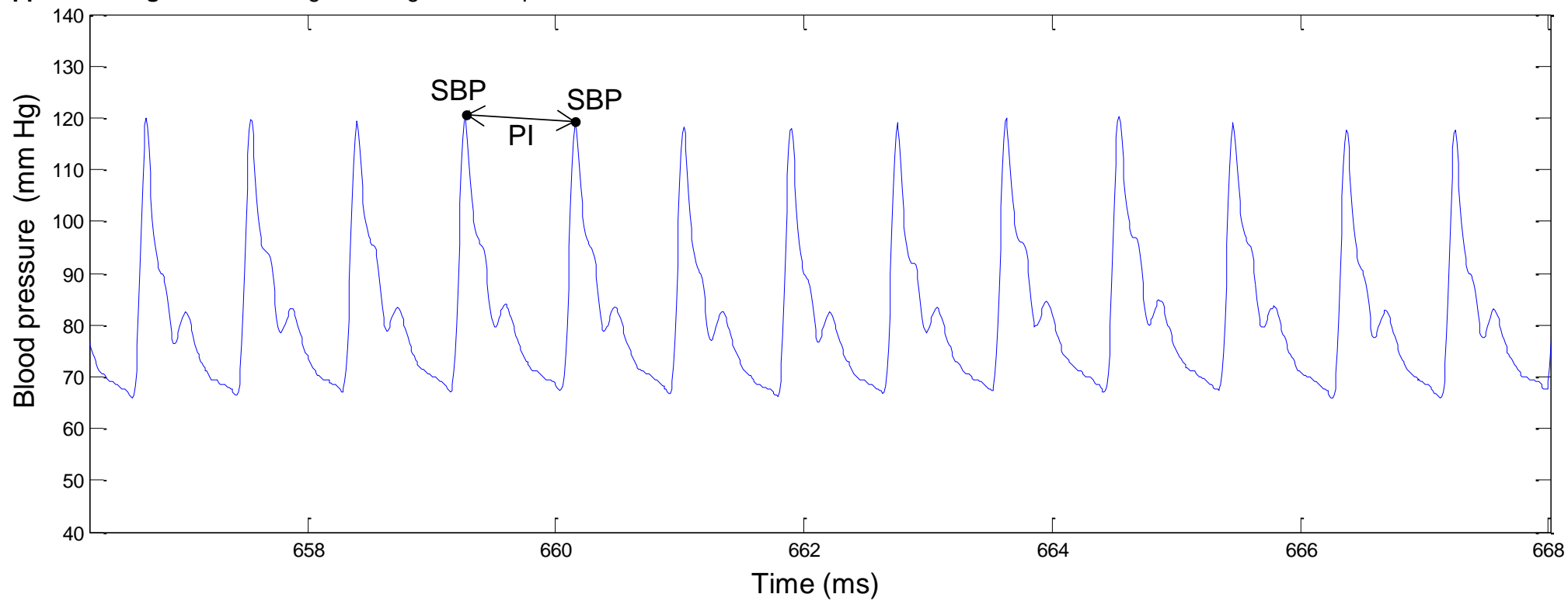
SBP; systolic blood pressure; PI, pulse interval

Appendix 4 Figure 2 – Tachogram and power spectra of pulse interval and systolic blood pressure signals[1]
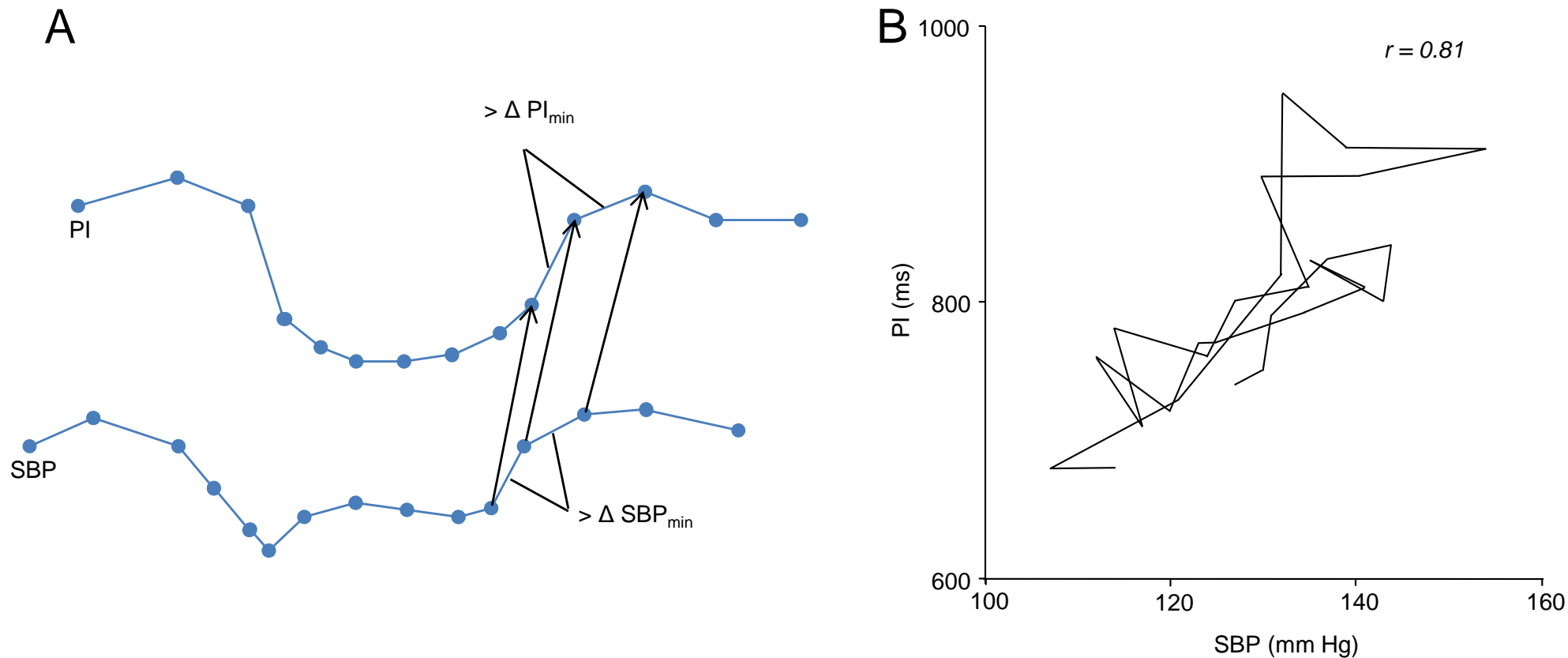

Abbreviations: PI, pulse interval; SBP, systolic blood pressure; $AUC_{0.05-0.015}$, area under the curve (i.e. power) in the low frequency band; αBRS, low frequency α-index indicating baroreflex sensitivity

Appendix 4 Figure 3 – Analysis of cross correlation baroreflex sensitivity

A: Sequence of signals where an increase in systolic blood pressure is followed by a baroreflex induced increase in pulse interval. (Figure adapted from [215])
B: Cross-correlation baroreflex sensitivity (xBRS) is calculated as the regression slope between SBP and PI over a 10 seconds sliding windows and with a PI delay of 0 to 5 seconds. Using the delay with the highest cross-correlation coefficient, the regression slope is accepted as a xBRS estimate if the correlation is significant at a *P*- value < 0.01. (Figure adapted from [289])
Abbreviations: PI, pulse interval, SBP, systolic blood pressure

Appendix 4. Quantified phenolic acids that were not flavanone metabolites

| Flavanone metabolite | mean ± SEM (nM) | | | P-value[1] |
| | Baseline | 5 h – baseline | | |
		Control	Orange juice	
Dihydroxy-benzoic acid	40 ± 7	4 ± 3	-1 ± 7	0.58
4-hydroxybenyaldehyde	113 ± 8	39 ± 10	45 ± 11	0.62
Salicyclic acid	876 ± 141	-586 ± 442	-229 ± 134	0.74
Methoxybenzoic acid	ND	ND	36 ± 0	0.34
Protocatechuic acid-4glucuronide	2 ± 0	17 ± 4	18 ± 2	0.80
Protocatechuic acid-glucuronide	7 ± 1	-1 ± 0	-2 ± 1	0.56
Methoxybenzoic acid-sulfate	ND	8 ± 8	23 ± 13	0.62
pCourmaric acid-glucuronide	28 ± 10	1 ± 2	-7 ± 2	<0.05
Hydroxyhippuric acid-glucuronide	2 ± 0	17 ± 4	18 ± 2	0.80

[1]5 h post intervention differences between the two dietary interventions were established using a linear mixed model for crossover studies with baseline values on each experimental period as the covariate. The statistical analysis was performed in R 3.0.1 (R Foundation for Statistical Computing) and a change was considered significant when $P < 0.05$.

Appendix 5. Significant Pearson's correlations between markers of CVD risk and circulating flavanone metabolites of the acute orange juice intervention

Marker of CVD risk (5h – BL)	flavanone metabolite (5h – BL)	r [a]	P
Heart rate	isovanillic acid-glucuronide	0.84	0.0003
Systolic BP	total phenolic acid metabolites	-0.62[b]	0.03
Pulse wave velocity	4-hydroxyphenylacetic acid	-0.66[b]	0.02
Cardiac baroreflex sensitivity	vanillic acid-glucuronide	0.60[b]	0.03
Plasma nitrite	hydroxyhippuric acid	0.63[b]	0.02
Serum soluble gp[91phox]	hesperetin-diglucuronide	-0.73[b]	0.005
Serum soluble gp[91phox]	isoferulic or ferulic acid-glucuronide	0.63	0.02

[a] r, Pearson's correlation coefficient (n = 13); [b] increased presence of flavanone metabolite has favourable effect on marker of CVD risk.

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