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The role of dietary intake of flavonoids and anthocyanins on vascular function, inflammation and other cardiovascular disease risk factors: From epidemiological to experimental evidence

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The role of dietary intake of flavonoids and anthocyanins on vascular function, inflammation and other cardiovascular disease risk factors: From epidemiological to experimental evidence

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ABSTRACT

Vascular health plays a major role in several human disorders, particularly in cardiovascular diseases (CVD) (1,2). The endothelium can be considered an organ that regulates vascular homeostasis by maintaining an appropriate vascular tone, platelet activity, leukocyte adhesion and angiogenesis. When one or more of these components is compromised due to impaired endothelial vascular signalling, endothelial dysfunction (ED) may occur (1). One of the main key metabolic manifestations of ED is reduced nitric oxide (NO) bioavailability. NO is an important signalling molecule that regulates various vascular processes, including through its main role as a vasodilator, acting through relaxation of smooth muscle cells (2,3). Impaired bioavailability of NO and up-regulation of various molecules involved in vascular function are partly explained by the cooperative and synergistic action of inflammation and oxidative stress on ED (4–6). This is particularly evident in older adults, as several studies support a new immune-metabolic viewpoint for age-related diseases, termed "inflammaging" which is characterized by a chronic low-grade inflammation (7). Thus, interventions aiming to regulate the immune response and to prevent the accumulation of deleterious reactive species, have been considered to be a relevant therapeutic target to improve vascular health (8).

Nutrition plays a major role in regulating the inflammatory state and enhancing endogenous antioxidant defences (9). Many dietary patterns have been associated with the prevention of diseases associated with inflammation in epidemiological studies (10), while at the same time a large range of dietary interventions have been explored in clinical practice to treat pathological conditions such as ED (11). Flavonoids, a class of dietary polyphenols, are bioactive compounds that have potential to both prevent and treat conditions related to a pro-inflammatory and oxidative stress state (12,13). Meta-analysis of controlled trials and cohort

studies demonstrate a protective effect of flavonoid intake on CVD (14–18), particularly on hypertension (19,20). Anthocyanins, a subclass of flavonoids, are emerging as a potential therapeutic option for CVD risk factors (21). Anthocyanins are the largest class of water-soluble plant pigments, that are responsible for the blue, purple and red colour of many fruits and vegetables, such as blueberries, blackberries, red grapes, plums and eggplants (22). The beneficial effects of anthocyanins on CVD risk factors are related to their antioxidant and immunomodulatory effects, thereby attenuating the synergistic deleterious effects of oxidative stress and inflammation in CVD (21,23). In humans, anthocyanin intake has been shown to be associated with a lower risk of cardiovascular events (24,25). Intervention studies using anthocyanins have demonstrated improvements in vascular function (26) and biomarkers related to oxidative stress (27–30), as well as antioxidant status (28,30–32), lipid profile (33–35) and inflammatory response (36,37) in both long-term and acute designs.

Despite these promising findings, there are still several gaps in the literature regarding the potential health benefits of flavonoids and anthocyanins. At the epidemiological level, a few populations have been investigated in appropriate large-scale studies. Epidemiological studies require a specific approach to quantify flavonoid content in foods due to significant variation between crops grown in different geographic areas, as well as consideration of the variety of diet patterns, both within and between countries. At the clinical level, a number of studies have investigated the potential effect of anthocyanins in attenuating and preventing pathological conditions. The immediate effect of anthocyanins in the postprandial state has been evaluated in several studies using a high-fat high-energy meal challenge. Acute feeding studies allow investigation of the ability of these bioactive compounds to attenuate the deleterious effects following a stressor meal; however, the findings have not been adequately collated to facilitate translation into dietary guidance. Such studies have not included a robust investigation of sensitive measures of vascular function in the postprandial state, which along

with the immune response are important predictors of CVD. Additionally, there are still several gaps to be addressed regarding the effect of anthocyanins on vascular health and inflammation in older adults, especially among those with neurodegenerative conditions, such as mild cognitive impairment (MCI), which share risk factors with CVD.

This thesis addresses the following research questions:

1. Is dietary intake of flavonoids, including anthocyanins, associated with the incidence of hypertension in Australian women?
2. What is the current level of evidence on the postprandial effects of anthocyanins on CVD risk factors in high-fat high energy (HFHE) meal challenge studies?
3. What are the post-prandial effects of anthocyanins on micro- and vascular function, and other CVD risk factors in overweight older adults following a HFHE challenge?
4. What are the chronic effects of anthocyanins on microvascular function, inflammatory biomarkers and 24 h ambulatory blood pressure in older adults with diagnosis of MCI?

Four studies were conducted to address these research questions. At the epidemiological level, nationally representative cohort study data was used to estimate the total dietary intake of flavonoids and their subclasses in Australian women. This provided novel data that demonstrated an association between flavonoids subclasses with a lower risk of hypertension in Australian women. This contributes to the body of evidence that informs nutrition messaging and policies for improved cardiovascular health in this population.

At the clinical level, knowledge synthesis was conducted using a systematic literature review approach to collate information on the postprandial effects of anthocyanins on CVD risk factors in high-fat meal challenge studies. A total of 13 eligible randomized clinical trials reported beneficial effects of anthocyanins, with most the promising results evident for the

attenuation of deleterious postprandial effects on oxidative stress and antioxidant status, triacylglycerol and total cholesterol concentrations, vascular endothelial function and inflammatory biomarkers. Post-prandial changes in blood pressure and lipoproteins were least affected by acute anthocyanin consumption.

A randomised crossover, placebo controlled clinical trial was subsequently designed to investigate the postprandial effects of anthocyanins following a HFHE meal challenge, but using a novel and robust evaluation of vascular function, namely macro and microvascular parameters. Methodologies combined both classical techniques (flow-mediated dilatation (FMD) with the novel imaging technology Laser Speckle Contrast Imaging (LSCI). To our knowledge, this is the first study to conduct this type of protocol in studies of nutritional interventions. Fruit-based anthocyanins attenuated the postprandial detrimental effects of a HFHE challenge on parameters of vascular and microvascular function, and inflammatory biomarkers in 16 overweight older adults.

A second randomised, placebo controlled clinical trial was conducted to investigate the longer term effects (8 weeks) of two different doses of anthocyanins provided by Queen Garnet plum on inflammatory markers associated with CVD risk factors, along with analysis of microvascular function and 24-hour ABP. The innovative aim of this study was to investigate such parameters in an older population diagnosed with mild cognitive impairment (MCI), a neurodegenerative condition that shares pathological mechanisms with CVD. A daily high dose (201 mg/day) of fruit-based anthocyanins consumed for 8 weeks significantly reduced tumour necrosis alpha (TNF-α), but did not alter interleulin-6 (IL-6), interleukin-1 beta (IL-1β), high-sensitivity C-reactive protein (hs-CRP), microvascular function nor blood pressure parameters. No effects were observed in the low dose (45mg/day) group.

This body of research contributes novel data on the role of flavonoids, and specifically anthocyanins, on vascular function, inflammation and CVD risk across various levels of evidence, including:

- Epidemiological evidence: analytical work conducted in national representative cohort of Australian women.
- Knowledge synthesis: systematic literature review.
- Experimental evidence: Two randomised controlled clinical trials with innovative design that allows interpretation of both acute and chronic effects of anthocyanin intake.

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Finally, I would like to acknowledge my family and friends (old ones back home, and new ones in this Country), that always supported me and encourage me to conduct this journey with a lot of will and dedication.

Certification

I, Vinicius Andre do Rosario declare that this thesis submitted in fulfilment of the requirements for the conferral of the degree Doctor of Philosophy, from the University of Wollongong, is wholly my own work unless otherwise referenced or acknowledged. This document has not been submitted for qualifications at any other academic institution.

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Vinicius Andre do Rosario

Date 15 / 01 / 2020

Dedication

This work is dedicated to my family who have always supported me and made this journey possible.

List of Abbreviations

ABP	Ambulatory blood pressure
ALSWH	Australian Longitudinal Study on Women's Health
ARR	adjusted relative risk
BKCa	Large conductance calcium-activated potassium channels
BMI	Body mass index
CAD	Coronary arterial disease
cGMP	Cyclic guanosine monophosphate
CHD	Coronary heart disease
CRP	c-reactive protein
COX	Cyclooxygenase
CVD	Cardiovascular disease
DPPH	2,2-diphenyl-1-picrylhydrazyl
ED	Endothelial dysfunction
eNOS	Endothelial nitric oxide synthase
FFQ	Food frequency questionnaire
FMD	Flow-mediated dilatation
FRAP	Ferric reducing antioxidant power assay
GEE	Generalised Estimating Equation
HDL-c	High-density lipoprotein cholesterol
HFM	High-fat meal
HFMC	High-fat meal challenge
HFHE	High-fat high energy
HR	Hazard ratio
HRT	hormone replacement therapy

hs-CRP	High-sensitivity c-reactive protein
IL-1β	Interleukin-1 beta
IL-1RA	Interleukin-1 receptor antagonist
IL-6	Interleukin-6
IL-8	Interleukin-8
iNOS	Inducible nitric oxide synthase
LDF	Laser Doppler Flowmetry
LDI	Laser Doppler Imaging
LDL-c	Low-density lipoprotein cholesterol
LSCI	Laser speckle contrast imaging
MDA	Malondialdehyde
MCI	Mild cognitive impairment
MET	total metabolic equivalent
MI	Myocardial infarction
NADP	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa-light-chain-enhancer
NO	Nitric oxide
NOS	Nitric oxide synthase
OCP	oral contraceptive pill
ORAC	Oxygen radical absorbance capacity
OxLDL-c	Oxidized low-density lipoprotein cholesterol
PAD	Peripheral arterial disease
PUFA	Polyunsaturated fatty acid
PWV	Pulse wave velocity
RHI	reactive hyperaemia index

RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RR	Relative risk
TAG	Triacylglycerol
TAS	total antioxidant status
TC	Total cholesterol
TNF-α	Tumour necrosis factor alpha
TRAP	Telomerase repeated amplification protocol
UA	Uric acid
XO	Xanthine oxidase

Publications constituting this Thesis

Peer reviewed publications:

- **do Rosario VA**, Spencer J, Weston-Green K, Charlton K. The Postprandial Effect of Anthocyanins on Cardiovascular Disease Risk Factors: a Systematic Literature Review of High-Fat Meal Challenge Studies. Current Nutrition Reports (2020) (Cardiovascular disease) (https://doi.org/ 10.1007%2Fs13668-020-00328-y)
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- **do Rosario VA**, Chang C, Spencer J, et al. Anthocyanins attenuate vascular and inflammatory responses to a high fat high energy meal challenge in overweight older adults: a cross-over, randomized, double-blind clinical trial. Clinical Nutrition (2020) (https://doi.org/10.1016/j.clnu.2020.09.041)
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Conference abstracts

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CHAPTER 1: Introduction

1.1 Vascular function and cardiovascular diseases

The health of the vascular system plays a major role in several human disorders, particularly in CVD (1,2). CVD is a major cause of mortality globally and is the leading cause of death for Australian men and women, responsible for over 43,500 deaths (27%) in the year 2017 (38). Following a pattern that is also similar worldwide, about 1 in 3 Australians aged 18 years and over (34%) have high blood pressure, comprised of 23% adults with uncontrolled high blood pressure and 11% whose blood pressure was controlled using medication (39).

A common characteristic among CVDs is the alteration of vascular function and/or structure. The endothelium, which is comprised of cells that line the internal surface of the lumen of blood vessels, is an organ that regulates vascular homeostasis by maintaining an appropriate vascular tone, platelet activity, leukocyte adhesion and angiogenesis through the controlled release of mediators such as NO and other regulatory factors (3,40). When one or more of these components becomes dysregulated due to impaired endothelial vascular signalling, a pathological state named endothelial dysfunction (ED) occurs. In the case of ED, there is attenuated vasodilation, augmented vasoconstriction, and remodelling of the vessel structure that occurs simultaneously in multiple vascular beds (3,41).

Determining the aetiology of ED is complex issue, as this condition shares causes and outcomes with several other diseases. For example, classical CVD risk factors such as smoking, high blood pressure, obesity, dyslipidaemia and glucose intolerance are associated with the occurrence of ED (1,40). Furthermore, because the degeneration of vascular beds occurs over time, ageing is an independent risk factor for ED (42). After ED manifests, it worsens the clinical status in people who have pre-existing diseases such as diabetes, peripheral vascular diseases, stroke and other vascular events, hypertension and atherosclerosis (1,2). In CVD for

instance, ED is involved in its pathogenesis by increasing its coexistent risk factors, correlating with disease progression and predicting cardiovascular events (43–47).

One of the main key metabolic manifestations of ED is the partial functional loss of endothelial-derived vasodilators, such as NO, in which an aberrant accumulation of reactive oxygen species (ROS) is one of the leading causes (3). The bioavailability of NO depends on its proper synthesis, which involves the enzyme nitric oxide synthase (NOS). NOS is a family of enzymes that catalyse a complex reaction involving L-arginine, NADPH, H+ and O_2 into citrullline, H_2O, NADP and NO, besides other cofactors. Endothelial production of nitric oxide synthase (eNOS) is key to the maintenance of vascular health. In a state of exacerbated oxidative stress, several intracellular interactions may occur in the endothelium, resulting in a phenomenon called "eNOS uncoupling". This attenuated and/or altered activity of the enzyme leads to a switch from the generation of NO to the generation of superoxide anions and hydrogen peroxide, thus creating a vicious cycle in regard to the production of ROS and down-regulation of eNOS (5,48). The outcome of this process is an endothelial phenotype consisting of arterial stiffness, altered vasomotion, increased cytokine synthesis, chemokine secretion, leucocyte adherence, LDL oxidation, platelet activation, smooth cell proliferation and migration and up-regulation of adhesion molecules. This dysregulation in eNOS signalling and remodelling of vasculature are the earliest pathological findings in atherosclerosis, a first step to CVD development(49,50).

1.2 Inflammation and oxidative stress: Implications for vascular health and cardiovascular diseases

Despite the development of successful treatments for dyslipidaemia and hypertension, and knowledge of well-established risk factors, CVD still accounts for one third of all deaths

worldwide. The multifactorial background makes it difficult to detect initial pathological events, which are usually subclinical during the early phase of disease (51). Oxidative stress and inflammation are known to contribute to endothelial dysfunction and vascular damage, and their roles in the pathophysiology of hypertension and atherosclerosis are now well accepted (52,53).

Inflammation is a complex series of coordinated events regulated by a diverse number of features of the immune system including cytokines, enzymes, lipid mediators and vasoactive mediators. In non-diseased conditions, inflammatory responses are resolved when pro-inflammatory factors are no longer active, resulting in maintenance of a highly active and well-regulated balanced state. However, in certain conditions, inflammation may not be completely resolved, resulting in persistent and low intensity stimulation that produces continuous inflammatory responses (54,55). This sustained pro-inflammatory state, also called low-grade inflammation, can enhance ED (55). ED is an early marker and one of most predominant risk factors in the development of atherosclerosis and can be detected before any structural change affects the vessel walls (3,40,56).

The traditional view is that atherosclerosis represents a consequence of lipid accumulation as a degenerative process associated with ageing, where cholesterol is indisputably recognized as an environmental and genetic driver of the disease. However, this does not explain the entire pathophysiological process. Robust evidence shows that inflammation participates centrally in all stages of the disease, from initial lesions to end-stage thrombotic complications, and current research is addressing questions about which inflammatory mechanisms are involved, as well as identification of therapeutic interventions (53,57,58). As the endothelium progresses to a dysfunctional state, anti-thrombotic properties and endothelial permeability are impaired, along with upregulation of pro-inflammatory cytokines and expression of adhesion molecules, thereby facilitating leukocyte adhesion to the

endothelium. Subsequently, leukocytes cross the endothelium and migrate into the intima, mediated by chemo-attractants. Upon reaching the intima, monocytes transform into macrophages and express receptors that facilitate uptake of lipids, leading to the transformation into foam cells, which initiate an atherosclerotic lesion (59,60).

In addition to the more specific vascular inflammatory process described above, which involve several mediators such as vascular adhesion molecules (intercellular adhesion molecule 1 and vascular adhesion molecule 1), proteases (plasminogen activator inhibitor-1 and metalloproteinases), endothelins, chemokines (monocyte chemo-attractant protein-1) and others, the role of peripheral inflammatory biomarkers such as C-reactive protein (CRP), tumour necrosis alpha (TNF-α), interleukin-6 (IL-6) and interleukin-1 beta (IL-1β) have been associated with an increased CVD risk (55,61,62). This is particularly evident in older adults, as several studies support a new immune-metabolic viewpoint for age-related diseases, termed "inflammaging", which is characterized by a chronic, low-grade inflammatory response in the absence of a pathogen (7). Additionally, excessive adiposity across all ages is associated with an up-regulation of a pro-inflammatory state, as the accumulation of adipose tissue mass promotes the secretion and release of inflammatory mediators, including C-reactive protein (CRP), interleukin-6 (IL-6), interleukin 1 beta (IL-1β) and tumour necrosis factor alpha (TNF-α) (63,64). This process also leads to chronic low-grade inflammation that is driven by a nutrient excess and/or overnutrition and has the same mechanisms as those underpinning "inflammaging" (7).

CRP has emerged as a major marker of vascular inflammation, playing a direct role in promoting ED (65,66) and the development of CVD (67,68). CRP is an acute-phase reactant considered to be a reliable biomarker of underlying systemic inflammation due its long half-life and clinically-available bioassay (53). Although CRP levels may increase up to 1000-fold in response to trauma or relevant infections, levels are remarkably stable over long periods of

time when measured in asymptomatic individuals, and a high-normal range concentration of CRP has been identified as an independent predictor of future vascular events (55). Increasing evidence suggests that CRP directly participates in the inflammatory process of atherogenesis, with a high expression in the atherosclerotic plaque and an important role in plaque vulnerability (69). In a study of 302 autopsies of an atherosclerosis phenotype, it was found that the lowest high-sensitivity c-reactive protein (hs-CRP) concentrations were observed in those who had died of non-cardiac causes (70). Stable plaques showed a modest elevation, while erosive plaques showed a greater elevation, and a marked elevation in hs-CRP was seen in ruptured plaques. The highest quartile of hs-CRP concentration was associated with a 1.5 to 1.7-fold increase in relative risk of symptomatic atherosclerosis. In all cases of sudden cardiac death, high concentrations of hs-CRP were identified, regardless of the presence or absence of thrombosis, suggesting that hs-CRP identifies lesions rich in lipids and macrophages, and that it is associated with a risk of a vascular event, even in patients with clinically stable coronary heart disease (70).

CRP levels may be useful for short-term prognosis and long-term risk assessment after a cardiovascular event (69). Therefore, several studies have investigated associations between CRP levels and the risk of a wide range of CVD events. A number of studies have investigated the predictive role of CRP in myocardial infarction (MI).The Honolulu Heart Study found that the odds of MI increased in the first few years of follow-up in participants with high CRP concentrations, and that trend was similar after 20 years of follow-up, showing that inflammation may play both an early and late role in the atherosclerotic process (71). In another study, among 1,086 men followed up for over 8 years, those with the highest quartile of hs-CRP had a 2.9-fold greater relative risk for MI ($p < 0.001$) and 1.9-fold greater relative risk for stroke ($p = 0.02$) compared to men in the lowest quartile, that was independent of traditional cardiovascular risk factors (72). Another study conducted in 82,544 adults [66,796 men and

15,748 women; mean (SD) age 55.1 (9.86) y] without prior cardiovascular diseases or cancer at baseline reported 714 incident MI cases over 6 years of follow-up (61). Higher baseline and cumulative average concentrations of hs-CRP were consistently associated with increased risk of MI ($p<0.001$ for both). A longitudinal increase in hs-CRP was also associated with a higher future risk of MI, after adjustment for baseline values and other covariates ($p<0.001$). Each 1mg/L increment per year in hs-CRP was associated with a 9.3% increase in risk for future MI [hazard ratio (HR) = 1.09, 95% CI, 1.03; 1.17]. Participants with high-grade inflammatory status (hs-CRP ≥10 mg/L) had a higher risk of MI occurring <3 months versus those compared with those that had hs-CRP concentrations <0.5 mg/L (HR = 6.64; 95% CI, 1.49-29.6), and with MI occurring ≥4 years (HR = 2.95; 95% CI, 0.90, 9.65) (61). A meta-analysis investigated the ability of CRP to predict major cardiovascular events in 5401 individuals with peripheral arterial disease (PAD). PAD patients with higher CRP had a significantly higher risk of major cardiovascular events compared with those with lower CRP concentrations (HR 2.26, 95% CI 1.65 to 3.09, $p < 0.001$). The HR for major cardiovascular events was 1.38 (95% CI 1.16 to1.63, $p < 0.001$) per unit increase in CRP concentration (73). Concerning coronary heart disease (CHD), a meta-analysis was conducted presenting a body of evidence of good quality, consistency, and applicability. For studies that adjusted for all Framingham risk variables, the relative risk for incident CHD was 1.58 (95% CI, 1.37 to 1.83) for CRP levels greater than 3.0 mg/L compared with levels less than 1.0 mg/L(74).

TNF-α is a cytokine with a wide range of pro-inflammatory activities and is primarily produced by macrophages, endothelial cells, and smooth muscle cells of atherosclerotic arteries (75). TNF-α may influence the atherosclerotic process both by causing metabolic perturbations and by increasing the expression of surface leukocyte adhesion molecules, chemokines and enhancing the production of other cytokines and growth factors. TNF-α also stimulates new vessel formation and induces features characteristic of developing atheroma. High

concentrations of TNF-α have been associated with premature coronary artery disease, acute MI, peripheral arterial disease, and congestive heart failure (75). Concerning the predictive role of TNF-α in CVD, a case-cohort study comprising 105 coronary artery disease (CAD) cases and 638 individuals randomly selected from a cohort of 5,404 participants aged 35–74 years (mean follow-up of 6.1 years) reported that TNF-α was significantly and independently associated with CAD (adjusted HRs=1.87;1.31–2.66) (75). Furthermore, there is evidence from a large-scale prospective cohort study (2225 participants aged 70-79 years old without baseline CVD) that were assessed for incident coronary heart disease, stroke and congestive heart failure events during an average follow-up of 3.6 years. TNF-α was significantly associated with CHD (per TNF-α SD increase: RR, 1.22; 95% CI, 1.04-1.43) and congestive heart failure (per TNF-α SD increase: RR, 1.59; 95% CI, 1.30 to 1.95) (76).

IL-6 is a pleiotropic cytokine with both anti and pro-inflammatory roles that regulates a plethora of immune and metabolic responses; however, high concentrations of this cytokine have been associated with CVD and mortality (76,77). IL-6 is highly expressed by the vascular endothelium and the pharmacological inhibition of IL-6 improves endothelial function (78) Such findings are clinically meaningful considering the predictive roles of IL-6 concentrations in CVD risk supported by a number of studies. Hazard ratios of 1.80 have been reported according to each 1-SD increase in IL-6 for risk of first-ever cerebrovascular events in individuals with vascular risk factors but without any pre-existing cardiovascular disease (79). Further, in a meta-analysis of 17 prospective studies investigating clinical coronary outcomes (i.e., myocardial infarction or coronary death), an odds ratio of 1.61 (95% CI 1.42–1.83) was found per 2 SD increase in baseline IL-6 (80). Another meta-analysis of 17 studies comprising 288,738 healthy individuals reported significantly higher IL-6 concentration in CVD cases compared to non-CVD controls [standardized mean difference of 0.14, (95% CI) 0.09-0.20]/mean difference of 0.36 [0.28-0.44] pg/mL) (81).

IL-1β also plays a central role in CVD development, representing one of the most potent inducers of innate immunity and acts as an upstream regulator in the inflammatory cascade (82). IL-1β synthesis is significantly upregulated after cardiovascular events such as myocardial infarction, as well as in advanced plaque formations in atherosclerotic disease, thus it has been investigated as a therapeutic option in secondary and tertiary prevention of CVD (83). Intrinsic vascular wall cells and lesional leukocytes alike can produce this cytokine. Local stimuli in the plaque induce the generation of active IL-1β through the action of a molecular assembly known as the inflammasome (84). The convincing links between IL-1β and pro-inflammatory diseases, such as atherosclerosis, indicates this cytokine to be a potential therapeutic target to improve cardiovascular outcomes (84). In this matter, an anti-IL-1β therapy was investigated in a large randomized, double-blind, placebo-controlled trial of including 10,061 patients (median follow-up of 3.7y) with a history of myocardial infarction and CRP concentrations ≥ 2 mg/L. The treatment with canakinumab (anti-IL-1β monoclonal antibody) led to 15% reduction in major adverse cardiovascular events (p=0.007) (85). Furthermore, a meta-analysis including 6 cohort studies with 1,855 CVD cases and 18,745 non-cases with (follow-up times between 5-16y) investigated the effect on incident CVD of an Interleukin-1 receptor antagonist (IL-1RA), which counter-regulates IL-1β as an endogenous inhibitor in vivo by blocking the binding site for IL-1β. A pooled standardized hazard ratio (95% CI) for incident CVD of 1.11 (1.06–1.17) was found after adjustment for age, sex, anthropometric, metabolic, and lifestyle factors (P<0.0001) (82).

Oxidative metabolism is essential for aerobic life, as nutrients provide energy through oxidative phosphorylation, while intermediary metabolism adds direct incorporation of oxygen atoms from molecular oxygen (O_2) into biomolecules. Any molecules or atoms generated by this process containing 1 or more unpaired electrons (free radical) are highly reactive (86). This biological process involving generation of oxidative breakdown products can interact and lead

to oxidation of DNA, proteins, carbohydrates and lipids. On the other hand, the human metabolism has several strategies of defence against oxidative damage, such as enzymatic and non-enzymatic antioxidants, besides adaptive responses (87,88).

The imbalance between oxidants and antioxidants in favour of oxidants, potentially leading to tissue damage and/or triggering cell death pathways, is called oxidative stress (50). Reactive species or free radicals include reactive oxygen and nitrogen species (RNS), which are also important components of intracellular signalling cascades. Thus, the deleterious oxidative overload in cells, organs or the entire organism is a condition that can be characterized not only by an aberrant quantity, but also by the quality (source) of these molecules, as aforementioned in the eNOS uncoupling example (5,88).

ROS molecules include free oxygen radicals, such as superoxide, hydroxyl and peroxyl, as well as non-radicals, such as hydrogen peroxide. The non-radicals are either oxidizing agents or are easily converted into radicals. RNS are molecules containing nitrogen such as NO, peroxynitrite, and nitrogen dioxide. The source of these molecules may be from by-products of endogenous compounds or xenobiotics through mechanisms, such as the electron transport chain, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase (XO), metabolism of the arachidonic acid and cytochrome P-450 (89,90).

In a normal physiological state, a sufficient supply of antioxidants is provided during metabolic processes, as well from dietary sources, that neutralizes harmful activity of reactive species. However, several factors can increase the concentration of such harmful molecules by depleting antioxidant defences leading to oxidative stress. These include smoking, sleep deprivation, acute microbial infections, being overweight, high sugar and/or fat intake, and exposure to metals and air pollutants (5,87). Oxidative stress can lead to injury of the endothelium and impairment of NO bioavailability, as well as reduce other functions, such as

regulation of blood clotting, local immune responses, control of the fluid volume and transportation of electrolytes and other substances between blood vessels and tissues (1,91).

The immune response plays a major role in ED pathogenesis and also acts directly in oxidative balance. Myeloid cells and T lymphocytes protect the host organism from pathogens by attacking with bursts of ROS. While these ROS may help to preserve the vascular tone, an aberrant production of ROS triggered by immune cells in the absence of any hemodynamic insult can lead to damage to the endothelium. Nonetheless, an oxidative stressed state in cardiovascular control organs may likewise potentiate inflammatory responses and augment ED. Therefore, inflammation and oxidative stress act as cooperative and synergistic partners in the pathogenesis of ED (4,5).

1.3 Assessment of vascular and microvascular function

There is increased interest in methods to assess ED to better understand the pathogenesis of CVD and other diseases. With the simultaneous advance of knowledge and technology in this field, there are already many non-invasive options available in both clinical practice and research. Traditional techniques, such as flow-mediated vasodilation (FMD), pulse wave velocity (PWV), and carotid intima media thickness are highly correlated with cardiovascular outcomes and are useful to track disease progression and assess the efficacy of therapeutic interventions in overall vascular health (11). The CVD risk prediction of FMD was addressed in a meta-analysis of 23 studies that included 14,753 subjects finding an overall 8 % reduction in CVD risk (RR= 0.92; 95%CI: 0.88; 0.95) for each percentage increase in FMD (92). However, these methods evaluate conduit artery function, therefore providing limited information with regard to mechanisms of systemic microvascular physiopathology.

The microcirculation comprises arteries with low resistance, arterioles, capillaries, and venules. While the capillary network acts on nutrient and gas exchanges between blood and tissues, arterioles play a major role in blood flow regulation by mechanisms including arteriolar myogenic response, flow-induced vasodilation, metabolic and neural mediators (93). These mechanisms, as well as pathological processes that might affect them are evident in the cutaneous microcirculation and can represent ED features and mechanisms upon other vascular beds. Therefore, considering that the skin is readily accessible, several imaging techniques have been developed in order to use cutaneous microcirculation to investigate the mechanisms of systemic microcirculatory function and dysfunction in various diseases (94,95).

The first imaging techniques for this purpose were developed using Laser Doppler technology. Laser Doppler flowmetry (LDF) assesses blood flow over a small volume (< $1mm^3$) and detects and quantifies relative changes in skin blood flow in response to a given stimulus. However, this technique presents a relatively poor reproducibility due to the significant spatial variability by a heterogenic capture of skin perfusion. Laser Doppler Imaging (LDI), the subsequent developed technology, decreased this spatial variability drawback, but it is much slower than LDF, thereby, recording rapid changes in skin blood flow over the larger areas becomes particularly challenging (95,96). Laser Speckle Contrast Imaging (LSCI) is a recent technique based on speckle contrast analysis that provides an index of blood flow. LSCI supports a continuous rate of high frame assessment of skin perfusion over wide areas, combining advantages of LDF and LDI, and thereby providing good reproducibility of tests such as the post-occlusive reactive hyperaemia (PORH) and local thermal heating (LTH) challenges (97,98).

Microvascular reactivity is assessed by stimulating microvessels with various physiological or pharmacological challenges. The most common tests that are used in

combination with imaging techniques are iontophoresis of vasoactive drugs, such as acetylcholine and sodium nitroprusside, PORH and thermal challenges, such as LTH (93).

Iontophoresis is a non-invasive method of transdermal delivery of drugs based on the transfer of charged molecules using electric currents. Acetylcholine and sodium nitroprusside iontophoresis have been widely used to assess endothelial-dependent and -independent microvascular vasodilation, respectively (95,99). Exact proportions of mechanisms that are predominant in the acetylcholine-induced vasodilation are still not clear; however, C-fiber (axon reflexes), COX-dependent pathways and NO contribute to this response. In addition, endothelial-derived hyperpolarization also contributes to acetylcholine-mediated vasodilation in a dose dependent manner (100). In order to avoid non-specific vasodilatory effects, one group of researchers reviewed several protocols of iontophoresis of acetylcholine and sodium nitroprusside, showing that the type of diluent used for each vasoactive agent, intensity of current and the method of electrical current delivery are important to maintain good reproducibility (101).

PORH is a microvascular reactivity test commonly conducted in the forearm when a transient increase in cutaneous blood flow occurs following release of a brief occlusion in the brachial artery. There is still no standardized protocol for this test; however, most studies use a method in which a cuff, placed above the antecubital fossa, is inflated 50-60mmHg above systolic pressure for 2-5 minutes (93). The mechanisms involved in this induced vasodilation are still being elucidated, with some studies showing inconsistent results. Many mediators seem to contribute to PORH vasodilation and although most of these responses are endothelial-dependent, NO and COX pathways appear not to exert significant influence (102,103). The major contributors to peak and time course of this microvascular reactivity are sensory nerves through an axon reflex response (96). The endothelium-derived hyperpolarizing factors (EDHF) are also involved, including activity of large-conductance calcium activated potassium

channels (BKCa) by epoxyeicosatrienoic acids (95,104). In addition to the abovementioned endothelial challenges that allow the evaluation of specific mechanisms, the postprandial state is a condition that presents a particular modulation of endothelial function.

There is emerging evidence that metabolic imbalances in the postprandial state, particularly after a high-energy meal rich in fat, are important contributing factors to development of CVD (105,106). Overall, the underlying mechanism involves a sharp increase in triacylglycerol along with an aberrant production of pro-oxidant molecules leading to an oxidative stress state. This may impair vascular and endothelial functions, and mediate the onset of an inflammatory response, which further contributes to the generation of more free radicals, thus creating a deleterious vicious cycle (105–107). Dietary fats comprise heterogeneous molecules with diverse structures, which affect diverse cell processes such as transcription regulation, cellular and organelle membrane structure and function, ion channel activity and electrophysiology. Responses vary depending on both the fatty acid composition of the food source, as well interactions with accompanying nutrients, the food matrix and process (108). Modification of the type of dietary fat in a food or overall meal has been shown to result in postprandial effects on appetite (109), lipaemia and markers for inflammation and endothelial activity (110).

The high-fat meal (HFM) challenge is one way to investigate the imbalances that are promoted on a daily basis in Western diets (105). Considering that a significant part of the day, usually two thirds of day time, is spent in the postprandial state, this is an important focus for therapeutic investigations. Further details of this type of study and its implications in CVD are provided in Chapter 3 of this thesis.

Taken together, the assessment of macro and microvascular function, including techniques that evaluate structural changes in these vascular beds, such as FMD and PORH combined with LSCI technology, along with evaluation of biomarkers related to the immune

response and oxidative stress, provide reliable information to investigate prediction, severity and responsiveness of treatment of several diseases, in particular CVD. Using these methods, a wide range of dietary interventions can be explored as potential therapeutic options.

1.4 Flavonoids and implication on cardiovascular diseases

Diet is one of the most important factors in modulating metabolic and immune responses by its direct effect on bioenergetics, body weight, gut microbiota and many other systemic body regulation mechanisms. Among different dietary components, polyphenols are bioactive compounds that naturally occur in various fruits, vegetables, cereals and beverages. They partly contribute to the bitterness, astringency, colour, flavour, odour and oxidative stability of such foods (111). Among the vast number of phenolic compounds, the most common and widely distributed class are flavonoids, present virtually present in all plants. In foods, flavonoids occur as aglycones, methylated derivatives and mostly as glycosides, also providing prevention of fat oxidation and protection of vitamins and enzymes. There are more than 9,000 flavonoids present in nature with different physical, chemical and physiological properties, characteristics that delineate their biological activities. They are found in subclasses such as flavanols, flavonols, flavanones, flavones, isoflavones and anthocyanins(112).

Dietary intake of flavonoids have been associated with the prevention and incidence of several diseases in epidemiological studies. Dietary flavonoid interventions have been explored in clinical trials to treat pathological conditions, such as ED (12,13). In the endothelium, dietary flavonoids can exert physiological effects as both antioxidant and as signalling molecules. The antioxidant activity is due to their ability to donate hydrogen, bind metal ions, resonance stabilization of phenoxyl radicals, thereby acting as reducing agents, metal chelators, ROS scavengers, chain-breaking antioxidants, quenchers of singlet oxygen formation and protectors of endogenous ascorbic acid. The signalling properties of flavonoids

are attributed to interactions with enzymes, kinases and cellular receptors in regulatory pathways mediating physiological responses or altering gene expressions (113,114). For instance, certain types of flavonoids can modify protein kinase-mediated signal transduction and up-regulate antioxidant and anti-inflammatory gene expression (115,116), as well as down-regulate inflammatory gene expression and improve blood pressure (117).

The association between dietary flavonoid intake and potential health benefits in large studies can present methodological challenges. Besides the fact that flavonoid content in foods is likely to be influenced by seasonality and geographical areas, there are some relevant discrepancies between food composition databases with regard to flavonoid content in foods. Additionally, the methods used to assess dietary intake of participants are generally insensitive to accurately characterise flavonoid consumption (118). Nonetheless, some large prospective studies and pooled analyses of clinical trials show positive results in relation to total dietary flavonoid and certain subclasses. In a total of 2,087 fatal coronary heart disease (CHD) events among 7 prospective cohorts, subjects in the higher tertile of dietary flavonol intake presented a combined risk ratio of 0.80 in comparison to subjects in the lower tertile, after adjustment for disease and dietary factors. The main flavonol sources that could be extracted from these cohorts were from a small number of fruits and vegetables, tea and red wine (16). A meta-analysis of ten studies investigated mortality by CVD events. The relative risk (RR) of all-cause mortality among subjects in the higher category of total flavonoid intake was 0.82 in relation to subjects in the lower category intake. A trend was also found for risk of death from CVD (RR: 0.85 and P=0.099) and CHD (RR: 0.74 and P=0.069). A dose-response analysis showed that the lowest risk of all-cause mortality was lower in subjects consuming >200mg/d of total flavonoids (13). The effects of cocoa flavonols was analysed in a meta-analysis of 35 studies, involving 40 treatment comparisons. This pooled analysis with moderate quality evidence showed a modest, but significant lowering of both systolic and diastolic blood

pressure of 1.8 mmHg on average (19). Furthermore, another subclass of flavonoids, quercetin, has also been found to be effective in lowering blood pressure; however, a meta-analysis of 7 clinical trials only reached significance with dosages of >500mg/day (28). Another study evaluated the dietary intake of total flavonoids and subclasses on incidence of hypertension. In a prospective cohort of 40,574 healthy women, 9,350 cases of hypertension were observed during a follow-up of approximately 14 years. A 10% lower rate of hypertension was found in individuals within the higher quintile of flavonol intake when compared to the lower quintile [hazard ratio (HR): 0.90, p=0.031]. Proanthocyanidin and anthocyanins also showed a similar effect of lowering the incidence of hypertension by 9% between the highest and lowest quintile of consumption (HR: 0.91, P= 0.0075 and HR: 0.91, P= 0.0051, respectively)(119). In this matter, anthocyanins are emerging as a potential therapeutic option for CVD due to its effects on ED mechanisms, mainly on oxidative stress and immune response.

1.5 Anthocyanins

Anthocyanins are the largest class of water-soluble plant pigments, which are responsible for the blue, purple and red colour of many fruits and vegetables. Anthocyanins are formed by the coupling of sugars to anthocyanidins, whilst anthocyanidins are their sugar-free analogues. There are more than 300 known anthocyanins, but considering the different possibilities for their glycosylated part, this can number more than 8,000 types. The most common anthocyanins in foods are from six subclasses, namely cyanindin, (comprising 50% of anthocyanins), pelargonidin (12%), peonidin (12%), delphinidin (12%), petunidin (7%) and malvidin (7%). Degradation of anthocyanins in foods can occur by several food processing and storage factors including pH, temperature, exposure to light and oxygen or interactions with foods components, such as ascorbic acid, sugar and metal ions (120,121).

In large population studies, the estimated average intake of anthocyanins is even more challenging than assessing of total flavonoids due to a lack of valid and reliable data on the food composition of dietary sources. Nonetheless, a multi-centre investigation in Europe estimated the average daily intake of anthocyanins for men to be 19.83 mg and 64.88 mg in Holland and Italy, respectively. Among women, the results showed an average daily intake of 18.73 mg in Spain and 44.08 mg in Italy (122). Besides the difficulties in obtaining accurate information on the anthocyanin content of foods and challenges with the assessment of dietary intake, seasonality and geographic variation also complicate such analysis. In Australia, a food frequency questionnaire was validated to measure flavonoid intake in older adults, and an Australian-specific anthocyanin food composition database for dietary studies is being developed (123).

Cell culture, animal model, human clinical trials and epidemiological studies have shown the potential effect of anthocyanins to improve vascular function, inflammatory response and other CVD risk factors (26,121,124–137). The essential feature of these benefits refers to the major role of anthocyanins modulating the immune response. A large number of in vitro and animal studies (121,127,138–140) explain the mechanisms related to the anti-inflammatory actions of anthocyanins. These include: (1) modulation of arachidonic acid metabolism, in which lipid mediators that regulate inflammation (e.g. prostaglandins and leukotrienes) have their key enzymes cyclooxygenases and lipoxygenases inhibited by anthocyanins; (2) decreased activity of the NF-κB pathway, which is a transcription factor responsible for triggering and regulating inflammatory processes, leading to the expression of pro-inflammatory cytokines and enzymes; and (3) suppression of acute pro-inflammatory genes that regulate inducible nitric oxide synthase (iNOS), which is responsible for preventing excessive production of nitric oxide (NO).

The beneficial effects of anthocyanins on ED may also be related to their ability to mitigate the oxidative stress-related damage to the endothelium and their action in endothelium-dependent vasodilation signalling pathways. Antioxidant mechanisms of anthocyanins are exerted by direct and indirect pathways. Anthocyanins provide direct free radical scavenging activity by electron donation (hydrogen), as well as by improving endogenous antioxidant defences, such as restoring or enhancing antioxidant enzyme activity, such as superoxide dismutase and glutathione peroxidase, as well as up-regulating gene expression of glutathione peroxidase (22,138). This antioxidant activity appears to be superior to that of other conventional antioxidants such as α-tocopherol, trolox and catechin (138,141,142). The chemical structure of each anthocyanin subclasses also influences its potential antioxidant effect, which is related to the number and position of hydroxyl groups, conjugation groups, degree of glycosylation and the capacity of the aromatic group of donating electrons. Thus, different anthocyanins may show different levels of scavenging activity towards ROS and reactive nitrogen species (RNS), or other adverse metabolic products (138,143).

The increase in bioavailability of NO, through increased expression of eNOS, has been investigated in cell culture studies. Edirisinghe et al. (126), pre-treated human umbilical vein endothelial cells with different concentrations of blackcurrant juice and for different time periods, independent of vitamin C. The results showed an up-regulation of eNOS that was activated via the Akt/PI3 kinase pathway, while the effect was not vitamin C-dependent. An increased eNOS expression was also demonstrated in bovine artery endothelial cells treated with cyanidin-3-glucoside. This increase, exerted via the Src-ERK1/2-Sp1 signalling pathway, was in a dose and time-dependent manner. Significant results were observed after an incubation time of only 8 hours, while after 24 hours of incubation NO output increased twofold (144).

In animal models, studies using anthocyanins as a dietary intervention confirmed many of the aforementioned effects observed in cell culture studies, including regulation of the immune response and reduced oxidative stress, as well as mediation of lipid transport and accumulation, lowering of blood pressure and improved vascular reactivity. In two acute experimental mice models of peritonitis and paw oedema, anthocyanins provided from wild mulberry inhibited carrageenan-induced inflammation by suppressing mRNA as well as protein levels of COX-2 (145). In a model of hypercholesterolemia, mice fed with a high-fat diet and treated with 2% açai pulp had improved oxidative stress-related biomarkers and better lipid profiles, as well as reduced superoxide dismutase activity and increased paraoxonase activity (protection of lipoproteins and membranes to oxidative damage) (146). Regarding weight loss and adiposity, another study of rodents fed with a high-fat diet for 12 weeks along with 40 and 200 mg/kg of cherry anthocyanins attenuated weight gain by 5.2% and 11.2%, respectively. This was associated with a lower concentration of plasma leptin, glucose, triacylglycerol, total cholesterol and LDL cholesterol, along with a decreased gene expression of Il-6 and TNF in this tissue, and a reduction in adipose cell size (147). Cholesterol efflux signalling can also be influenced by anthocyanins; however, this effect is attributed to its downstream metabolites. For example, protocatechuic acid, a gut microbiota metabolite in mice, exerts this cholesterol efflux from macrophages; however, anthocyanins in physiologically reachable concentrations did not show this effect (148).

Parameters related to vascular function such as blood pressure and vascular reactivity have also been investigated in animal models. In stroke-prone rats, treatment with a 3% blueberry diet for 8 weeks resulted in a significant decrease in systolic blood pressure of 19% and 30% in weeks 4 and 6, respectively, along with reduced markers of renal oxidative stress, such as proteinuria and kidney nitrite (149). In vascular reactivity parameters, rats fed with blueberries for 7 weeks showed diminished vasoconstrictor response to an L-phenylephrine

challenge, while inhibition of NOS, but not COX caused a higher vasoconstriction in the blueberry group. This was also evidenced as the endothelium-dependent vasorelaxation induced by acetylcholine, mediated by the NO pathway, was greater in the anthocyanins group compared to the placebo (150). Moreover, another study showed improvements in the aortic endothelium-dependent vasorelaxation response, also induced by acetylcholine, in mice fed with 2g/kg of cyanidin-3-glucoside for 8 weeks. This result was followed by an increase in cGMP concentration and eNOS phosphorylation at Ser 1177 in the aorta (151).

A few studies have also advanced in nutrigenomic analysis of anthocyanin effects in mice models. A mouse model of accelerated atherosclerosis development was fed with 0.02% of the diet comprising bilberry anthocyanins extract. The attenuated atherosclerotic lesions in the anthocyanins group was followed by a modulation in expression of 1,261 genes, which were related mainly to different cellular processes such as oxidative stress, inflammation, regulation of adhesion molecules, cell to cell adhesion, paracelullar permeability and angiogenesis (152). The same research group previously found altered expression of 2,289 genes in liver tissue, in the same experimental model provided with the same diet. Transcriptional analyses showed that these genes were involved in bile acid synthesis and cholesterol uptake into the liver, and downregulation of pro-inflammatory cytokines (153).

The translation of findings from anthocyanin intervention studies is complex. This is because the potential effects of these compounds depends on their complex absorption, breakdown to phenolic metabolites and subsequent various actions in cells. Despite promising findings to date, many of the mechanistic pathways of their effects are not fully elucidated in humans. The method of delivery of anthocyanins also differs between studies and they may be provided in whole foods, juices, extracts or as purified anthocyanins. While there is homogeneity regarding some outcomes, many other parameters are not consistent between studies. Table 1 summarizes several clinical trials using different forms of anthocyanins, and

for several conditions related to ED. Studies evaluating parameters related to oxidative stress, inflammation, lipid profile, glucose homeostasis, blood pressure, hemodynamic and vascular outcomes were included. However, conditions such as inflammatory and infectious diseases, cancers, as well as studies evaluating specific groups of individuals such as professional athletes, smokers, children, and pregnant or breastfeeding women were not included.

Overall, chronic supplementation of anthocyanins showed a relevant role in oxidative stress, lipid peroxidation, antioxidant status and vascular outcomes with the majority of studies reporting improvements in related parameters. A moderate beneficial effect, with some heterogeneity among studies, was evidenced for lipid profile, glucose homeostasis, vascular inflammatory markers and hemodynamic factors. Although some studies showed improvements in outcomes such as blood pressure, peripheral inflammatory markers and anthropometry, these were the parameters with the highest inconsistency between studies using anthocyanins in medium or long-term dietary interventions. On the other hand, postprandial studies show the most promising results in the attenuation of deleterious postprandial effects

Table 1-1. List of clinical trials with chronic and acute supplementation of anthocyanins

CHRONIC SUPPLEMENTATION OF ANTHOCYANINS								
Study	**Subjects/ condition**	**Sex**	**Age**	**Sample size**	**Study design**	**Intervention/daily dose – anthocyanins content**	**Duration (days)**	**Results (intervention vs control)**
Traustadóttir et al. 2009(154)	Healthy	F/ M	61-75	12	Double blind, placebo controlled, crossover	Tart cherry juice (480mL) - 59.5 mg of total anthocyanins	14	↓forearm ischemia-reperfusion F2-isoprostane response ↓ urinary 8-hydroxy-2 9-deoxyguanosine; 8-hydroxyguanosine ↔ urinary isoprostanes
Lee et al. 2016 (155)	Body mass index >23 kg/m^2	F/ M	19-65	63	Double blind, placebo controlled, RCT	Extract of black soybean - 31.45 mg of total anthocyanins	56	↓abdominal fat; TG; LDL-c; non-HDLc
Davinelli et al. 2015(156)	Healthy, overweight and smokers	F/ M	45-65	42	Double-blind, placebo-controlled, RCT	Extract of maqui berry - 468 mg of total anthocyanins	28	↓ plasma OxLDL; urinary F2-isoprostanes ↔ anthropometry; blood pressure; lipid profile
Li et al. 2015(157)	Diabetic	F/ M	>18 (39.8 ± 13.8)	58	Double-blind, placebo-controlled, RCT	Billberry and blackcurrant purified anthocyanins – 160mg of total anthocyanins	168	↓LDL-c; TG; apolipoprotein B-48; apolipoprotein C-III; glucose; HOMA-IR ↑ HDL-c ↓F2-isoprostanes; 13-hydroxyoctadecadienoic acid; carbonylated proteins ↑ total radical-trapping antioxidant parameter; FRAP
Habanova et al. 2016(158)	Healthy	F/ M	48.3 ± 5.64	36	Non-randomized, pre-post intervention study	65g of frozen bilberries – 194mg of total anthocyanins	42	↓ TC; TG; LDL-c; glucose; ↑ HDL-c ↔ anthropometry; blood pressure
McAnulty et al. 2014(159)	Healthy, postmenopausal	F/ M	18-50	25	Placebo-controlled, RCT	Blueberry extract (equivalent to 250 g rehydrated berries) – total anthocyanins value not provided	42	↓ AIx; aortic systolic pressure ↔ anthropometry; overall blood pressure ↓ diastolic blood pressure (sub-analyses) ↑ absolute NK cells ↔ ORAC; FRAP

Kuntz et al. 2014(160)	Healthy	F	23-27	30	Double blind, placebo controlled, crossover, two intervention groups + palcebo	Juice – 277.5mg of total anthocyanins; smoothie - 324.7mg of total anthocyanins	14	↑plasma superoxide dismutase; catalase activity; TEAC ↔ plasma glutathione peroxidase and erythrocyte superoxide dismutase ↓ plasma and urinary MDA ↔IL-2, -6, -8 and -10; CRP; sCD40;TNF-α; MCP-1; CAMs
Study	**Subjects/ condition**	**Sex**	**Age**	**Sample size**	**Study design**	**Intervention/daily dose – anthocyanins content**	**Duration (days)**	**Results (intervention vs control)**
Kardum et al. 2014 (161)	Healthy	F	25-49	29	Non-randomized, pre-post intervention study	Chokeberry juice - 25mg of total anthocyanins	84	↓ TBARS; PAB; TOS ↑ PON1; TAC ↔ anthropometry; blood pressure; biochemical parameters
Lynn et al. 2014(162)	Healthy	F/M	35-50	47	Double-blind, placebo-controlled, RCT	Tart cherry juice concentrate - 273.5mg total anthocyanins	42	↔ arterial stiffness; CRP; blood pressure; TC; HDL-c ↑ FRAP
Wright et al. 2013(163)	BMI: 32.8 ± 4.6 kg/m^2	M	53.1 ± 7.6	16	Double-blind, placebo-controlled, RCT	Dried purple carrot- 118.5 mg of total anthocyanins	28	↔ anthropometry; LDL-c; TC; blood pressure; CRP
Broncel et al. 2010(164)	Healthy (n=22) and metabolic syndrome (n=25)	F/M	42-65	47		Aronia extract - 3-O-cyanidin-galactoside (64.5%), 3-O-cyanidin-arabinoside (28.9%), 3-O-cyanidin-xyloside (4.2%), and 3-O-cyanidinglucoside (2.4%) – mg?	60	↓ systolic and diastolic blood pressure; endothelin-1; TBARS; antioxidant enzymes catalase ↓TC; LDL-c; TG; ↑ superoxide dismutase; glutathione peroxidase; fibrinogen ↔ CRP
Basu et al. 2011(165)	Metabolic syndrome	F	52.0 ±8.0	36	Double-blind, placebo-controlled, RCT	Cranberry juice - 12.4 mg of total anthocyanins; 119mg of total Proanthocyanidins	56	↔blood pressure; lipid profile; glucose; CRP; IL-6 ↓ serum MDA and 4-hydroxynonenal; plasma OxLDL ↑ plasma antioxidant capacity

Basu et al. 2010(166)	Metabolic syndrome	F/ M	50.0 ± 3.0	66	Single-blind, placebo-controlled, RCT	Blueberry beverage – 742mg of total anthocyanins	56	↓ diastolic and systolic blood pressure; ↓ plasma MDA; 4-hydroxynonenal; OxLDL ↔ lipid profile; glucose; $HbA_{1C;}$ CRP; CAMs; IL-6; myeloperoxidase
Dohadwala et al. 2011(167)	Coronary artery disease	F/ M	62.0 ± 10.0	44	Double blind, placebo controlled, crossover, RCT	Cranberry juice – 94mg of total anthocyanins	28	↔ blood pressure; FMD; carotid-radial PWV ↓ carotid-femoral PWV ↔ lipid profile; glucose; insulin; CRP, ICAM-1
Kent et al. 2017(168)	Mild to moderate dementia		70+	49	Double-blind, placebo-controlled, RCT	Cherry juice – 138 mg of total anthocyanins	84	↓ Systolic and diastolic blood pressure ↔ CRP; IL-6
Study	**Subjects/ condition**	**Sex**	**Age**	**Sample size**	**Study design**	**Intervention/daily dose – anthocyanins content**	**Duration (days)**	**Results (intervention vs control)**
Thompson et al. 2017(169)	Healthy	F/ M	38.0 ± 12.0	16	Double blind, placebo controlled, crossover, RCT	Blackberry and billberry extracts - 320mg of total anthocyanins	28	↓ monocyte-platelet aggregate formation; platelet endothelial cell adhesion molecule-1; procaspase activating compound-; P-selectin; ADP-induced whole blood platelet aggregation ↔ blood pressure; fibrinogen; lipid profile, CRP
Zhu et al. 2011/2013 (170,171)	Hypercholesterolemic	F/ M	40-65	150	Double blind, placebo controlled, crossover, RCT	Purified anthocyanins - 320mg of total anthocyanins	84	↑ FMD; serum cGMP ↔ blood pressure; TG; TC; glucose; insulin ↓ VCAM-1; CRP; IL-1b; LDL-c ↑ HDL-c
Hasselund et al. 2012/2013 (172,173)	Healthy; blood pressure>140/ 90 mm Hg; not medicated	M	35-51	31	Double blind, placebo controlled, crossover, RCT	Purified anthocyanins – 640mg of total anthocyanins	28	↔ blood pressure ↑ HDL-c; von Willebrand factor, ↔ TC; TG; LDL-c; glucose; CRP; MCP-1; P-selectin; CAMs; IL-6 (trend); TNF-α; IL-4; L-arg/ADMA; FRAP

ACUTE SUPPLEMENTATION OF ANTHOCYANINS								
Study	**Subjects/condition**	**Sex**	**Age**	**Sample size**	**Study design**	**Intervention/daily dose – anthocyanins content**	**Sample collection (hours)**	**Results (intervention vs control)**
Park et al. 2016 (174)	Individuals with insulin resistence	F/M	56-67	21	Randomized controlled, four-arm, dose-response, crossover trial	High fat and carbohydrate meal with freeze-dried whole strawberry powder (0, 10, 20, 30 and 40g)	0, 0.5, 1, 1.5, 2, 3, 4, 5 and 6	↓ insulin; insulin;glucose; OxLDL ↔ IL-6
Toaldo et al. 2015 (175)	Healthy	F/M	20-55	30	Double-blind, placebo-controlled, cross-over, two intervention groups	Organic red grape juice – 159.2 mg of total anthocyanins / conventional red grape juice – 42 mg of total anthocyanins	0-1h	↓ TBARS; lipid hydroperoxides
Frank et al. 2012(176)	Healthy	F/M	22-27	8	Randomized, open-label, two-way crossover	*Hibiscus sabdariffa L.* aqueous extract - 130.25mg of total anthocyanins	0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8 and 10h	↑ plasma FRAP AUC; plasma ascorbic acid ↓ plasma uric acid ↓ urinary MDA ↑ urinary ascorbic acid
Edirisinghe et al. 2011 (177)	Overweight; 25>BMI>33.5 k/m^2	F/M	50.9 ± 15.0	24	Randomised, single-blind, placebo-controlled, cross-over trial	high-carbohydrate, moderate-fat meal + strawberry beverage - 120.69mg of total anthocyanins	0, 0.5, 1, 1.5, 2, 3, 4, 5 and 6	↓ CRP; IL-6; insulin ↔ IL-1β; PAI-1; TNF-α Glucose;
Study	**Subjects/condition**	**Sex**	**Age**	**Sample size**	**Study design**	**Intervention/daily dose – anthocyanins content**	**Sample collection (hours)**	**Results (intervention vs control)**
Alqurashi et al. 2016 (178)	Healthy, overweight 25>BMI>30k/m^2	M	30-65	23	Randomized, controlled, double-blind, crossover	Açai-based smoothie – 493mg of total anthocyanins	0, 1, 2, 3, 4, 5, 6 and 7h	↑ FMD ↓ total peroxide oxidative status AUC ↔ blood pressure; glucose
Dohadwala et al. 2011 (167)	Coronary artery disease	F/M	62.0 ± 8.0	15	Open label	Cranberry juice – 94mg of total anthocyanins	0, 2 and 4h	↔ blood pressure; ↑ FMD (%); FMD (mm)

Keane et al. 2016 (179)	Pre-hypertension (SBP>130 mm Hg, DBP >80 mm Hg, or both)	M	31.0 ± 9.0	16	Placebo-controlled, single-blinded, crossover, randomized Latin square design	Montmorency tart cherry juice – 73.5 mg of total anthocyanins	0, 1, 2, 3, 5 and 8h	↓ systolic blood pressure; DVP-RI; PWV; peripheral blood pressure; mean arterial pressure ↔ microvascular reactivity [endothelium (trend) and non-endothelium dependent] ; heart rate; DVP-SI(trend); AIx
Jin et al. 2011 (180)	Healthy	F/ M	44.5 ±13. 3	20	Randomised, double-blind, placebocontrolled cross-over acute	Blackcurrant juice – 50.5mg of total anthocyanins	0 and 2h (vascular reactivity) , 0-8 (plasma and urine), 8-24 (urine)	↑ plasma ascorbic acid; uric acid; insulin ↔ microvascular reactivity ↔ plasma FRAP; ORAC; TG; ICAM-1; VCAM-1; glucose;
Kent et al. 2016 (181)	Young and older adults	M/ F	18-35 / 55+	13	Pilot cross-over study	High-flavonoid cherry juice (300mLx1 and 100mLx3) -	0, 2 and 6h	↓ systolic and diastolic blood pressure; heart rate
Rodriguez -Mateoz et al. 2013 (182)	Heathy	M	18-40	21	Randomized, controlled, double-blind, crossover	Five blueberry beverages – 129, 258, 310, 517 and 724mg of total anthocyanins	0, 2, 4 and 6h	↑ FMD ↓NADPH oxidase activity ↔ Aix; PWV; DVP-SI; DVP-RI; ↔ peripheral and central blood pressures
Zhu et al. 2011 (170)	Hypercholesterolemic	F/ M	40-65	12	Randomized, controlled, double-blind, crossover	Purified anthocyanins - 320mg of total anthocyanins	0, 1, 2 and 4h	↑ FMD; serum cGMP

Symbols: ↑ Higher than control treatment ↓ Lower than control treatment ↔ No significant effect. **Abbreviations:** RCT, random clinical trial; LDL-c, low-density lipoprotein cholesterol; OxLDL, oxidized low-density lipoprotein; TBARS, thiobarbituric acid reactive substances; ORAC, oxygen radical absorbance capacity; FRAP, ferric ion reducing antioxidant power; TEAC, trolox equivalent antioxidant capacity; MDA, malondialdehyde; TNF-α, Tumour necrosis factor alpha; MCP-1, monocyte chemoattractant protein-1; CAMs, cell adhesion molecules; TAC, total antioxidative capacity; TOS, total oxidative status; PON1, paroxonase-1 activity; PAB, pro-oxidant-antioxidant balance; AUC, area under the curve; CAT, antioxidant enzymes catalase; FMD, flow-mediated dilation; HbA_{1C}, glycated haemoglobin; PWV, pulse wave velocity; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular adhesion molecule-1; DVP-RI, digital volume pulse reflection index; DVP-SI, digital volume pulse stiffness index; AIx, augmentation index; L-arg/ADMA, L-arginine/asymmetric dimethyilarginine ratio; cGMP, cycluc guanosine monophosphate.

on oxidative stress and antioxidant status, triacylglycerol and total cholesterol concentrations, vascular endothelial function and inflammatory biomarkers. Post-prandial changes in blood pressure and lipoproteins were least affected by anthocyanins (more details are provided in *Chapter 3: The postprandial effect of anthocyanins on cardiovascular disease risk factors: a systematic literature review of high-fat meal challenge*).

In addition to the findings reported in Table 1, other studies investigated the effect of anthocyanins in a large numbers of subjects, both in large prospective cohorts and systematic reviews with or without meta-analysis. Cassidy et al (183–185), conducted three different analyses in the Nurses' Health Study II evaluating the anthocyanin intake and incidence of certain conditions and events related to ED. The flavonoid and anthocyanin intakes were calculated using validated food-frequency questionnaires. In one cohort analysis of 93,600 healthy women aged between 25 and 42 years, an inverse association between intake of anthocyanins (highest versus lowest quintiles) and risk of myocardial infarction was observed (HR: 0.68; 95% CI, 0.49-0.96; P=0.03) after multivariate adjustment. The combined intake of blueberries and strawberries, in individuals consuming more than three serving per week versus a lower intake, tended to be associated with a decreased risk of myocardial infarction (HR: 0.66; 95% CI, 0.40-1.08; P=0.03). Anthocyanins were the only flavonoid class to show positive benefits in relation to the incidence of these CVD events (184). Furthermore, while following 43,880 healthy men with no prior diagnosis of CVD for 24 years, a total of 4046 myocardial infarction and 1572 stroke cases occurred, in which all of these events were confirmed by medical records. After multivariate adjustment, a higher anthocyanin intake was inverse associated with non-fatal myocardial infarction (HR: 0.81; 95% CI: 0.69-0.96; P= 0.03) in normotensive individuals, whilst a borderline significant trend was observed in all individuals (HR: 0.87; 95% CI: 0.75-1.00; P = 0.04; P= 0.098). Fatal myocardial infarction and stroke was not associated with anthocyanin intake in that analysis (183). In another

investigation, Cassidy et al.(185) examined the association between flavonoid intake and incidence of hypertension by grouping results from three different cohorts. A total of 133,914 women from the Nurses' Health Study I and II, as well as 23,043 men from the Health Professionals Follow-Up Study were followed-up for 14 years, resulting in 29,018 cases of hypertension in women and 5629 cases of hypertension in men. A reduction of 8% in risk of hypertension (RR: 0.92; 95% CI: 0.86, 0.98; $P < 0.03$) was found comparing individuals from the highest quintile versus lowest quintile of anthocyanins intake. This effect was even higher among individuals under 60 years of age (RR: 0.88; 95% CI: 0.84, 0.93; $P < 0.001$).

Regarding the pooled analyses of anthocyanin interventions in clinical trials, systematic reviews and meta-analysis have been conducted for outcomes related to ED such as blood pressure, CVD risk factors and vascular function, with inconsistent results. A meta-analysis evaluating the effect of berries on CVD risk factors among healthy individuals or patients with CVD (n = 1,251 subjects from 21 randomized clinical trials) reported that berry consumption lowered LDL-c, systolic blood pressure, fasting glucose, body mass index, haemoglobin A1c and TNF-α, whilst no significant effects were demonstrated for HDL-c, triglycerides, total cholesterol and diastolic blood pressure. It is important to note that not all berries have anthocyanins as their primary source of polyphenols; however, the majority of dietary interventions from the included studies were rich in anthocyanins (186). Another systematic review of randomized controlled trials evaluated the effect of purified anthocyanins and anthocyanin-rich extracts on markers of CVD. A total of twelve clinical trials with both healthy and individuals with CVD were included in this review, but the large heterogeneity prevented the conduct of a meta-analysis. Considering LDL-c, HDL-c, total cholesterol, triglycerides and blood pressure, an improvement was only evident for LDL-c among diseased individuals or those with elevated concentrations of this biomarkers (131).

Parameters such as vascular reactivity and stiffness have also been evaluated in a systematic review and meta-analysis of 24 studies, including both acute and chronic dietary interventions of anthocyanin-rich foods or extracts(26). Among the acute studies, FMD was reported in 4 studies, while reactive hyperaemia index by peripheral arterial tonometry was evaluated only in 2 studies. The pooled analysis showed a significant improvement for FMD, mainly observed 1-8 hours after consumption of anthocyanin doses, which ranged from 7 to 724mg. There was no improvement in reactive hyperaemia index; however, when the microvascular reactivity outcome was analysed collectively along with FMD, there was a significant improvement after anthocyanin provision. Arterial stiffness, which was evaluated through pulse wave velocity, was also improved by acute anthocyanins intake, whilst no changes were observed for augmentation index. Once again, considering both outcomes for a pooled analysis of vascular stiffness, there was a trend towards an improvement of this parameter following anthocyanins intake. Among longer term studies ranging from one week to six months, with anthocyanin daily doses of 12 to 320mg, an improvement in FMD was found. The increase in reactive hyperaemia index did not reach significance; however, taken together along with FMD for a vascular reactivity measure, the pooled analysis showed an improvement in this parameter. In relation to arterial stiffness, the results from both pulse wave velocity and augmentation index showed no improvements when analysed individually; however, there was a trend toward an improvement in vascular stiffness when these two parameters were analysed collectively (26).

Taken together, this body of pre-clinical, clinical and epidemiological evidence support the potential beneficial effects of anthocyanins on vascular function and other CVD risk factors, and warrant the conduction of clinical trials with innovative methodologies for a robust investigation of such parameters.

1.6 Problem statement

Several gaps in the literature remain regarding the association of flavonoids and their potential health benefits. At the epidemiological level, only a few populations have been investigated in appropriate large-scale studies. This type of evidence requires consideration of specific differences in the flavonoid content of foods that exist between different geographical areas and crops, as well as the variety of dietary patterns and cuisines between populations will affect the amount and type of consumption of flavonoid-rich food items, while differing lifestyle factors and life expectancies will also influence the contribution of flavonoids to the incidence of hypertension and other chronic diseases. These methodological challenges limit extrapolation of findings from epidemiological studies of flavonoid intake and incidence of disease outcomes from one population to another. Still, previous analysis of dietary patterns, conducted in the Australian population identified differences in the level of consumption of individual food groups by age (187). Therefore, considering the limited evidence from longitudinal studies that assess the association between the incidence of hypertension and consumption of flavonoids in the Australian population, a life course approach is necessary.

At the clinical level, a number of studies have investigated the effect of specific flavonoids across a wide range of conditions. Among the different groups of flavonoids, anthocyanins are particularly promising in terms of their potential benefits in attenuating and preventing pathological conditions, mainly through their potential anti-inflammatory, anti-oxidant and signalling effects. The effect of anthocyanins in the postprandial state have been evaluated by several studies using the HFHE meal challenge. This method allows investigation of whether these bioactive compounds are capable of attenuating the deleterious effects following a HFHE meal; however, such findings have not been adequately collated and synthesized regarding the scope of the impact of acute anthocyanin dietary intake on CVD risk

factors. Within this range of studies, another gap that was identified is the lack of robust investigation of the vascular function in the postprandial state, which along with the immune response is an important predictor of CVD. This is particularly evident in older adults, as several studies support a gradual loss of vascular health, and a new immune-metabolic viewpoint for age-related diseases, termed ‘inflammaging’, characterized as a chronic low-grade inflammation. Additionally, there are still several gaps to be addressed regarding the effect of anthocyanins in this vascular-inflammatory axis in older adults, especially among those with neurodegenerative conditions that share risk factors with CVD.

1.7 Hypothesis

Following the problem statement, this thesis has three main hypotheses, namely:

1) A higher dietary intake of flavonoids is associated with a lower incidence of hypertension in Australian women;
2) An acute dietary intervention with food anthocyanins is capable of attenuating potential postprandial deleterious effects of a high-fat high energy-meal on vascular function, immune response and other CVD risk factors;
3) A chronic dietary intervention with food anthocyanins will improve microvascular health, and reduce inflammation and blood pressure in older adults with a diagnosis of MCI.

1.8 Research Objectives and Conceptual Framework

To test the study hypotheses, the following objectives were developed:

1) To determine if there is an association between higher dietary intake of flavonoids and subclasses and the incidence of hypertension in two cohorts of Australian women;
2) To collate and synthesize the current evidence of the postprandial effects of anthocyanins on CVD risk factors in high-fat meal challenge studies;
3) To determine if consumption of food anthocyanins has postprandial effects on the macro and microvascular function, inflammatory and oxidative stress biomarkers, and lipid profile following a high-fat high-energy meal in overweight or obese older adults;
4) To determine if chronic consumption of food anthocyanins has beneficial effects on the microvascular function, inflammatory biomarkers and 24 h ambulatory BP in older adults with a diagnosis of MCI.

In order to test the above hypothesis and address the research objectives, four studies were developed across three levels of evidence, including:

- Epidemiological evidence: Secondary analysis of data from a nationally representative cohort of Australian women.
- Knowledge synthesis: Systematic Literature Review.
- Experimental evidence: Two randomised placebo-controlled Clinical Trials (RCTs) with an innovative design that allowed interpretation of both acute and chronic effects of anthocyanin intake.

The Conceptual Framework for the thesis, demonstrating the four studies across the three levels of evidence is shown in Figure 1-1.

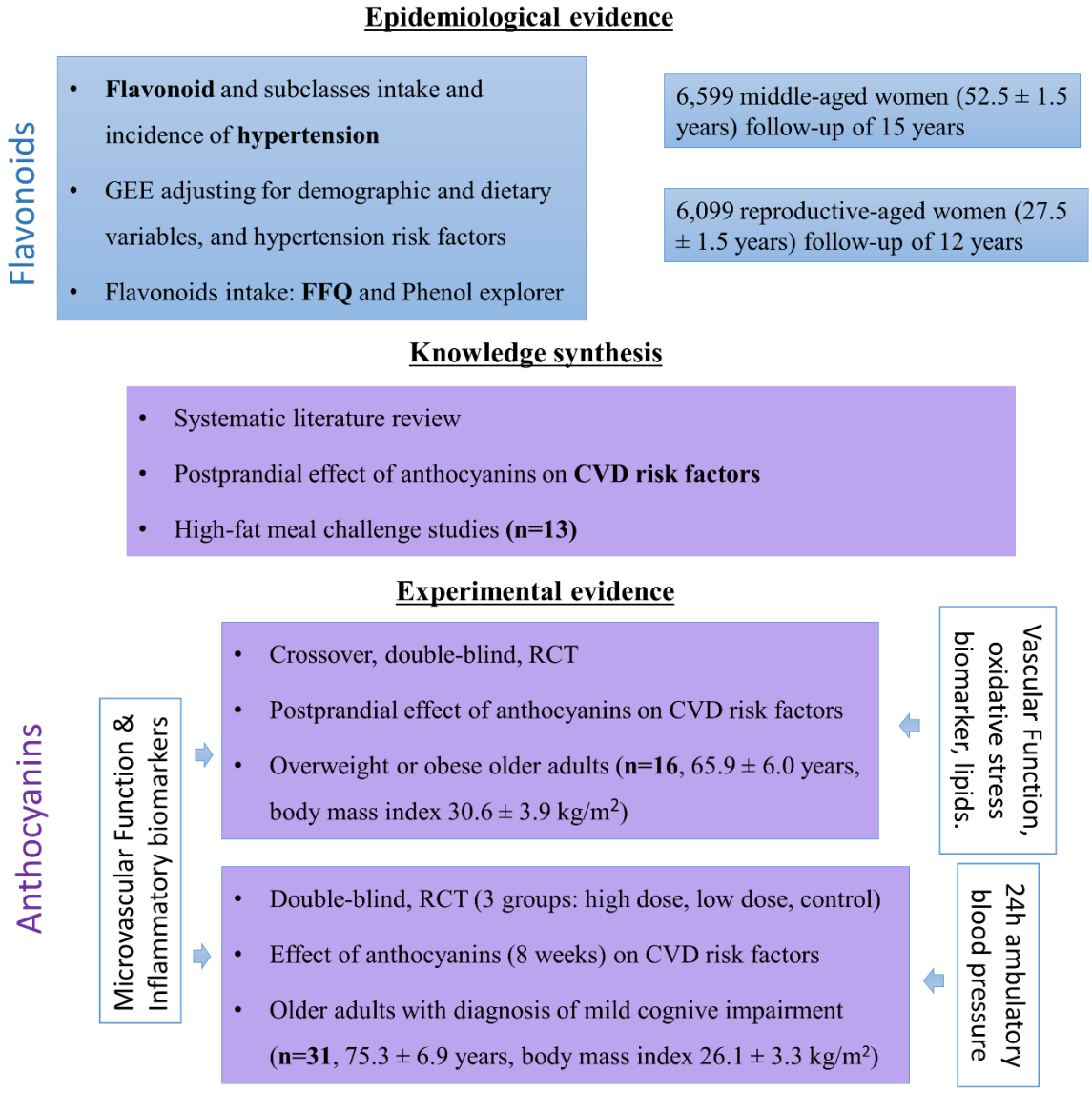


Figure 1-1. Thesis Conceptual Framework
GEE, generalized estimated equations; FFQ, food frequency questionnaire; CVD, cardiovascular disease.

1.9 Significance of the Research

Flavonoids are emerging as potential dietary bioactive components that exert beneficial effects on certain pathological conditions, as well as having a preventive role in several chronic and degenerative diseases. This thesis aims to explore gaps in the literature in both cases. Regarding the epidemiological aspect, this thesis addresses the association between daily consumption of flavonoids and the incidence of hypertension. A population-based study was

conducted to estimate the total intake of flavonoids and their subclasses in the Australian female population by exploring data collected in a nationally representative cohort study. This analysis found an association between flavonoids and the occurrence of hypertension, which is a novel finding. This provides evidence that is useful for nutrition messaging and policies targeting improved cardiovascular health in this population.

A robust review of pre-clinical literature showed that anthocyanins are capable of improving vascular endothelial function, and have relevant anti-inflammatory and anti-oxidant effects; however, clinical evidence of these effects in humans is still lacking in many aspects. This thesis aims to advance this field, by conducting a systematic literature review to synthesize and summarise the published literature relating to the acute effect of anthocyanins on postprandial responses to a high energy, high fat stressor meal, as well as by undertaking two randomised, placebo- controlled clinical trials to add to knowledge inquiry.

The two innovative clinical trials were conducted in older adults. The first investigated the postprandial effects of anthocyanins following a HFHE meal challenge using a more robust evaluation of vascular function than has been conducted in previous similar studies. Vascular function was measured by exploring macro and microvascular parameters through a combination of classical and novel techniques using the latest imaging technologies, including FMD and LSCI. To our knowledge, this was the first study of its kind to conduct this protocol using an anthocyanin dietary intervention. As well as the assessment of vascular function, classical CVD biomarkers were included, such as lipid profile, blood pressure, and inflammatory biomarkers. Another clinical trial was conducted to investigate the longer term effects (8 weeks) of anthocyanin supplementation provided through the Australian-grown Queen Garnet plum on inflammatory markers associated with CVD risk factors, along with analysis of microvascular function and 24-hour ABP. The aim of this study was to investigate such parameters in an older population with a diagnosis of mild cognitive impairment, which

is a neurodegenerative condition that shares pathological mechanisms with CVD. Considering the major role of the inflammatory response and vascular function in CVD and cognitive decline, this data may be of clinical relevance for this high-risk group.

Chapter 2: Association between flavonoid intake and risk of hypertension in two cohorts of Australian women: a longitudinal study

Large prospective studies and pooled analyses of clinical trials have shown health benefits of flavonoids on CVD risk factors and incidence. However, only a few populations were investigated by appropriate large-scale studies, including a comprehensive assessment of flavonoid subclasses and food sources. This type of evidence requires a specific approach as flavonoid content in foods can vary significantly among different geographic areas and crops, as well as considering the cultural diversity in cuisine and dietary patterns within a country and worldwide. Therefore, epidemiological studies associating flavonoid intake and incidence of diseases can hardly be extrapolated from one population to another. In this matter, the present population-based study estimated the association between intake of total flavonoids and its subclasses and incidence of hypertension in two population-based cohorts of Australian women, including reproductive-aged and middle-aged individuals. Flavonoid intake was associated with a lower incidence of hypertension in both cohorts. Higher intakes of flavones, isoflavones and flavanones, attributed mainly to orange, orange juice, apples and soy milk, were associated with a reduced risk of hypertension among middle-aged women followed-up over 15 years. Higher intakes of flavanols, attributed mainly to red wine and apples, were associated with a reduced risk of hypertension among reproductive-aged women followed-up for 12 years. These findings can be used in nutritional messages and policies aimed at improving the cardiovascular health of women of different life stages

The majority of this chapter forms the substantive content of a published article (Appendix C)

2.1 Introduction

Cardiovascular disease (CVD) is a major cause of mortality globally and is the leading cause of death for Australian men and women. In 2018, CVD was responsible for over 41,800 deaths in Australia (26% of all deaths)(188). Following a similar pattern to that observed worldwide, the prevalence of hypertension among adults was 33.7% (36.0% among men and 31.4% among women in 2017-2018[1]. This prevalence increased with age, from less than 10% in the 18-34 year age group to almost 50% in people aged over 85 years(188).

Hypertension is an independent risk factor for CVD and does not have a defined aetiology. Several factors play a role in increased blood pressure including modifiable factors, such as smoking, body weight, physical activity, alcohol, high sodium intake and other dietary imbalances(189). Among the various pathophysiological mechanisms involved in hypertension, the co-operative and synergistic action of inflammation and oxidative stress play an important role(190–192).

Nutrition plays a major role in enhancing endogenous antioxidant defences and regulating the inflammatory state(193). Epidemiological studies have identified many dietary patterns that are associated with the prevention of diseases associated with inflammation(194). Among the various protective dietary components are polyphenols, which are bioactive, plant-based compounds that naturally occur in fruits, vegetables, cereals and beverages(195). Of the large number of polyphenolic compounds found in foods, the most common and widely distributed class are flavonoids, present in virtually all plants. Flavonoids are classified into six subclasses, namely flavanols (including proanthocyanidins), flavonols, flavanones, flavones, isoflavones and anthocyanins(196). In the endothelium, dietary flavonoids can exert physiological effects as both antioxidants and as signalling molecules(196,197).

Several large prospective studies and pooled analyses of clinical trials show health benefits for total dietary flavonoid intake and some of its subclasses. A meta-analysis of five

prospective cohort studies, comprising 200,256 individuals and 45,732 cases of hypertension, showed a non-significant lower risk of hypertension for higher total flavonoid intake, while dietary anthocyanin intake was associated with an 8% reduction in risk of hypertension when comparing highest vs. lowest intake(198). The effects of cocoa flavonols were analysed in a meta-analysis of 35 randomized clinical trials studies, showing significant lowering of both systolic and diastolic blood pressure by 1.8 mm Hg(199). Furthermore, a meta-analysis of 7 trials involving 587 participants found that intake of quercetin, a flavanol subclass, resulted in a significant reduction in blood pressure(200). However, differing dietary patterns and cuisines between populations will affect the amount and type of consumption of flavonoid-rich food items, while differing lifestyle factors and life expectancies will also influence the contribution of flavonoids to the incidence of hypertension and other chronic diseases. Previous analysis of dietary patterns in the ALSWH identified differences in the level of consumption of individual food groups in the middle-aged and reproductive-aged cohorts (187). Therefore, considering the limited evidence from longitudinal studies that assess the association between the incidence of hypertension and consumption of flavonoids in the Australian population, a life course approach is necessary.

This study aims to evaluate the association between intake of total flavonoids and its subclasses and incidence of hypertension in two population-based cohorts of Australian women that included reproductive-aged and middle-aged individuals.

2.2 Methods

Study population

The Australian Longitudinal Study on Women's Health (ALSWH) includes a representative sample of more than 40,000 women that were recruited in 1996. Women were randomly sampled from the National Health Insurance scheme (Medicare) that includes all Australian citizens and permanent residents(201). The reproductive-aged and middle-aged

cohorts (including women born in 1973–78 and 1946–51, respectively) were used for the purpose of this study, as these cohorts included validated assessment of dietary intake. Further details of the recruitment methods, response rates, retention and attrition have been described elsewhere (202).

These cohorts have been surveyed seven and eight times at approximately three-year intervals, respectively, since 1996. The initial sample response rate from the middle-aged cohort was n = 13,714 for Survey 1, with subsequent Surveys 2 to 8 yielding samples of *n* = 12,338 (90%), *n* = 11,221 (81.8%), *n* = 10,905 (79.5%), *n* = 100 10,638 (77.6%), *n* = 10,011 (73.0%), *n* = 9,151 (66.7%) and *n* = 8,622 (62.9%), respectively. In the reproductive-aged cohort, the initial sample comprised n = 14,247, while response rates for Surveys 2 to 7 were *n* = 9,688 (68%), *n* = 9,081 (63.7%), *n* = 9,145 (64.2%), *n* = 8,200 (27.6%), *n* = 8,126 (57%) and *n* = 7,186 (50%), respectively. Dietary intake was assessed at Surveys 3 and 5 in the reproductive-aged cohort, and at Surveys 3 and 7 in the middle-aged cohort. Survey 3 was therefore used as baseline for both cohorts. The Human Research Ethics Committees of the University of Newcastle and the University of Queensland approved the study methods. All participants signed a consent form before joining the study.

Dietary intake

Dietary intake over the past 12 months was assessed through a validated FFQ, The Dietary Questionnaire for Epidemiological Studies, version 2 (DQES v2)(203,204), which has been validated against seven days of weighed food records with correlation coefficients ranging from 0.28 for vitamin A to 0.78 for carbohydrates(204). Participants reported their usual daily frequency of intake and portion size for 101 individual food items according to a 10-point scale ranging from ‘Never’ to ‘Three or more times per day’. The Australian Food Composition Database (NUTTAB95) was used to calculate energy, macro and micronutrient intakes(205).

In order to determine flavonoid intake, each food item was assigned a total flavonoid and subclasses content value using the 'PhenolExplorer' polyphenol food composition database. The PhenolExplorer is a comprehensive and freely available database that contains more than 35,000 content values for 500 different polyphenols in over 400 foods(206). Each selected food item from the FFQ was manually cross-referenced with this database. Foods listed in the FFQ that were not in PhenolExplorer were assumed to contain no flavonoids. Of the foods in the FFQ, 42 foods were assigned a flavonoid content. Flavonoid and subclass intakes from each food were measured by multiplying the consumption (g/day) for each food by its flavonoid content (per gram edible weight). Individual flavonoids from the six subclasses were summed to provide a total value for each subclass, and data for total flavonoids were calculated as the sum of these subclasses. A daily dietary intake of each flavonoid subclass and total flavonoid intake was calculated for each individual. The food item 'tea', which is an important source of flavonoids in this population, was not included in this version of the FFQ, but a relevant question was present in another part of the survey. However, the rate of responses and missing values differed from the FFQ data, thus the inclusion of "tea" and "herbal tea" flavonoids was addressed in a sub-analysis.

Dietary intake was not assessed at every survey. Based on previous research showing that diet quality is stable in these age cohorts(207,208), dietary intake data from Survey 3 were applied to survey 4 in the reproductive-aged cohort and to Surveys 4 to 6 in the middle-aged cohort. Survey 5 dietary data were applied to subsequent surveys in the reproductive-aged cohort to account for any changes in intake.

Incidence of hypertension

The occurrence of hypertension was determined from self-reported data on doctor-diagnosed hypertension available in each survey from the following question: *"In the past three*

years, have you been diagnosed or treated for: High blood pressure (hypertension)". For incidence estimation, hypertension was defined as new onset reported at Survey 4 onwards, while participants that reported hypertension in the first three surveys were excluded from our analysis. A previous study showed an agreement of 89% between self-reported hypertension and antihypertensive medication use in the middle-aged cohort utilised in the present study(209).

Covariates

Data on a wide range of demographic, socio-economic factors and hypertension risk factors were collected in each survey for both cohorts. The following variables were included as covariates: education (low – no formal qualifications or school or intermediate certificate or equivalent; intermediate – high school or leaving certificate, trade/apprenticeships, or certificate or diploma; or high – university degree); income management (impossible/difficult all the time; difficult some of the time; or easy); body mass index (BMI)[underweight and normal weight (BMI up to 25 kg/m^2); overweight (BMI 25-30 kg/m^2); or obese (BMI >30 kg/m^2)]; smoking status (never been a smoker; former smoker; or current smoker); alcohol consumption [rarely & non-drinker (<1 drink per week); low risk (up to 14 drinks per week); or risky (15 to 28 drinks per week) & high risk (more than 28 drinks per week)]; doctor-diagnosed diabetes type I & II combined (no or yes). Physical activity scores were derived from validated questions on frequency and duration of walking, and on moderate- and vigorous-intensity activity, and were categorised as: sedentary/low (<500 total metabolic equivalent (MET) minutes/week); moderate (500 - <1000 MET minutes/week); or high (>1000 MET minutes/week)(210). In the middle-aged cohort, menopause status was determined using questions regarding the occurrence of hysterectomy, oophorectomy, hormone replacement therapy (HRT) and menstrual pattern, and categorised as 'surgical menopause', 'not defined

due to HRT or oral contraceptive pill, 'pre-menopausal', 'peri-menopausal' or 'post-menopausal'. Four dietary intake variables derived from the FFQ, (fibre, cholesterol, vitamin C and sodium) were also included as covariates.

Data analysis

Generalised Estimating Equation (GEE) analyses investigated associations of quintiles of total flavonoid and subclass intake with incident hypertension, adjusting for demographic and dietary variables, and hypertension risk factors. GEE was the chosen statistical approach to enable accounting for repeated exposure and outcome measures in the same individual. Survival analysis could not be performed due to lack of data on date of hypertension diagnosis. An exchangeable correlation matrix, a binomial distribution of dependent variables and a log link function were selected to conduct the GEE. Potential confounders were defined *a priori* based on literature and on the available data. Four models were used to adjust for potential confounders: (1) adjusted for energy intake and age; (2) additionally adjusted for hypertension risk factors (diabetes, smoking, physical activity, alcohol intake and menopause status) and demographics (education and income management); (3) additionally adjusted for dietary intake variables related to flavonoid intake and hypertension risk (fibre, vitamin C, sodium and cholesterol); and (4) additionally adjusted for BMI (potential mediator).

Sub-analyses were conducted by adding the total flavonoids, flavanols and flavonols related to tea consumption for each individual. Another sub-analysis was conducted to determine the influence on the results of additionally adjusting for gestational diabetes and hypertension among women who reported a live birth during the study period. All analyses were conducted using the software STATA/SE 15.1 (StataCorp LLC, TX, USA).

2.3 Results

In the middle-aged cohort, 11,221 women completed Survey 3 and after exclusion for hypertension reported prior to Survey 4 (n=3,334), missing data on dietary intake (n=407), hypertension (n=339) and covariates (n=463), as well as implausible energy intake (<500kcal or >5000 kcal/day) (n=48), 6,630 women were included for data analyses (Figure 2-1). In the reproductive-aged cohort, 9,081 women completed Survey 3 and after exclusion for hypertension reported prior to Survey 4 (n=693), missing data on dietary intake (n=140), hypertension (n=1,367) and covariates (n=737), and implausible energy intake (n=45), 6,099 women were included for data analyses (Figure 2-2). No differences were found in baseline characteristics between women in- and excluded for analyses in the middle-aged and reproductive-aged cohort (Table 2-S1 and 2-S2).

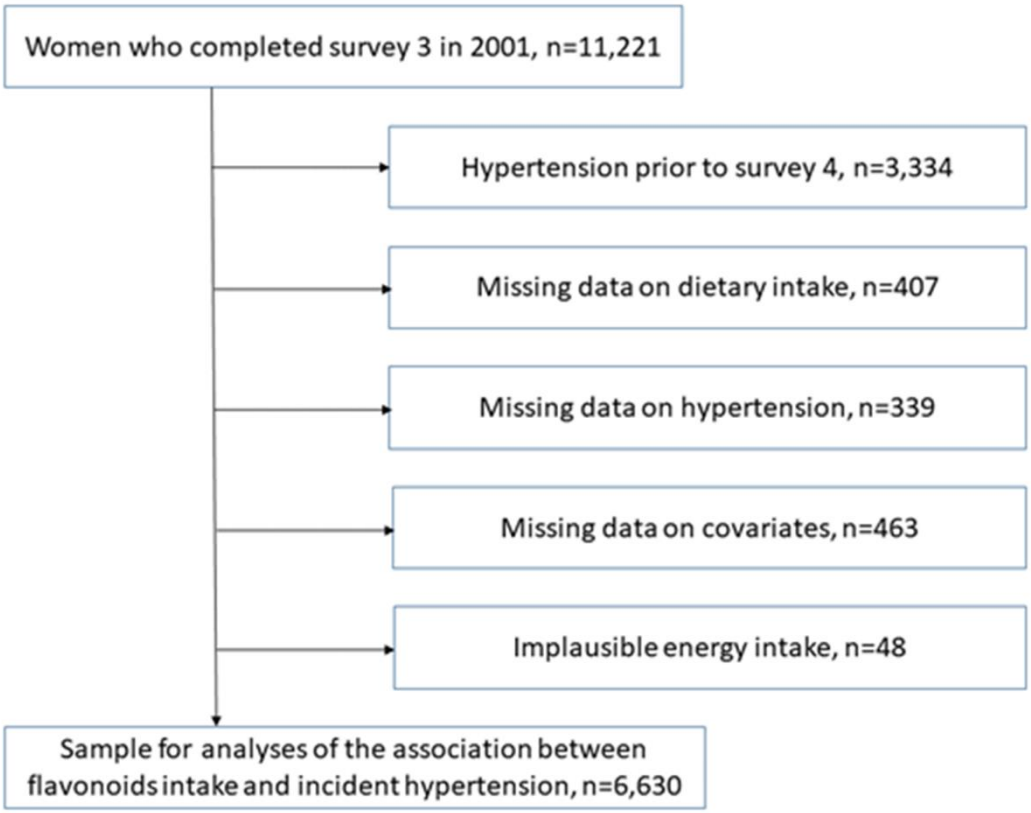


Figure 2.1. Flow diagram of the sample for analyses of the association between flavonoids intake and incidenct hypertension in middle-aged women in the Australian Longitudinal Study on Women's Health, **n=6,630**

All baseline characteristics are presented according to quintiles of total flavonoid intake (Table 2-1 and 2-2 for middle-aged and reproductive-aged cohorts, respectively). There was a higher level of education and physical activity, and a lower BMI, according to higher quintiles of total flavonoid intake, in both cohorts. In the middle-aged cohort, there was a decreasing number of current smokers across the quintiles of total flavonoids consumption, which was not observed in the reproductive-aged cohort. Comparing the 5th quintile of intake between cohorts, there was more than double the number of current smokers in the younger cohort compared to the middle-aged cohort (20.8% vs 9.9%). Alcohol consumption had a similar non-linear pattern

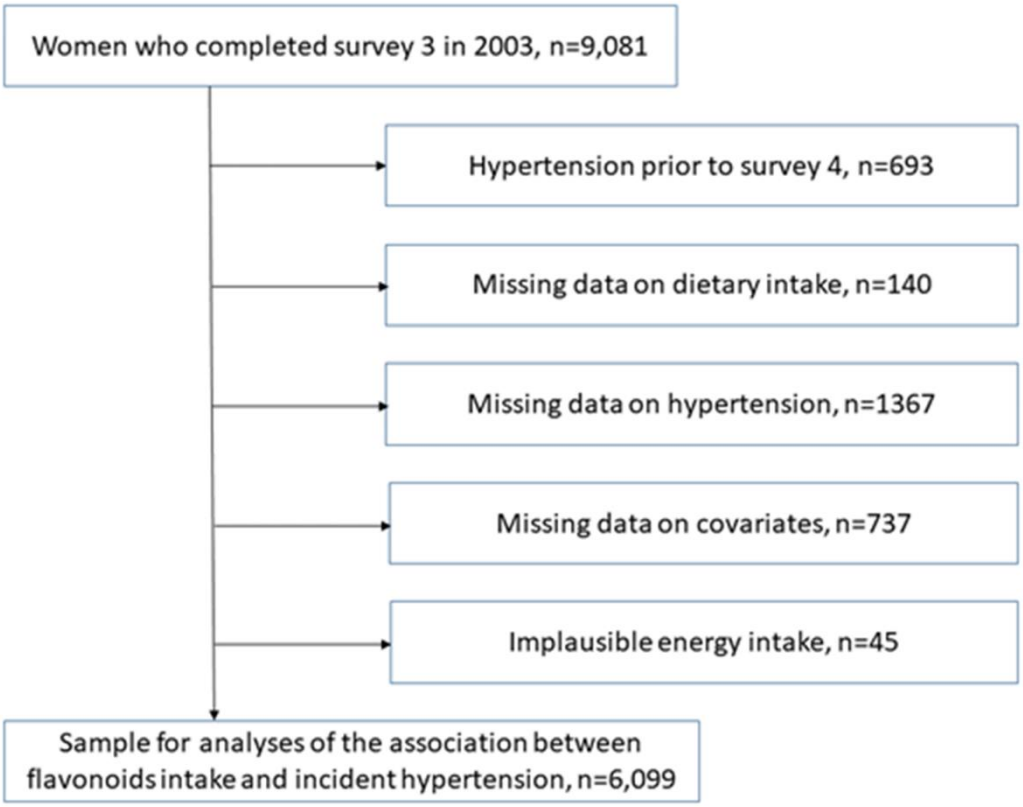


Figure 2-2. Flow diagram of the sample for analyses of the association between flavonoids intake and incidenct hypertension in reproductive-aged women in the Australian Longitudinal Study on Women's Health, **n=6,099**

across quintiles in both cohorts for the 'risky & high risk" alcohol intake category, however individuals in the 5th quintile had higher values in both cohorts, which could be attributed to the high flavonoid content of red wine. Concerning income management, the category "easy" presented an increase across quintiles of flavonoid intake, while a decrease was observed in the category "impossible/difficult all the time", showing that higher income is associated with higher flavonoid intake.

Table 2-1. Baseline characteristics of middle-aged women in the Australian Longitudinal Study on Women's Health according to quintiles of total flavonoids intake, **n=6,630**

		Total flavonoid intake					
Variables	Categories	**Quintile 1 n =1289**	**Quintile 2 n =1304**	**Quintile 3 n =1315**	**Quintile 4 n =1367**	**Quintile 5 n =1353**	p-value[a]
Mean age (years) (SD)	-	52.3 (1.44)	52.4 (1.42)	52.4 (1.44)	52.5 (1.45)	52.5 (1.46)	0.0027
Education[b] (%)	low	74.31	66.08	62.48	54.93	53.72	< 0.0001
	intermediate	15.55	19.93	22.06	24.09	23.70	
	high	10.14	13.99	15.46	20.98	22.58	
Income management (%)	impossible/difficult all the time	12.80	11.03	8.05	7.16	6.67	< 0.0001
	difficult some of the time	26.67	26.97	23.33	26.20	22.41	
	not too bad	43.11	44.49	48.10	44.14	47.39	
	easy	17.42	17.52	20.52	22.49	23.52	
BMI[c] (%)	normal weight	46.26	49.58	51.18	51.47	54.92	< 0.0001
	overweight	32.68	30.21	32.28	32.60	31.14	
	obese	21.06	20.20	16.55	15.92	13.94	
Smoking status (%)	never smoker	52.46	58.29	65.82	65.63	61.76	< 0.0001
	former smoker	25.10	25.30	21.88	24.09	28.31	
	current smoker	22.44	16.40	12.30	10.28	9.92	
Physical activity[d] (%)	sedentary or low	57.09	51.81	45.84	44.23	39.78	< 0.0001
	moderate	21.16	18.91	23.69	23.67	25.41	
	high	21.75	29.29	30.47	32.10	34.82	
Alcohol consumption (%)	rarely & non-drinker	46.46	39.94	37.88	30.83	23.78	< 0.0001
	low risk	47.64	53.01	58.32	64.70	65.53	
	risky & high risk	5.91	7.04	3.80	4.47	10.69	
Menopause status (%)	surgical menopause	29.23	29.19	25.77	27.13	22.67	0.039
	not defined due to HRT/OCP	18.60	19.46	19.98	18.96	18.99	
	pre-menopausal	9.55	9.36	10.49	8.85	10.44	
	peri-menopausal	19.39	17.33	17.99	19.55	22.67	
	post-menopausal	23.23	24.65	25.77	25.53	25.24	
Diabetes type I & II (%)	no	97.24	97.50	97.02	96.38	98.03	0.167
	yes	2.76	2.50	2.98	3.62	1.97	

BMI, body mass index; HRT, hormone replacement therapy; OCP, oral contraceptive pill. [a]P-value of chi-square test. [b]Education: low, none or school certificate; intermediate, high school certificate, trade/apprenticeship, certificate/diploma; high: university or higher university degree. [c]BMI: normal weight, 16-25 kg/m^2; overweight, 25-30 kg/m^2, obese, >30 kg/m^2. [d]Physical activity: sedentary & low, <500 metabolic equivalents (MET) per week; moderate, 500-1000 MET per week; high, >1000 MET per week.

Table 2-2. Baseline characteristics of reproductive-aged women in the Australian Longitudinal Study on Women's Health according to quintiles of total flavonoids intake, **n=6,099**

		Total flavonoid intake					
Variables	Categories	**Quintile 1 n =1177**	**Quintile 2 n =1203**	**Quintile 3 n =1245**	**Quintile 4 n =1236**	**Quintile 5 n =1239**	p-value[a]
Mean age (years) (SD)	-	27.6 (1.45)	27.6 (1.46)	27.4 (1.47)	27.6 (1.43)	27.6 (1.44)	0.0177
Education[b] (%)	low	13.42	9.72	7.03	5.24	6.02	< 0.0001
	intermediate	51.60	47.11	43.06	37.69	34.81	
	high	34.98	43.18	49.90	57.07	59.17	
Income management (%)	impossible/difficult all the time	12.07	10.99	9.92	7.81	9.47	< 0.0001
	difficult some of the time	33.64	30.62	24.86	27.79	25.44	
	not too bad	37.77	39.94	39.79	41.54	39.74	
	easy	16.51	18.45	25.43	22.85	25.35	
BMI[c] (%)	normal weight	58.93	63.98	65.61	67.36	68.74	< 0.0001
	overweight	22.60	22.47	20.42	21.66	20.51	
	obese	18.47	13.54	13.97	10.98	10.75	
Smoking status (%)	never smoker	57.38	63.98	62.04	62.12	60.36	< 0.0001
	former smoker	16.82	15.41	17.15	17.51	18.84	
	current smoker	25.80	20.61	20.81	20.38	20.81	
Physical activity[d] (%)	sedentary or low	51.39	44.16	39.11	33.04	30.37	< 0.0001
	moderate	22.08	24.83	25.53	24.33	23.87	
	high	26.52	31.01	35.36	42.63	45.76	
Alcohol consumption (%)	rarely & non-drinker	46.54	38.96	32.18	24.83	20.71	< 0.0001
	low risk	50.77	58.98	65.41	72.50	73.27	
	risky & high risk	2.68	2.06	2.41	2.67	6.02	
Diabetes type I & II (%)	no	98.86	98.92	98.65	98.81	99.01	0.956
	yes	1.14	1.08	1.35	1.19	0.99	

BMI, body mass index. [a]P-value of chi-square test. [b]Education: low, none or school certificate; intermediate, high school certificate, trade/apprenticeship, certificate/diploma; high: university or higher university degree. [c]BMI: normal weight, 16-25 kg/m^2; overweight, 25-30 kg/m^2, obese, >30 kg/m^2. [d]Physical activity: sedentary & low, <500 metabolic equivalents (MET) per week; moderate, 500-1000 MET per week; high, >1000 MET per week.

Baseline nutrient and food group intake, according to quintiles of total flavonoid intake, are presented in the Supplementary material (Table 2-S3 and 2-S4 for middle-aged and reproductive-aged cohorts, respectively). There was an increasing consumption of the majority of dietary variables (energy, PUFA, protein, carbohydrates, vitamin C, fibre, calcium, iron, low fat dairy, vegetables, legumes, fruits and fruit juice) across quintiles in both cohorts, while

'high fat dairy' was the only variable which decreased across quintiles. The consumption of tea increased with the increasing consumption of total flavonoids in the middle-aged cohort, but not in the reproductive-aged cohort.

The baseline proportional intake of flavonoid subclasses that contributed to total flavonoids for both cohorts are presented in the Supplementary material (Figure 2-S1). The addition of tea substantially increased total flavonoid intake (89.8 to 244.7 mg and 71.5 to 167.2 mg in the middle-aged and reproductive-aged cohorts, respectively) as well as the relative contribution of flavonols to total flavonoid intake (47 to 79%, and 32 to 70%, in the middle-aged and reproductive-aged cohorts, respectively). Baseline food sources of flavonoid subclass intakes are presented in the Supplementary material (Figure 2-S2 and 2-S3 for middle-aged and reproductive-aged cohorts, respectively). The top two contributors to flavonols, flavanols and isoflavones, along with the three top contributors to sources of anthocyanins, flavones and flavanones, were identical in both cohorts. Red wine and apples were the main source of flavanols, while strawberries and red wines were the main source of anthocyanins. Orange and orange juice were the main sources of flavanones, while orange juice and watermelon were the major source of flavones. Red wine, onion and spinach were the main sources of flavonols, while soymilk was the major source of isoflavones.

In the middle-aged cohort, there were 1,645 cases (24.9%) of hypertension during a maximum of 15 years follow-up. Higher intakes of flavones [adjusted relative risk (ARR) for intake quintile 5 vs 1: 0.82, 95% CI: 0.70-0.97], isoflavones (0.86, 0.75-0.99) and flavanones (0.83, 0.69-1.00) were associated with a lower risk of hypertension (Table 2-3). Intakes of total flavonoids, anthocyanins, flavanols and flavonols were not associated with incidence of hypertension. In the reproductive-aged cohort, there were 336 cases (5.5%) of hypertension during a maximum of 12 years follow-up. In this cohort, the relative risks for total flavonoids, flavanols and flavonols were lower when comparing quintile 4 versus quintile 1. A higher

intake of flavanols (ARR for intake quintile 4 vs 1: 0.70, 95% CI: 0.49-0.99) was associated with a 30% lower risk of hypertension (Table 2-4). In both cohorts when including tea in the analysis, intake of total flavonoids and flavanols, were not associated with incidence of hypertension (Table 2-S5 and 2-S6). A sub-analysis was conducted in the reproductive-aged cohort among women that gave birth to children. In addition to the fully adjusted model from previous analyses, a model additionally adjusted for gestational diabetes and gestational hypertension did not change the results (Table 2-S7).

Table 2-3. Relative risks for associations of total flavonoids and subclasses intake with incident hypertension in middle-aged women in the Australian Longitudinal Study on Women's Health, **n=6,630**

	Total Flavonoids				
	Quintile 1 n =1289	**Quintile 2 n =1304**	**Quintile 3 n =1315**	**Quintile 4 n =1367**	**Quintile 5 n =1353**
Mean intake (mg) (SD)	27.9 (10.0)	52.4 (5.8)	74.0 (7.1)	102.9 (10.4)	169.6 (48.5)
N (%) cases	312 (24.2)	351 (26.9)	328 (24.9)	342 (25.0)	301 (22.2)
Model 1	1.00	1.02 (0.89 – 1.16)	0.90 (0.78 – 1.03)	0.88 (0.77 – 1.01)	0.76 (0.66 – 0.87)
Model 2	1.00	1.07 (0.93 – 1.22)	0.99 (0.86 – 1.13)	0.99 (0.86 – 1.14)	0.86 (0.74 – 0.99)
Model 3	1.00	1.07 (0.93- 1.22)	0.98 (0.86 – 1.13)	0.99 (0.85 – 1.14)	0.84 (0.71 – 1.00)
Model 4	1.00	1.08 (0.95 – 1.24)	1.02 (0.88 – 1.17)	1.03 (0.89 – 1.19)	0.90 (0.76 -1.06)
	Flavanols				
Mean intake (mg) (SD)	9.7 (4.4)	23.9 (3.8)	36.0 (2.7)	47.6 (4.3)	86.6 (34.8)
N (%) cases	303 (23.5)	341 (26.1)	319 (24.2)	359 (26.2)	312 (23.1)
Model 1	1.00	0.97 (0.85 – 1.11)	0.94 (0.82 – 1.08)	0.97 (0.85 – 1.11)	0.81 (0.70 – 0.93)
Model 2	1.00	1.02 (0.89 – 1.16)	0.97 (0.85 – 1.11)	1.05 (0.92 – 1.20)	0.88 (0.77 – 1.02)
Model 3	1.00	1.04 (0.91 – 1.19)	0.99 (0.86 – 1.13)	1.10 (0.96 – 1.26)	0.94 (0.81 – 1.09)
Model 4	1.00	1.03 (0.90 – 1.18)	1.01 (0.88 – 1.15)	1.10 (0.96 – 1.26)	0.97 (0.83 – 1.12)
	Flavonols				
Mean intake (mg) (SD)	3.4 (1.5)	5.5 (2.6)	6.3 (3.9)	6.4 (3.9)	15.0 (8.3)
N (%) cases	318 (24.7)	304 (23.3)	348 (26.5)	341 (24.9)	323 (23.9)
Model 1	1.00	0.90 (0.78 – 1.03)	0.95 (0.83 – 1.08)	0.96 (0.84 – 1.09)	0.86 (0.76 – 0.99)
Model 2	1.00	0.94 (0.82 – 1.08)	1.00 (0.88 – 1.15)	1.00 (0.88 – 1.14)	0.93 (0.81 – 1.07)
Model 3	1.00	0.96 (0.83 – 1.10)	1.03 (0.90 – 1.17)	1.02 (0.90 – 1.17)	0.98 (0.85 – 1.13)

Model 4	1.00	0.97 (0.84 – 1.11)	1.04 (0.91 – 1.19)	1.04 (0.91 – 1.18)	1.02 (0.89 – 1.18)
	Anthocyanins				
Mean intake (mg) (SD)	0.61 (0.3)	1.6 (0.3)	2.9 (0.5)	5.3 (0.99)	12.4 (5.8)
N (%) cases	308 (23.9)	341 (26.1)	354 (26.9)	311 (22.7)	320 (23.6)
Model 1	1.00	0.99 (0.86 – 1.13)	1.02 (0.90 – 1.17)	0.85 (0.74 -0.98)	0.92 (0.80 – 1.05)
Model 2	1.00	1.03 (0.90 – 1.18)	1.08 (0.95 – 1.24)	0.95 (0.82 – 1.09)	0.99 (0.86 – 1.14)
Model 3	1.00	1.04 (0.91 – 1.19)	1.10 (0.96 – 1.26)	0.97 (0.84 – 1.11)	1.03 (0.89 – 1.19)
Model 4	1.00	1.02 (0.90 – 1.17)	1.08 (0.94 – 1.23)	0.94 (0.81 – 1.08)	0.99 (0.86 – 1.15)
	Flavones				
Mean intake (mg) (SD)	1.54 (0.7)	3.9 (0.7)	6.8 (0.9)	10.7 (1.4)	21.0 (8.5)
N (%) cases	347 (26.9)	305 (23.4)	335 (25.5)	357 (26.1)	290 (21.4)
Model 1	1.00	0.83 (0.73 – 0.95)	0.86 (0.76 – 0.98)	0.88 (0.77 – 1.00)	0.72 (0.63 – 0.83)
Model 2	1.00	0.88 (0.77 – 1.00)	0.94 (0.82 -1.07)	0.98 (0.86 – 1.11)	0.81 (0.70 – 0.93)
Model 3	1.00	0.87 (0.76 – 1.00)	0.93 (0.81 – 1.07)	0.97 (0.84 – 1.12)	0.82 (0.69 – 0.97)
Model 4	1.00	0.88 (0.77 – 1.00)	0.94 (0.82 – 1.08)	0.96 (0.84 – 1.11	0.81 (0.69 – 0.96)
	Isoflavones				
Mean intake (mg) (SD)	0.22 (2.5)	0.31 (3.4)	0.30 (2.8)	0.61 (4.0)	13.8 (16.6)
N (%) cases	361 (28.0)	340 (26.1)	318 (24.2)	325 (23.8)	290 (21.4)
Model 1	1.00	0.92 (0.81 -1.05)	0.85 (0.74 – 0.97)	0.84 (0.74 – 0.97)	0.70 (0.61 – 0.80)
Model 2	1.00	0.92 (0.81 – 1.04)	0.86 (0.76 -0.98)	0.89 (0.78 – 1.01)	0.77 (0.68 – 0.89)
Model 3	1.00	0.92 (0.81 – 1.05)	0.87 (0.76 – 0.99)	0.90 (0.80 – 1.04)	0.81 (0.71 -0.94)
Model 4	1.00	0.91 (0.80 – 1.04)	0.87 (0.76 - 0.99)	0.95 (0.84 – 1.09)	0.87 (0.76 – 1.00)
	Flavanones				
Mean intake (mg) (SD)	6.1 (9.2)	13.9 (10.2)	22.6 (9.8)	35.4 (11.3)	64.7 (27.9)
N (%) cases	343 (26.6)	322 (24.7)	339 (25.8)	322 (23.5)	308 (22.8)
Model 1	1.00	0.85 (0.74 – 0.97)	0.88 (0.77 – 0.99)	0.82 (0.72 – 0.94)	0.77 (0.67 – 0.88)
Model 2	1.00	0.90 (0.79 – 1.03)	0.96 (0.84 – 1.10)	0.92 (0.80 – 1.05)	0.87 (0.76 – 1.00)
Model 3	1.00	0.90 (0.79 – 1.03)	0.94 (0.82 – 1.08)	0.88 (0.75 – 1.03)	0.79 (0.65 – 0.96)
Model 4	1.00	0.91 (0.80 – 1.04)	0.95 (0.83 -1.09)	0.90 (0.77 – 1.04)	0.83 (0.69 – 1.00)

Model 1: adjusted for total energy intake and age; Model 2: model 1 + additionally adjusted for hypertension risk factors (smoking status, diabetes, physical activity, alcohol intake, menopause status) and demographics variables (education, income management); Model 3: model 2 + additionally adjusted for dietary intake variables (fibre, cholesterol, vitamin C, sodium); Model 4: model 3 + additionally adjusted for body mass index.

Table 2-4. Relative risks for associations of total flavonoids and subclasses intake with incident hypertension in reproductive-aged women in the Australian Longitudinal Study on Women's Health, **n=6,099**

	Total Flavonoids				
	Quintile 1 n =1177	**Quintile 2 n =1203**	**Quintile 3 n =1245**	**Quintile 4 n =1236**	**Quintile 5 n =1239**
Mean intake (mg) (SD)	32.6 (27.9)	49.2 (29.3)	63.2 (30.6)	83.8 (34.2)	126.0 (54.4)
N (%) cases	74 (6.3)	79 (6.1)	70 (5.3)	51 (4.1)	62 (4.6)
Model 1	1.00	0.97 (0.71 – 1.32)	0.85 (0.62 – 1.17)	0.60 (0.42 – 0.86)	0.74 (0.53 – 1.05)
Model 2	1.00	1.02 (0.75 – 1.39)	0.92 (0.66 – 1.27)	0.66 (0.46 – 0.95)	0.79 (0.55 – 1.12)
Model 3	1.00	0.99 (0.72 – 1.36)	0.87 (0.62 – 1.23)	0.61 (0.42 – 0.91)	0.68 (0.44 – 1.06)
Model 4	1.00	1.05 (076. 1.43)	0.93 (0.66 – 1.30)	0.68 (0.46 – 1.01)	0.77 (0.50 – 1.20)
	Flavanols				
Mean intake (mg) (SD)	7.6 (11.8)	12.5 (14.2)	17.1 (13.8)	23.9 (15.0)	48.3 (31.8)
N (%) cases	82 (7.0)	77 (6.4)	69 (5.5)	53 (3.9)	55 (4.1)
Model 1	1.00	0.89 (0.66 – 1.21)	0.76 (0.56 – 1.04)	0.58 (0.41 – 0.81)	0.61 (0.43 – 0.85)
Model 2	1.00	0.93 (0.69 – 1.27)	0.81 (0.59 – 1.12)	0.63 (0.44 – 0.89)	0.61 (0.43 – 0.88)
Model 3	1.00	0.94 (0.69 – 1.28)	0.82 (0.59 – 1.14)	0.64 (0.45 – 0.92)	0.64 (0.44 – 0.93)
Model 4	1.00	0.98 (0.72 – 133)	0.90 (0.65 – 1.24)	0.70 (0.49 – 0.99)	0.72 (0.49 – 1.04)
	Flavonols				
Mean intake (mg) (SD)	3.4 (1.5)	5.5 (2.6)	6.3 (3.9)	6.4 (3.9)	15.0 (8.3)
N (%) cases	71 (5.5)	82 (6.3)	57 (4.3)	50 (3.7)	55 (4.1)
Model 1	1.00	1.06 (0.78 – 1.45)	0.75 (0.54 – 1.06)	0.65 (0.45 – 0.92)	0.72 (0.51 – 1.01)
Model 2	1.00	1.10 (0.81 – 1.51)	0.81 (0.57 – 1.14)	0.69 (0.48 – 0.99)	0.74 (0.52 – 1.06)
Model 3	1.00	1.10 (0.80 – 1.50)	0.80 (0.56 – 1.15)	0.69 (0.48 – 1.00)	0.74 (0.51 – 1.08)
Model 4	1.00	1.12 (0.82 – 1.54)	0.88 (0.61 – 1.25)	0.79 (0.54 – 1.14)	0.86 (0.59 – 1.26)
	Anthocyanins				
Mean intake (mg) (SD)	1.8 (2.9)	2.9 (2.8)	4.1 (2.9)	5.8 (3.4)	10.5 (7.5)
N (%) cases	70 (5.4)	69 (5.3)	68 (5.2)	68 (5.0)	61 (4.5)
Model 1	1.00	0.92 (0.66 – 1.28)	0.89 (0.64 – 1.24)	0.92 (0.66 – 1.27)	0.86 (0.61 – 1.20)
Model 2	1.00	0.97 (0.70 – 1.34)	0.95 (0.68 – 1.32)	0.98 (0.70 – 1.37)	0.89 (0.63 – 1.26)
Model 3	1.00	0.98 (0.70 – 1.36)	0.96 (0.69 – 1.33)	0.99 (0.71 – 1.40)	0.93 (0.65 -1.33)
Model 4	1.00	0.97 (0.70 – 1.35)	0.93 (0.67 – 1.30)	0.99 (0.71 – 1.39)	0.88 (0.62 – 1.26)
	Flavones				
Mean intake (mg) (SD)	1.5 (0.7)	3.9 (0.7)	6.8 (0.9)	10.7 (1.4)	21.0 (8.5)
N (%) cases	75 (6.4)	58 (4.8)	78 (6.3)	57 (4.6)	68 (5.5)
Model 1	1.00	0.76 (0.55 – 1.07)	0.99 (0.73 – 1.37)	0.69 (0.49 – 0.97)	0.83 (0.59 – 1.17)

Model 2	1.00	0.80 (0.57 – 1.11)	1.04 (0.77 – 1.43)	0.75 (0.53 – 1.05)	0.89 (0.64 – 1.26)
Model 3	1.00	0.80 (0.57 – 1.12)	1.03 (0.75 – 1.43)	0.73 (0.50 – 1.05)	0.84 (0.54 – 1.32)
Model 4	1.00	0.79 (0.57 – 1.12)	1.06 (0.77 – 1.46)	0.73 (0.51 – 1.05)	0.84 (0.54 – 1.30)
	Isoflavones				
Mean intake (mg) (SD)	0.3 (3.1)	0.3 (2.9)	0.5 (3.9)	1.0 (4.8)	9.5 (14.1)
N (%) cases	71 (6.0)	62 (5.1)	82 (6.6)	68 (5.5)	53 (4.3)
Model 1	1.00	0.86 (0.61 – 1.20)	1.16 (0.84 – 1.58)	0.91 (0.65 – 1.26)	0.68 (0.48 – 0.96)
Model 2	1.00	0.90 (0.64 – 1.25)	1.20 (0.88 – 1.64)	0.99 (0.71 – 1.37)	0.76 (0.53 – 1.08)
Model 3	1.00	0.90 (0.64 – 1.25)	1.22 (0.89 – 1.66)	1.01 (0.72 – 1.41)	0.83 (0.57 – 1.20)
Model 4	1.00	0.92 (0.66 – 1.28)	1.30 (0.95 -1.77)	1.12 (0.80 – 1.57)	1.03 (0.71 – 1.49)
	Flavanones				
Mean intake (mg) (SD)	11.2 (15.9)	17.1 (15.0)	24.8 (16.5)	33.7 (18.4)	57.1 (31.2)
N (%) cases	69 (5.9)	70 (5.8)	51 (4.1)	71 (5.7)	68 (5.5)
Model 1	1.00	1.01 (0.73 – 1.40)	0.81 (0.58 – 1.15)	0.98 (0.70 – 1.35)	0.95 (0.68 – 1.33)
Model 2	1.00	1.06 (0.76 – 1.46)	0.86 (0.61 – 1.21)	1.06 (0.76 – 1.48)	1.03 (0.73 – 1.45)
Model 3	1.00	1.07 (0.77 – 1.49)	0.87 (0.61 – 1.24)	1.09 (0.76 – 1.56)	1.03 (0.64 – 1.66)
Model 4	1.00	1.13 (0.82 – 1.57)	0.90 (0.63 – 1.28)	1.13 (0.79 – 1.63)	1.08 (0.67 – 1.73)

Model 1: adjusted for total energy intake and age; Model 2: model 1 + additionally adjusted for hypertension risk factors (smoking status, diabetes, physical activity, alcohol intake) and demographics variables (education, income management); Model 3: model 2 + additionally adjusted for dietary intake variables (fibre, cholesterol, vitamin C, sodium); Model 4: model 3 + additionally adjusted for body mass index.

2.4 Discussion

Findings from this population-based prospective study of Australian women showed an association between a higher dietary intake of flavonoid subclasses and lower incidence of hypertension. In the middle-aged cohort, a higher intake of flavone, flavanone and isoflavone subclasses of flavonoids were associated with a lower incidence of hypertension. In the reproductive-aged cohort, higher intakes of flavanols were associated with lower incidence of hypertension. Inconsistencies in the present findings between cohorts may be explained by generational differences in food intake, as previously demonstrated by differences in the level of consumption of individual food items in the ALSWH middle-aged and reproductive-aged cohorts(187). Our analyses have also shown differences in the contribution of foods to

flavonoid intake, both in the diversity and in the percentage of food items for each subclass, which may further contribute to explaining the differences in associations between the cohorts. Additionally, the generational differences on hypertension risk factors(211) may also partly explain the inconsistencies found for associations between subclasses of flavonoids and incidence of hypertension in the two different age-range cohorts in our study. Similar analyses have been conducted in other populations. Cassidy et al.(212) examined the association between flavonoid intake and incidence of hypertension in a combined grouping of three different cohorts that included 133,914 women from the Nurses' Health Study I and II, and 23,043 men from the Health Professionals Follow-Up Study. Across 14 years of follow-up, there were 29,018 cases of hypertension in women and 5629 cases of hypertension in men. A reduction of 8% in the risk of hypertension (RR: 0.92; 95% CI: 0.86-0.98) was found, comparing individuals from the highest quintile versus lowest quintile of anthocyanin intake. This association was stronger among individuals younger than 60 years (RR: 0.88; 95% CI: 0.84-0.93). Other subclasses were not associated with hypertension; however, a pooled analysis for individual compounds suggested a 5% (95% CI: 0.91-0.99) lower risk for the highest compared with the lowest quintiles of intake of the flavone apigenin. In participants ≤60 y, a 6% (95% CI: 0.88-0.97) lower risk was observed for the flavanol catechin(212). Another study conducted in women found that the highest quintile of flavonol intake was associated with a 10% lower rate of hypertension compared to the lowest quintile (HR: 0.90; 95% CI: 0.84-0.97) after a follow-up of 14 years(213). Proanthocyanidin (polymerised flavanols) and anthocyanin subclasses also showed a similarly lowered risk of 9% in incidence of hypertension between highest and lowest quintiles of consumption (HR: 0.91; 95% CI: 0.84-0.97; and HR: 0.91, 95% CI: 0.85-0.97, respectively)(213).

In the present study, there were slight differences between the two age cohorts with regard to contribution of subclasses to total flavonoid intake, but few relevant differences compared

to the aforementioned studies conducted in other countries. In both the middle-aged and reproductive-aged cohorts, the four subclasses that were main sources of flavonoids were the same (in descending order: flavanols, flavanones, flavonols and anthocyanins) as those reported in USA cohorts(212,214). In a cohort of French women, anthocyanins were the subclass that provided the second highest amount of flavonoids(213). There were, however, pronounced differences between our study and other cohort studies in total amount of flavonoid intake. A limitation is that the FFQ in the ALSWH did not include tea, but this could be ascertained from questions in another part of the survey. The daily total flavonoid intake, including tea flavonoids, in the middle-aged and reproductive-aged cohorts was 245 and 167 mg/day, respectively. Comparing to cohort studies from other countries, our results were similar to the value of 190 mg/day reported in NHANES 1999–2002(214), but lower than the 358 to 415 mg/day reported in the analysis of the three Health Professionals cohort studies by Cassidy et al.(212) and the amount of 575 ±302 mg/day reported in a French cohort(213). Comparisons between such studies need to be interpreted with caution due to the use of different databases for flavonoid calculation, as well as considering that such databases are constantly being updated to reflect changes in the food supply. However, a previous study conducted in a cohort of women aged over 75 years (n=1063) observed that the application of the United States Department of Agriculture (USDA) and phenol-explorer source data yielded a high correlation of intake estimates for total-flavonoids, flavanols, flavanones and anthocyanidins, while a poorer correlation for flavonols and flavones was found(215). Both studies conducted in USA(212,214) used the United States Department of Agriculture (USDA) database, while the study conducted in France(213) used the European Phenol-Explorer database.

In both the middle-aged and reproductive-aged cohorts, total flavonoid intake increased with higher education, income and physical activity and lower BMI, with the same pattern

found for education(213,214), income(214), physical activity(212,214) and BMI(212,213) among other studies. A higher intake of total flavonoids found in our middle-aged, compared to reproductive-aged, cohort (245 vs 167 mg/day, respectively) is consistent with an increased flavonoid density of diets with age that is reported in other cohorts(213,214).

Three subclasses of flavonoids, flavones, isoflavones and flavanones, were associated with a lower risk incident hypertension in the middle-aged cohort. The primary contributor for flavones and flavanones was orange juice (66% and 45% respectively), while for isoflavones was soy milk (93%). Flavones have been shown to exert an anti-hypertensive effect, mainly through their vasorelaxation properties(216). Flavone derivatives possess an endothelium-dependent vasorelaxant effect, associated with an increased production of NO and prostacyclin PGI_2 in a concentration-dependent manner(216). For example, luteolin, a flavone found in high concentrations in food items evaluated in the present study (including watermelon, celery and pumpkin) has been shown to exert a direct effect on vasorelaxation by improving acetylcholine-induced NO generation(217).

The BP lowering potential of isoflavones is linked to their signalling properties in the endothelium. The soy isoflavones, genistein, daidzein and glycitein, activate endothelial nitric oxide synthase and increase the capacity of serum to stimulate prostacyclin release in human endothelial cells(218). However, their effects on blood pressure remain equivocal, mainly due to inter-individual differences in equol (a gut microbiota-derived isoflavone metabolite) production as well as different dietary sources of isoflavones(218,219).

The main flavanones, naringin, hesperidin, and eriodictoyl, present a wide range of beneficial properties, such antioxidant, anti-inflammatory, hypolipidemic, and anti-atherogenic activities(217). The main source of flavanones worldwide are citrus fruits(217), as was found in both cohorts in the present study. A few studies support our findings of the association between flavanone intake and reduced incident hypertension. Experimental studies indicate

that the antihypertensive activity of flavones are mediated by endothelium-dependent and endothelium-independent mechanisms. Not only can flavones increase NO generation, but can also reduce $[Ca^{2+}]_i$ and the consequent contraction of endothelial muscle cells(220).

The inclusion of tea flavonoids in our analyses resulted in the association of flavanols with lower incidence of hypertension not remaining longer significant in the reproductive-aged cohort. Some factors may explain this finding. The missing values and response rate of the FFQ did not match the questions about tea within the survey. Thus, the sub-analysis had a lower sample size and less statistical power. The percentage of total flavonoids and flavanols from tea is also much higher in the present study compared to other similar studies in the USA(212,214) and France(213), in which the annual per capita consumption of tea is 0.23 and 0.20 kg, compared to 0.75 kg in the Australian population. Lastly, the consumption of black tea alone was not associated with a lower incidence of hypertension in the present study (ARR for intake quintile 4 vs 1: 0.96, 95% CI: 0.74-1.59).

Limitations of this study include the use of self-reported data for hypertension. This may have caused misclassification of cases; however, a previous study(209) showed a high correlation (89%) between self-reported doctor-diagnosed hypertension and use of antihypertensive medication. Moreover, flavonoid content in foods is likely to be influenced by seasonality and geographic areas, and there may be discrepancies between food composition databases and flavonoid content in foods between different countries. Potential measurement error and selection bias based on the factors of seasonality and geographic areas were attenuated by the fact that the FFQ reflected 12 months of dietary intake and that recruitment of participants was proportional in all Australian states. In general, FFQs may lack detail about some flavonoid-rich food sources(221). The FFQ included in ALSWH was developed over two decades ago(204), and it may not have included relevant high-flavonoid food items that have increased in popularity over this period, such as blackberries, cherries, blueberries and

raspberries(222). This could have led to an underestimation of anthocyanin intake, as the mean intake in the present study was 4.7 mg/day in the middle-aged and 5.0 mg/day in the reproductive-aged cohort, compared to 24.2 mg/day reported in 2019 in the overall Australian population from a nationally representative sample that used two 24-hr recalls as the method of dietary assessment(222). The mean anthocyanin intakes in other cohort studies that have found a reduction in incident hypertension were 12.5 mg/day(212) and 71.0 mg/day(213), while another cohort from NHANES 1999–2002(214) reported a dietary intake of only 3.03 mg/day. Strengths of this study include the representative sample of Australian women across two age cohorts, the prospective design, and repeated measures.

Residual confounding is a known problem in all observational studies; however, adjustments were made for key confounding factors, in-line with previous studies. Potential confounders were extensively explored in our models, with access to a wide range of demographic, hypertension risk factor and dietary intake variables. The large sample size allowed for a stepwise regression with bidirectional elimination in order to create the models for such adjustments, therefore avoiding major multicollinearity and overfitting issues of the model. Results were not adjusted for multiple testing. A common limitation is the inability to adjust for all dietary intake variables that are highly related (collinearity). The main predictor variable in this study, flavonoids, represents bioactive compounds that are present in virtually all plants; therefore, adjusting for all dietary components such as fibre, potassium and inorganic nitrates, that are also present in plants and/or other high-flavonoid food items is not viable in the modelling. We included fibre in our model because of its high significance in the model, and the well-established contribution of fibre in overall diet quality that is associated with blood pressure, as well as its high linearity with potassium and inorganic nitrates. Lastly, there was a relevant number of missing survey data on both cohorts; however, there is only a small probability that such loss over time was selective, as it is unlikely that a person would be unable

or unwilling to complete the next survey as a result of being diagnosed with hypertension. Hypertension is a 'silent' condition that per se has no major cognitive or physical implications, and has a non-invasive and non-onerous treatment(189). Potential attrition bias could be due to hypertension being a risk factor for major adverse cardiovascular events (MACE), which can lead to debilitating complications(223); however, this would only have been a cause for selective drop-out if a MACE occurred without the diagnosis of hypertension, or after the diagnosis of hypertension and before the following survey (period of approximately 3 years). Still, there was a higher response rate for surveys conducted in the middle-aged cohort (62.9%) compared to the reproductive-aged cohort (50.0%), despite a 5-fold higher number of cases of hypertension in the former. Concerning the exclusion of women due to missing data, there was no significant change in any variable included in the models in both cohorts. Recruitment of participants was proportional to population size in all Australian states, thereby enabling generalizability of the study findings to Australian women.

2.5 Conclusion

Flavonoid intake was associated with a lower incidence of hypertension in two population-based cohorts of Australian women who were either of reproductive-age or middle-aged. Higher intakes of flavones, isoflavones and flavanones, attributed mainly to orange, orange juice, apples and soy milk, were associated with a reduced risk of hypertension among middle-aged women followed-up over 15 years. Higher intakes of flavanols, attributed mainly to red wine and apples, were associated with a reduced risk of hypertension among reproductive-aged women followed-up for 12 years. These findings can be used in nutritional messages and policies aimed at improving the cardiovascular health of women of different life stages.

2.6 Supplementary Material

Table 2-S1. Baseline characteristics of the study population comparing the sample with exclusions (n=6,630) with the sample with no exclusions (n=7,887) for missing data of middle-aged women in the Australian Longitudinal Study on Women's Health

Variables	Categories	n=6,630	n=7,887	*P*-value[a]
Mean age (years)	-	58.4 ±1.4	58.4 ±1.4	0.99
Total energy (kcal)	-	1583.3 ±539.8	1579.0 ±549.0	0.64
Total flavonoid intake	-	89.8 ±54.1	88.1 ±54.5	0.06
Total flavonoid intake (including tea)	-	244.7 ±143.1	243.3 ±144.1	0.54
Fibre	-	20.3 ±8.1	20.0 ±8.0	0.06
Cholesterol	-	240.3 ±107.5	241.3 ±110.1	0.58
Vitamin C	-	117.1 ±64.0	116.5 ±65.5	0.58
Sodium	-	2105.0 ±782.1	2098.3 ±790.3	0.61
Education[b] (%)	low	61.2	63.2	0.97
	intermediate	21.4	20.7	
	high	17.4	16.1	
Income management (%)	impossible/difficult all the time	9.7	10.6	0.99
	difficult some of the time	21.9	22.2	
	not too bad	45.5	45.4	
	easy	22.9	21.9	
BMI[c] (%)	normal weight	45.9	45.3	0.98
	overweight	33.8	33.9	
	obese	20.4	20.8	
Smoking status (%)	never smoker	60.8	60.3	0.97
	former smoker	29.0	28.8	
	current smoker	10.2	10.8	
Physical activity[d] (%)	sedentary or low	35.11	36.2	0.98
	moderate	23.7	23.5	
	high	41.1	40.3	
Alcohol consumption (%)	rarely & non-drinker	32.8	33.8	
	low risk	61.0	60.1	
	risky & high risk	6.2	6.1	
Menopause status (%)	surgical menopause	30.0	30.5	1.0
	not defined due to HRT/OCP	7.1	7.0	
	pre-menopausal	0.1	0.1	
	peri-menopausal	2.0	2.0	
	post-menopausal	60.7	60.3	
Diabetes type I & II (%)	no	94.8	94.8	1.0
	yes	5.2	5.2	

Values are mean and SD (±) or percentages (%). BMI, body mass index; HRT, hormone replacement therapy; OCP, oral contraceptive pill. [a]*P*-value of independent t-test or chi-square test. [b]Education: low, none or school certificate; intermediate, high school certificate, trade/apprenticeship,

certificate/diploma; high: university or higher university degree. [c]BMI: normal weight, 16-25 kg/m^2; overweight, 25-30 kg/m^2, obese, >30 kg/m^2. [d]Physical activity: sedentary & low, <500 metabolic equivalents (MET) per week; moderate, 500-1000 MET per week; high, >1000 MET per week.

Table 2-S2. Baseline characteristics of the study population comparing the sample with exclusions (n=6,099) versus the sample with no exclusions (n=8,388) for missing data of reproductive-aged women in the Australian Longitudinal Study on Women's Health

Variables	Categories	n=6,099	n=8,388	*P*-value[a]
Total energy (kcal)	-	1638.7 ±586.5	1638.9 ±604.7	
Total flavonoid intake	-	71.5 ±49.0	70.3 ±48.8	0.14
Total flavonoid intake (including tea)	-	167.2 ±123.5	165.5 ±123.4	0.41
Fibre	-	19.4 ±7.3	19.3 ±7.4	0.42
Cholesterol	-	266.9 ±115.0	269.3 ±122.1	0.23
Vitamin C	-	105.9 ±59.0	104.9 ±59.0	0.31
Sodium	-	2256.8 ±889.2	2267.4 ±925.3	0.49
Mean age (years) (SD)	-	33.7 ±1.4	33.7 ±1.5	0.21
Education[b] (%)	low	5.9	7.0	0.84
	intermediate	37.6	40.0	
	high	56.7	53.1	
Income management (%)	impossible/difficult all the time	10.1	11.2	0.98
	difficult some of the time	26.5	28.2	
	not too bad	41.2	39.7	
	easy	22.2	20.9	
BMI[c] (%)	normal weight	56.0	55.8	0.99
	overweight	25.5	25.5	
	obese	18.5	18.7	
Smoking status (%)	never smoker	65.2	62.2	0.85
	former smoker	23.3	24.6	
	current smoker	11.5	13.4	
Physical activity[d] (%)	sedentary or low	49.4	49.6	0.99
	moderate	22.1	22.5	
	high	28.4	27.8	
Alcohol consumption (%)	rarely & non-drinker	35.6	36.3	0.99
	low risk	60.3	59.6	
	risky & high risk	4.1	4.2	
Diabetes type I & II (%)	no	97.8	97.6	0.99
	yes	2.2	2.4	

Values are mean and SD (±) or percentages (%). BMI, body mass index. [a]*P*-value of independent t-test or chi-square test. [b]Education: low, none or school certificate; intermediate, high school certificate, trade/apprenticeship, certificate/diploma; high: university or higher university degree. [c]BMI: normal weight, 16-25 kg/m^2; overweight, 25-30 kg/m^2, obese, >30 kg/m^2. [d]Physical activity: sedentary & low, <500 metabolic equivalents (MET) per week; moderate, 500-1000 MET per week; high, >1000 MET per week.

Table 2-S3. Baseline nutrient and food group intake according to quintiles of total flavonoids intake in middle-aged women in the Australian Longitudinal Study on Women's Health, **n=6,630**

	Total flavonoid intake					
Intake per day	**Quintile 1 n =1289**	**Quintile 2 n =1304**	**Quintile 3 n =1315**	**Quintile 4 n =1367**	**Quintile 5 n =1353**	**p-value[1]**
Total flavonoids (mg)	27.9 ±10.0	52.4 ±5.8	74.0 ±7.1	102.9 ±10.4	169.6 ±48.5	< 0.0001
Energy (kcal)	1381.5 ±479.0	1476.2 ±484.0	1561.9 ±497.1	1655.7 ±526.1	1784.2 ±590.4	< 0.0001
Flavonoids per 1000 kcal	20.2 ±7.2	35.5 ±4.0	47.4 ±4.6	62.1 ±6.3	95.1 ±27.2	<0.0001
Total fat (g)	58.3 ±24.2	60.4 ±24.1	60.9 ±24.8	63.3 ±25.8	66.9 ±27.1	< 0.0001
Energy from diet (%)	38.0	36.8	35.1	34.4	33.7	
Saturated fat (g)	24.0 ±10.9	24.7 ±11.1	24.4 ±11.2	24.9 ±11.6	25.9 ±11.9	< 0.0001
PUFA (g)	8.8 ±4.5	9.0 ±4.3	9.5 ±4.8	10.2 ±4.8	11.0 ±5.5	< 0.0001
MUFA (g)	20.5 ±8.8	21.3 ±8.9	21.4 ±9.0	22.4 ±9.6	23.6 ±10.22	< 0.0001
Protein (g)	71.4 ±28.1	76.4 ±28.1	79.4 ±26.5	84.7 ±29.9	89.8 ±34.0	< 0.0001
Energy from diet (%)	20.7	20.7	20.3	20.5	20.1	
Carbohydrates (g)	144.2 ±50.2	158.1 ±52.9	175.4 ±54.5	187.8 ±58.4	206.7 ±70.4	< 0.0001
Energy from diet (%)	41.7	42.8	44.9	45.4	46.3	
Vitamin C (mg)	67.9 ±26.7	87.7 ±34.6	110.48 ±40.9	135.8 ±50.6	173.9 ±84.7	< 0.0001
Cholesterol (mg)	225.4 ±107.0	237.1 ±103.2	235.4 ±97.5	244.6 ±108.0	256.0 ±117.1	< 0.0001
Fibre (g)	15.1 ±5.8	17.2 ±6.1	20.0 ±6.6	22.4 ±7.2	25.3 ±9.3	< 0.0001
Calcium (mg)	751.8 ±282.2	807.3 ±284.7	858.8 ±287.5	913.3 ±306.7	931.5 ±318.9	< 0.0001
Iron (mg)	9.5 ±4.2	10.5 ±4.2	11.5 ±4.3	12.4 ±4.8	13.6 ±5.9	< 0.0001
High fat dairy (g)	123.3 ±180.0	98.0 ±163.9	82.1 ±145.5	77.8 ±152.6	66.0 ±135.5	< 0.0001
Low fat dairy (g)	168.7 ±181.1	207.3 ±183.2	231.5 ±178.0	250.9 ±188.8	260.3 ±185.2	< 0.0001
Vegetables (g)	83.4 ±45.0	90.6 ±44.9	98.3 ±42.9	107.2 ±46.7	118.3 ±53.2	< 0.0001
Legumes (g)	26.8 ±19.6	26.8 ±17.6	27.8 ±19.5	28.9 ±19.7	30.3 ±20.3	< 0.0001
Fruits (g)	102.7 ±72.8	139.8 ±84.6	197.1 ±99.0	249.8 ±112.9	312.4 ±152.2	< 0.0001
Fruit juice (g)	20.4 ±32.4	43.3 ±57.8	68.0 ±75.3	89.8 ±95.2	135.4 ±157.1	< 0.0001
Tea (g)	108.7 ±148.2	268.3 ±201.2	288.8 ±195.3	293.2 ±192.5	295.8 ±197.3	< 0.0001

Values presented in mean and standard deviation (±). [1]p-value based on one-way ANOVA. PUFA, polyunsaturated fatty acids; MUFA, monounsaturated fatty acids; CHO, carbohydrates; High fat dairy:

full cream milk, hard cheese, firm cheese, soft cheese, cream cheese, flavoured milk; Low fat dairy: ricotta, cottage cheese, low fat cheese, reduced fat milk, skim milk, soy milk, Vegetables: beetroot, broccoli, cabbage, capsicum, carrots, cauliflower, celery, garlic, lettuce, mushrooms, onion, pumpkin, spinach, tomatoes, zucchini; Legumes: baked beans, bean sprouts, green beans, other beans, chickpeas; Fruit: apples, pineapples, apricots, bananas, mango, watermelon, rockmelon, honeydew, oranges, peaches, pears, strawberries.

Table 2-S4. Baseline nutrient and food group intake according to quintiles of total flavonoids intake in reproductive-aged women in the Australian Longitudinal Study on Women's Health, **n=6,099**

Intake per day	Quintile 1 n =1177	Quintile 2 n =1203	Quintile 3 n =1245	Quintile 4 n =1236	Quintile 5 n =1239	p-value[1]
Total flavonoids (mg)	20.2 ±6.6	39.1 ±5.2	58.6 ±6.2	84.7 ±9.7	147.6 ±43.0	< 0.0001
Energy (kcal)	1436.7 ±526.3	1549.6 ±552.4	1647.6 ±576.4	1681.3 ±595.8	1897.4 ±684.3	<0.0001
Flavonoids per 1000 kcal	14.1 ±4.6	25.2 ±3.3	35.6 ±3.8	50.4 ± 5.8	77.8 ±22.7	<0.0001
Tota fat (g)	61.8 ±27.3	64.0 ±28.5	66.9 ±29.2	66.5 ±29.5	73.1 ±32.2	<0.0001
Energy from diet (%)	38.7	37.2	36.5	35.6	34.7	
Saturated fat (g)	26.6 ±12.6	27.1 ±13.2	27.9 ±13.0	27.2 ±13.5	29.3 ±14.7	<0.0001
PUFA (g)	8.3 ±4.3	8.8 ±4.4	9.4 ±5.0	9.7 ±4.7	11.0 ±5.4	<0.0001
MUFA (g)	21.6 ±9.8	22.4 ±10.3	23.6 ± 10.9	23.5 ±10.8	25.9 ±11.8	<0.0001
Protein (g)	71.8 ±27.0	77.6 ±29.1	81.9 ±30.9	83.1 ±31.9	91.6 ±36.6	<0.0001
Energy from diet (%)	20.0	19.4	19.9	19.8	19.3	
Carbohydrates (g)	150.0 ±54.5	167.3 ±56.9	180.9 ±59.5	189.0 ±63.6	219.6 ±49.6	<0.0001
Energy from diet (%)	41.8	43.2	43.0	45.0	46.3.	
Vitamin c (mg)	64.2 ±22.9	93.3 ±31.0	118.2±40.0	142.8 ±56.0	197.4 ±91.3	<0.0001
Cholesterol (mg)	231.2 ±101.3	239.7 ±107.8	249.0 ±111.8	248.0 ±116.2	269.8 ±133.1	<0.0001
Fibre	14.3 ±5.3	16.7 ±5.7	18.4 ±6.2	20.2 ±6.9	23.8 ±8.6	<0.0001
Calcium (mg)	764.1 ±273.5	824.2 ±268.2	848.0 ±276.6	877.9 ±289.4	912.6 ±328.7	<0.0001
Iron (mg)	9.3 ±3.8	10.6 ±4.4	11.4 ±4.7	11.9 ±4.9	13.5 ±5.5	<0.0001
High fat dairy (g)	150.0 ±196.1	125.2 ±176.8	108.6 ±165.3	91.2 ±149.3	92.2 ±158.1	<0.0001
Low fat dairy (g)	165.1 ±177.2	189.0 ±172.5	202.6 ±174.3	222.9 ±172.0	221.7 ±173.2	<0.0001
Vegetables (g)	72.9 ±42.4	87.8 ±46.1	91.9 ±45.3	98.7 ±46.8	109.4 ±54.8	<0.0001
Legumes (g)	23.6 ±20.7	25.7 ±21.1	26.5 ±21.3	27.7 ±22.0	30.6 ±25.8	<0.0001
Fruits (g)	73.5 ±48.1	110.1 ±65.7	145.0 ±86.9	196.0 ±112.1	267.1 ±153.5	<0.0001
Fruit juice (g)	20.7 ±24.3	60.7 ±54.9	99.1 ±78.2	129.3 ±115.0	222.6 ±210.8	<0.0001
Tea (g)	118.2 ±160.9	144.4 ±164.3	135.4 ±160.7	135.9 ±155.9	143.0 ±160.3	<0.0001

Values presented in mean and standard deviation (±). [1]p-value based on one-way ANOVA. PUFA, polyunsaturated fatty acids; MUFA, monounsaturated fatty acids; CHO, carbohydrates; High fat dairy: full cream milk, hard cheese, firm cheese, soft cheese, cream cheese, flavoured milk; Low fat dairy: ricotta, cottage cheese, low fat cheese, reduced fat milk, skim milk, soy milk, Vegetables: beetroot, broccoli, cabbage, capsicum, carrots, cauliflower, celery, garlic, lettuce, mushrooms, onion, pumpkin, spinach, tomatoes, zucchini; Legumes: baked beans, bean sprouts, green beans, other beans, chickpeas; Fruit: apples, pineapples, apricots, bananas, mango, watermelon, rockmelon, honeydew, oranges, peaches, pears, strawberries.

Table 2-S5. Relative risks for associations of total flavonoids and flavanols intake (including tea) with incident hypertension in middle-aged women in the Australian Longitudinal Study on Women's Health, **n=6,630**

	Total Flavonoids				
	Quintile 1 n =1289	**Quintile 2 n =1304**	**Quintile 3 n =1315**	**Quintile 4 n =1367**	**Quintile 5 n =1353**
Intake (mg), mean±SD	44.8 ±21.6	132.1 ±27.0	241.6 ±28.4	354.3 ±24.6	434.8 ±46.1
N (%) cases	315 (24.4)	312 (24.2)	343 (26.1)	335 (24.5)	329 (24.3)
Model 1	1.00	0.87 (0.76 – 1.00)	0.93 (0.81 – 1.06)	0.98 (0.86 – 1.12)	0.89 (0.78 – 1.02)
Model 2	1.00	0.93 (0.81 – 1.06)	0.99 (0.86 – 1.12)	1.01 (0.88 – 1.15)	0.96 (0.84 – 1.10)
Model 3	1.00	0.94 (0.87 – 1.14)	1.00 (0.89 – 1.16)	1.02 (0.89 – 1.16)	0.98 (0.85 – 1.13)
Model 4	1.00	0.95 (0.83 – 1.09)	1.02 (0.90 – 1.17)	1.05 (0.92 – 1.19)	1.02 (0.89 – 1.18)
	Flavanols				
Intake (mg), mean±SD	13.7 ±8.4	77.0 ±25.7	177.8 ±30.5	288.2 ±38.1	342.0 ±29.8
N (%) cases	314 (24.3)	309 (23.7)	343 (26.1)	325 (23.8)	343 (25.4)
Model 1	1.00	0.90 (0.79 – 1.03)	0.97 (0.85 -1.10)	0.96 (0.84 – 1.10)	0.96 (0.84 – 1.09)
Model 2	1.00	0.95 (0.83 – 1.09)	1.02 (0.89 – 1.16)	0.98 (0.86 – 1.12)	1.03 (0.90 – 1.18)
Model 3	1.00	0.98 (0.85 – 1.12)	1.04 (0.91 – 1.19)	0.99 (0.86 – 1.13)	1.08 (0.94 – 1.23)
Model 4	1.00	0.99 (0.86 – 1.13)	1.06 (0.92 -1.20)	1.03 (0.90 – 1.17)	1.11 (0.97 – 1.27)

Model 1: adjusted for total energy intake and age; Model 2: model 1 + additionally adjusted for hypertension risk factors (smoking status, diabetes, physical activity, alcohol intake, menopause status) and demographics variables (education, income management); Model 3: model 2 + additionally adjusted for dietary intake variables (fibre, cholesterol, vitamin C, sodium); Model 4: model 3 + additionally adjusted for body mass index.

Table 2-S6. Relative risks for associations of total flavonoids and flavanols intake (including tea) with incident hypertension in reproductive-aged women in the Australian Longitudinal Study on Women's Health, **n=5,340**

	Total Flavonoids				
	Quintile 1 n =1030	**Quintile 2 n =1049**	**Quintile 3 n =1090**	**Quintile 4 n =1088**	**Quintile 5 n =1083**
Intake (mg), mean±SD	34.6 ±13.5	77.8 ±13.3	131.6 ±15.7	212.0 ±32.9	370.3 ±72.7
N (%) cases	68 (6.6)	80 (7.6)	58 (5.3)	50 (4.6)	59 (5.4)
Model 1	1.00	1.12 (0.82 – 1.54)	0.80 (0.57 – 1.13)	0.69 (0.48 – 0.99)	0.80 (0.57 – 1.12)
Model 2	1.00	1.19 (0.87 – 1.64)	0.85 (0.61 -1.21)	0.75 (0.52 – 1.08)	0.85 (0.60 – 1.21)
Model 3	1.00	1.18 (0.85 – 1.63)	0.85 (0.60 -1.22)	0.75 (0.51 – 1.09)	0.86 (0.60 – 1.23)
Model 4	1.00	1.26 (0.91 – 1.74)	0.94 (0.66 – 1.34)	0.89 (0.61 – 1.30)	1.04 (0.72 – 1.48)
	Flavanols				
Intake (mg), mean±SD	8.0 ±4.3	30.1 ±9.1	75.9 ±18.5	146.4 ±38.4	300.1 ±67.0
N (%) cases	79 (7.7)	66 (6.3)	56 (5.1)	52 (5.7)	62 (5.7)
Model 1	1.00	0.80 (0.58 – 1.10)	0.69 (0.49 – 0.96)	0.63 (0.45 – 0.88)	0.73 (0.53 – 1.01)
Model 2	1.00	0.84 (0.61 – 1.16)	0.73 (0.52 – 1.02)	0.66 (0.47 -0.94)	0.78 (0.56 – 1.08)
Model 3	1.00	0.85 (0.62 – 1.18)	0.75 (0.53 – 1.05)	0.68 (0.48 – 0.97)	0.81 (0.58 – 1.13)
Model 4	1.00	0.88 (0.64 – 1.22)	0.82 (0.58 – 1.15)	0.79 (0.56 – 1.12)	0.95 (0.68 – 1.33)

Model 1: adjusted for total energy intake and age; Model 2: model 1 + additionally adjusted for hypertension risk factors (smoking status, diabetes, physical activity, alcohol intake) and demographics variables (education, income management); Model 3: model 2 + additionally adjusted for dietary intake variables (fibre, cholesterol, vitamin C, sodium); Model 4: model 3 + additionally adjusted for body mass index.

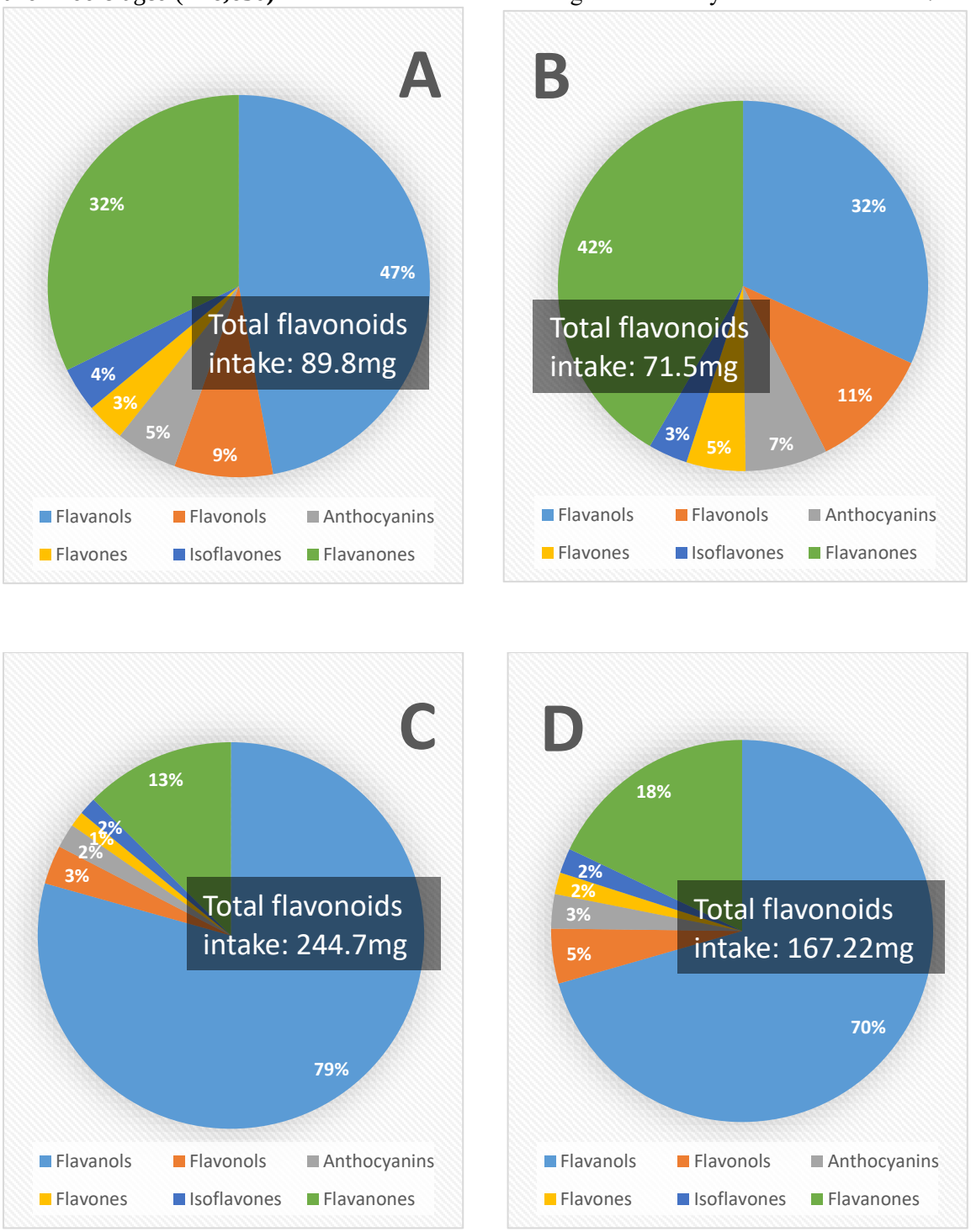
Table 2-S7. Relative risks for associations of total flavonoids and subclasses intake with incident hypertension in reproductive-aged women that gave birth to children in the Australian Longitudinal Study on Women's Health, **n=3,345**

	Total Flavonoids				
	Quintile 1 n =655	**Quintile 2 n =664**	**Quintile 3 n =671**	**Quintile 4 n =678**	**Quintile 5 n =677**
Intake (mg), mean±SD	28.5 ±21.9	45.5 ±24.4	62.1 ±25.1	84.1 ±29.6	132.4 ±51.1
N (%) cases	31	41	35	19	26
Model 1	1.00	1.40 (0.87 – 2.24)	1.20 (0.72 – 2.00)	0.65 (0.35 – 1.22)	0.97 (0.49 – 1.91)
Model 2	1.00	1.43 (0.89 – 2.28)	1.23 (0.74 – 2.04)	0.66 (0.35 -1.24)	0.99 (0.50 – 1.94)
	Flavanols				
Intake (mg), mean±SD	6.4 ±8.0	11.1 ±11.1	16.4 ±11.3	24.2 ±13.0	50.8 ±29.3
N (%) cases	30	38	35	25	24
Model 1	1.00	1.24 (0.77 -1.99)	1.19 (0.73 – 1.97)	0.91 (0.52 – 1.60)	0.87 (0.49 – 1.57)
Model 2	1.00	1.25 (0.78 – 2.01)	1.23 (0.75 – 2.04)	0.94 (0.54 – 1.64)	0.90 (0.50 – 1.61)
	Flavonols				
Intake (mg), mean±SD	3.7 ±2.3	5.4 ±2.6	7.2 ±3.4	8.4 ±4.4	12.6 ±7.1
N (%) cases	33	45	22	25	20
Model 1	1.00	1.40 (0.89 – 2.18)	0.81 (0.47 -1.40)	0.89 (0.52 – 1.52)	0.75 (0.41 – 1.35)
Model 2	1.00	1.42 (0.91 – 2.21)	0.84 (0.49 – 1.46)	0.90 (0.52 – 1.54)	0.76 (0.42 – 1.37)
	Anthocyanins				
Intake (mg), mean±SD	1.5 ±2.3	2.7 ±2.4	3.9 ±2.6	5.8 ±2.8	10.8 ±6.3
N (%) cases	37	35	31	31	18
Model 1	1.00	0.87 (0.55 – 1.37)	0.77 (0.78 – 1.23)	0.82 (0.51 – 1.34)	0.50 (0.28 – 0.90)
Model 2	1.00	0.87 (0.55 – 1.37)	0.78 (0.49 – 1.25)	0.84 (0.52 – 1.38)	0.52 (0.29 – 0.92)
	Flavones				
Intake (mg), mean±SD	1.2 ±1.8	1.9 ±2.0	2.8 ±1.9	4.2 ±2.1	7.6 ±4.4
N (%) cases	32	30	38	25	27
Model 1	1.00	1.03 (0.63 – 1.69)	1.27 (0.78 – 2.07)	0.82 (0.46 – 1.46)	0.91 (0.45 – 1.87)
Model 2	1.00	1.03 (0.63 – 1.69)	1.29 (0.79 – 2.09)	0.85 (0.48 – 1.50)	0.93 (0.45 – 1.89)
	Isoflavones				
Intake (mg), mean±SD	0.27 ±2.8	0.20 ±2.3	0.29 ±2.6	0.61 ±3.3	9.9 ±14.5
N (%) cases	39	29	39	24	21
Model 1	1.00	0.73 (0.46 – 1.17)	1.12 (0.72 – 1.74)	0.75 (0.44 – 1.25)	0.87 (0.50 – 1.51)
Model 2	1.00	0.73 (0.45 – 1.17)	1.12 (0.73 – 1.74)	0.75 (0.45 – 1.25)	0.88 (0.50 – 1.52)

	Flavanones				
Intake (mg), mean±SD	9.2 ±13.7	16.0 ±13.1	24.0 ±14.0	33.9 ±15.6	61.1 ±30.8
N (%) cases	32	34	28	32	26
Model 1	1.00	1.25 (0.77 – 2.02)	0.98 (0.58 – 1.66)	1.17 (0.67 – 2.04)	1.08 (0.51 – 2.31)
Model 2	1.00	1.27 (0.79 – 2.06)	1.01 (0.60 – 1.70)	1.19 (0.68 – 2.08)	1.10 (0.52 – 2.36)
Total flavonoids (including tea)					
Intake (mg), mean±SD	41.2 ±25.0	80.2 ±28.0	130.4 ±31.5	209.4 ±46.4	367.2 ±79.0
N (%) cases	36	36	26	23	24
Model 1	1.00	1.10 (0.69 – 1.76)	0.84 (0.50 – 1.40)	0.82 (0.47 – 1.41)	0.79 (0.46 – 1.36)
Model 2	1.00	1.10 (0.69 – 1.75)	0.85 (0.51 – 1.42)	0.84 (0.48 – 1.44)	0.79 (0.46 – 1.35)
Flavanols (including tea)					
Intake (mg), mean±SD	10.3 ±9.8	30.5 ±13.4	76.2 ±23.8	146.7 ±42.1	297.2 ±68.4
N (%) cases	37	34	21	24	29
Model 1	1.00	1.24 (0.77 – 1.99)	1.19 (0.73 – 1.97)	0.91 (0.52 – 1.60)	0.87 (0.49 – 1.57)
Model 2	1.00	1.25 (0.78 – 2.01)	1.23 (0.75 – 2.04)	0.94 (054 – 1.64)	0.90 (0.50 – 1.61)

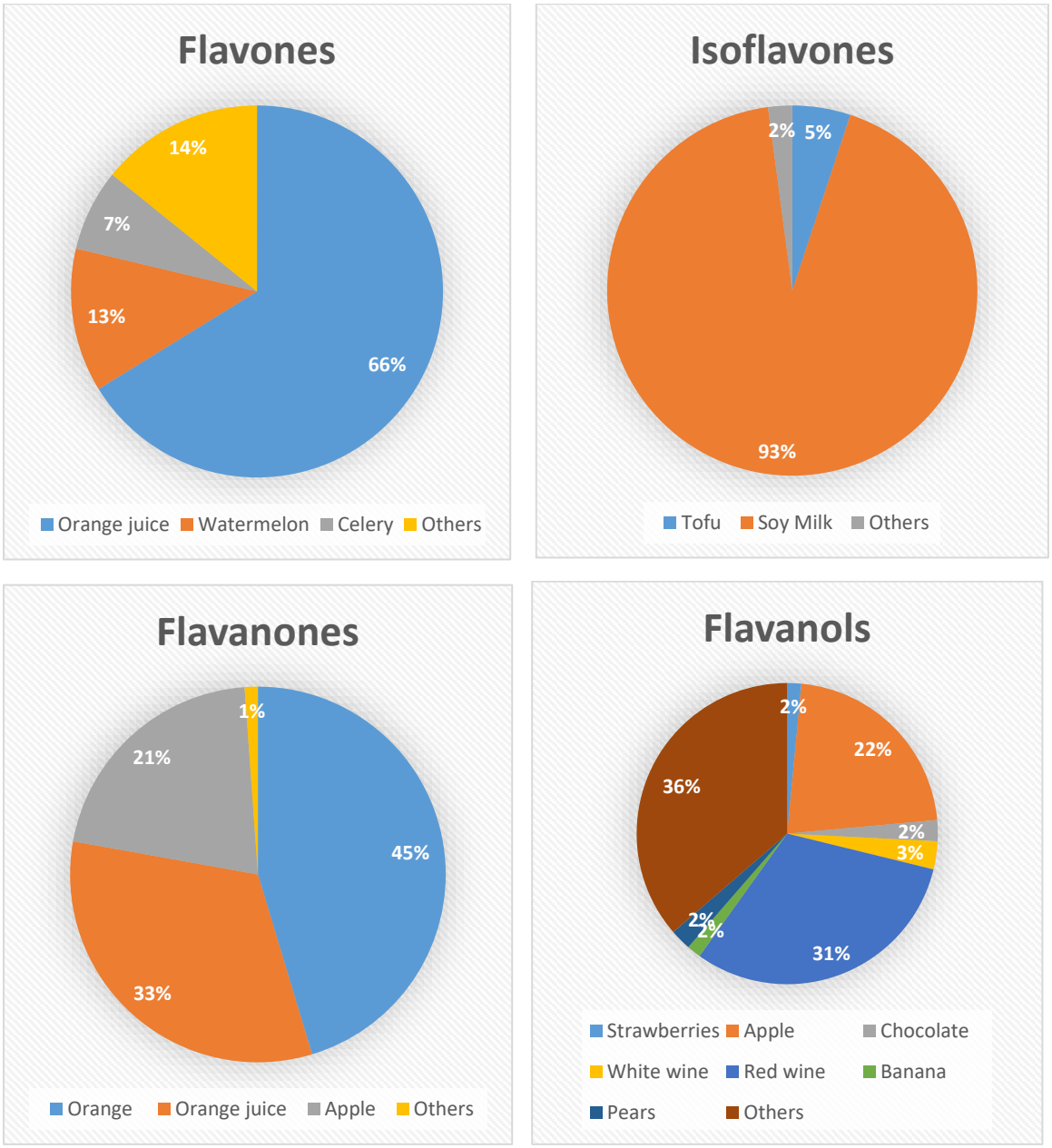
Model 1: adjusted for total energy intake, age, hypertension risk factors (smoking status, diabetes, physical activity, alcohol intake, demographics variables (education, income management), dietary intake variables (fibre, cholesterol, vitamin C, sodium) and body mass index; Model 2: Model 1 + additionally adjusted for gestational diabetes and gestational hypertension.

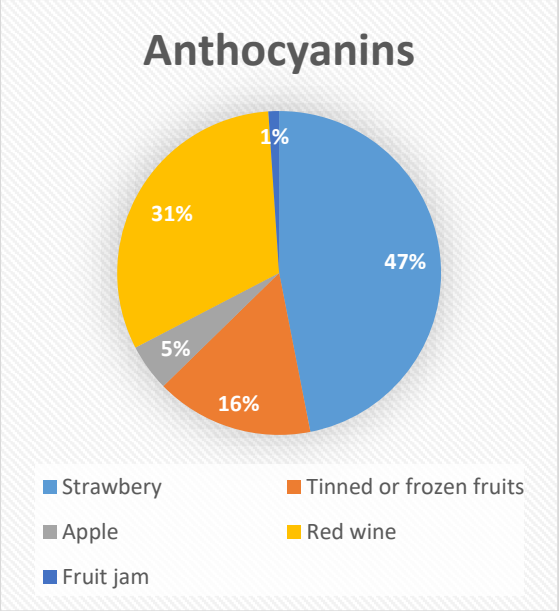
Figure 2-S1. Flavonoids subclasses intake among total flavonoids in reproductive-aged (**n=6,099)** and middle-aged (**n=6,630)** women in the Australian Longitudinal Study on Women's Health.



A, middle-aged cohort; B, reproductive-aged cohort; C, middle-aged cohort including tea flavonoids; D, reproductive-aged cohort including tea flavonoids.

Figure 2-S2. Food source of flavonoids subclasses intake in middle-aged women in the Australian Longitudinal Study on Women's Health, **n=6,630**




Anthocyanins
1%
31%
47%
5%
16%
Strawbery
Tinned or frozen fruits
Apple
Red wine
Fruit jam

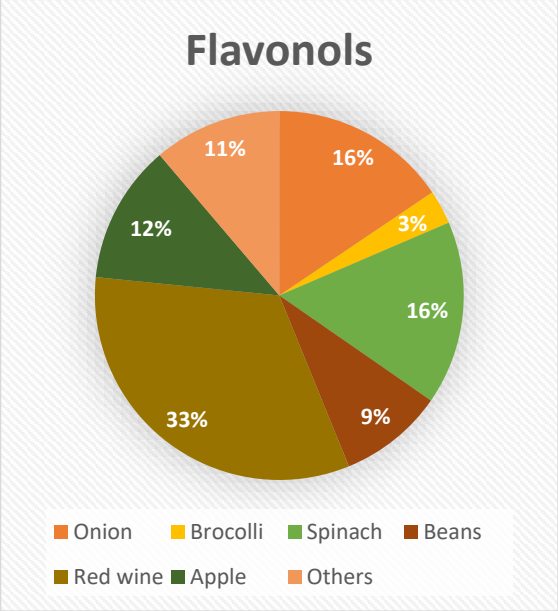
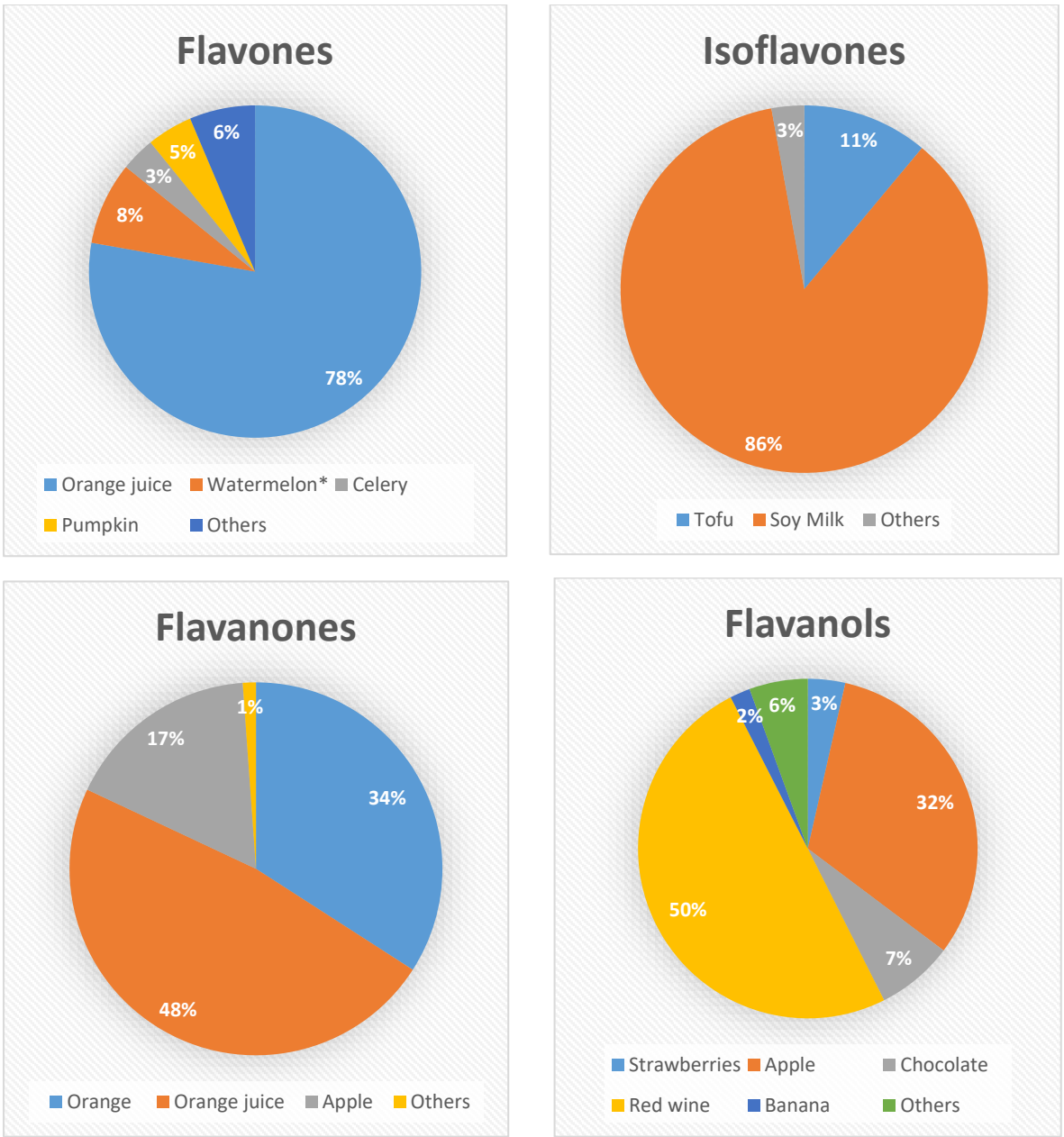
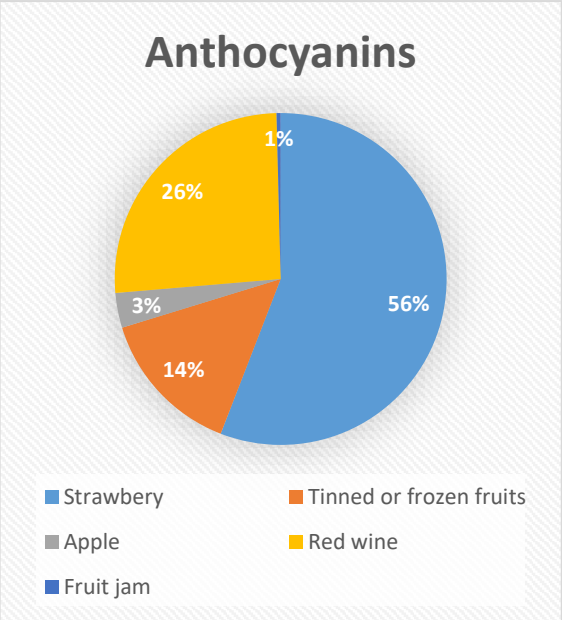
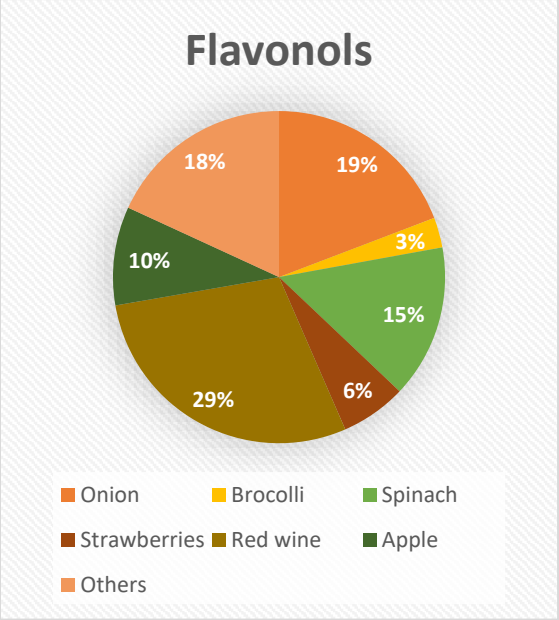

Flavonols
11%
16%
12%
3%
16%
33%
9%
Onion
Brocolli
Spinach
Beans
Red wine
Apple
Others

Figure 2-S3. Food source of flavonoids subclasses intake in reproductive-aged women in the Australian Longitudinal Study on Women's Health, **n=6,099**




Anthocyanins
1%
26%
3%
14%
56%
Strawbery
Tinned or frozen fruits
Apple
Red wine
Fruit jam


Flavonols
18%
19%
3%
10%
15%
29%
6%
Onion
Brocolli
Spinach
Strawberries
Red wine
Apple
Others

CHAPTER 3: The postprandial effect of anthocyanins on cardiovascular disease risk factors: a systematic literature review of high-fat meal challenge studies

The effect of anthocyanins in the postprandial state have been evaluated by several clinical trials using a HFHE meal challenge. This method allows to assess if these bioactive compounds are capable of attenuating the deleterious effects following a HFHE meal; however, such findings were not yet collated and synthetized with the scope of the impact of anthocyanins on CVD risk factors. Therefore, systematic research literature was conducted in this purpose. A total of 13 eligible studies were included and beneficial effects of anthocyanins were reported with most promising results indicating the attenuation of deleterious postprandial effects on oxidative stress and antioxidant status, triacylglycerol and total cholesterol concentrations, vascular endothelial function and inflammatory biomarkers. Post-prandial changes in blood pressure and lipoproteins were least affected by anthocyanins.

The majority of this chapter forms the substantive content of a published article (Appendix A)

3.1 Introduction

Cardiovascular diseases (CVD) are still the number one cause of death globally, representing 31% of deaths in 2016. Most CVDs can be prevented by addressing and managing behavioural risk factors such as diet (224). Several CVD risk factors are associated to the atherosclerotic process and other vascular dysfunctions closely linked to nutrition (225).

There is an emerging evidence that metabolic imbalances at the postprandial state, particularly after a high-energy meal rich in fat, are important contributing factors to development of CVD (105,106). Overall, the underlying mechanism involves a sharp increase in triacylglycerol along with an aberrant production of pro-oxidant molecules leading to an oxidative stress state, which may impair vascular and endothelial functions, as well as mediate the onset of an inflammatory response, which further contributes to the generation of more free radicals, thus creating a deleterious vicious cycle (105–107). Dietary fats comprise heterogeneous molecules with diverse structures, which affect diverse cell processes such as transcription regulation, cellular and organelle membrane structure and function, ion channel activity and electrophysiology. Responses vary depending on both the fatty acid composition of the food source, as well interactions with accompanying nutrients, the food matrix, and how it has been processed(108). Modification of the type of dietary fat in a food or overall meal has been shown to result in postprandial effects on appetite (109), lipaemia and markers for inflammation and endothelial activity (110).

The high-fat meal (HFM) challenge is one way to investigate these imbalances promoted in a daily basis in Western diets (105). Thus, this type of studies allows dietetic therapeutic opportunities to attenuate the harmful effects of aberrant production of pro-oxidant molecules. Nutrition plays a major role in enhancing endogenous antioxidant defences and regulating the inflammatory state (9) and dietary components consumed alongside a high fat meal may be beneficial in blunting a harmful postprandial response (226).

Anthocyanins, a subclass of flavonoids, are emerging as a potential therapeutic option for CVD risk factors(21). Anthocyanins are the largest class of water-soluble plant pigments, that are responsible for the blue, purple and red colour of many fruits and vegetables, such as blueberries, blackberries, red grapes, plums and eggplants (22). The positive results of anthocyanins on CVD risk factors are related to its antioxidant and immunomodulatory effects,

thereby attenuating the cooperative and synergistic deleterious effects of oxidative stress and inflammation in this condition (21,23). In humans, anthocyanins intake has been associated with a lower risk of cardiovascular events (24,25), as well as studies using anthocyanins as a diet intervention have shown improvements in vascular function (26) and in biomarkers related to oxidative stress (27–30), antioxidant status (28,30–32), lipid profile (33–35) and inflammatory response (36,37) in both long-term and acute designs.

To date, there has been no review of the effect of such compounds in studies using a HFM challenge. In order to address this question, a systematic literature review was undertaken using the procedures outlined in the PRISMA statement (227) aiming to evaluate if concomitant consumption of anthocyanins with a HFM attenuates the deleterious postprandial response of parameters known to be CVD risk factors, including blood pressure, vascular endothelial function, lipid profile and biomarkers related to oxidative stress, antioxidant status and immune response.

3.2 Methods

Search strategy

A systematic literature review was undertaken using the procedures outlined in the PRISMA statement (227). Five electronic databases were searched up to the period of 1st February 2020; Medline, Scopus, CINAHL, Web of Science and PubMed. Two researchers were responsible for studies selection, data extraction, quality assessment and synthesis. The search strategy was carried out in accordance with the database orientations using Boolean operators (OR and AND), parenthesis, quotation marks and asterisk. Quotation marks were used to search for exact terms or expressions; parenthesis were used to indicate a group of search terms or combine two groups of search terms enabling all possible combinations of

sentences; asterisks were used to search all words derived of the precedent inflected part. The groups of search terms used were: "endothelium function" or "endothelium dysfunction" or "laser speckle" or "laser doppler" or "flow-mediated dilatation" or FMD or LSCI or "arterial stiffness" or "pulse wave velocity" or PWV or "lipid profile" or LDL or VLDL or ox-LDL or "oxidative stress" or "lipid peroxidation" or "blood pressure" or cytokines or inflammation or immune or inflammatory or chemokine or adhesion or malondialdehyde or isoprostanes or nitrite or nitrate or ENO* "nitric oxide") AND ("postprandial" or "postprandial" or "post prandial" or "meal challenge" or "challenge meal" or "test meal") AND anthocyanins. The study selection, quality assessment, data extraction and synthesis were conducted by two researchers independently and then reviewed by all authors. The review was registered with PROSPERO (CRD42019126265).

Selection criteria

Selection criteria were formed using the Population Intervention Comparison Outcome Study design **(PICOS)** format (227). The criteria used to screen the titles and abstracts of literature returned through database searching (Table 3-1).

Table 3-1. PICOS (participants, interventions, comparisons, outcomes, and study design) criteria to define the research question

Parameter	Inclusion criteria
Participants	Young adults (18-59 years of age)
Interventions	Dietary intervention with anthocyanins in whole food or purified extract
Comparators	A comparison group receiving a control intervention
Outcomes	CVD risk factors including blood pressure, lipid profile, vascular and endothelial function, biomarkers related to inflammation, oxidative stress and antioxidant status

Study design	Randomised or cross-over clinical trials with high fat meal challenge for all groups.

Only articles published in English were included. Exclusion criteria: mean age of participants <18 or >59 years; no nutritional information of the HFM, placebo or dietary intervention; other conditions besides the HFM challenge. Reference lists of included articles were screened for further studies that may have been missed in initial database search.

Data extraction

Two independent reviewers extracted data and cross checked results to ensure consistency. The following data were extracted from each study and reported in a summary table; year of publication, author(s), participant demographics, sample size, anthocyanin source and dose, control used, test meal and study outcomes (Table 3-2). Authors were contacted if further information was required.

Risk of bias assessment

Studies included in this review were assessed for potential bias using the revised Cochrane risk-of-bias tool for randomised trials (228). Two researchers independently reviewed each study, performing evaluation across five risk-of-bias domains, with each domain rated either low risk, high risk or some concern of bias. The prescribed algorithm was used to determine domain ratings and overall risk-of-bias judgement for each study (228).

3.3 Results

Study selection

A total of 9135 articles returned through database searching. 1464 duplicates were removed and 7671 articles were excluded during title and abstract screening (Figure 3-1). The full-text of the remaining 29 articles were accessed and evaluated according the study selection criteria. Sixteen articles were excluded as they did not meet all criteria and the remaining 13 studies were eligible for the review.

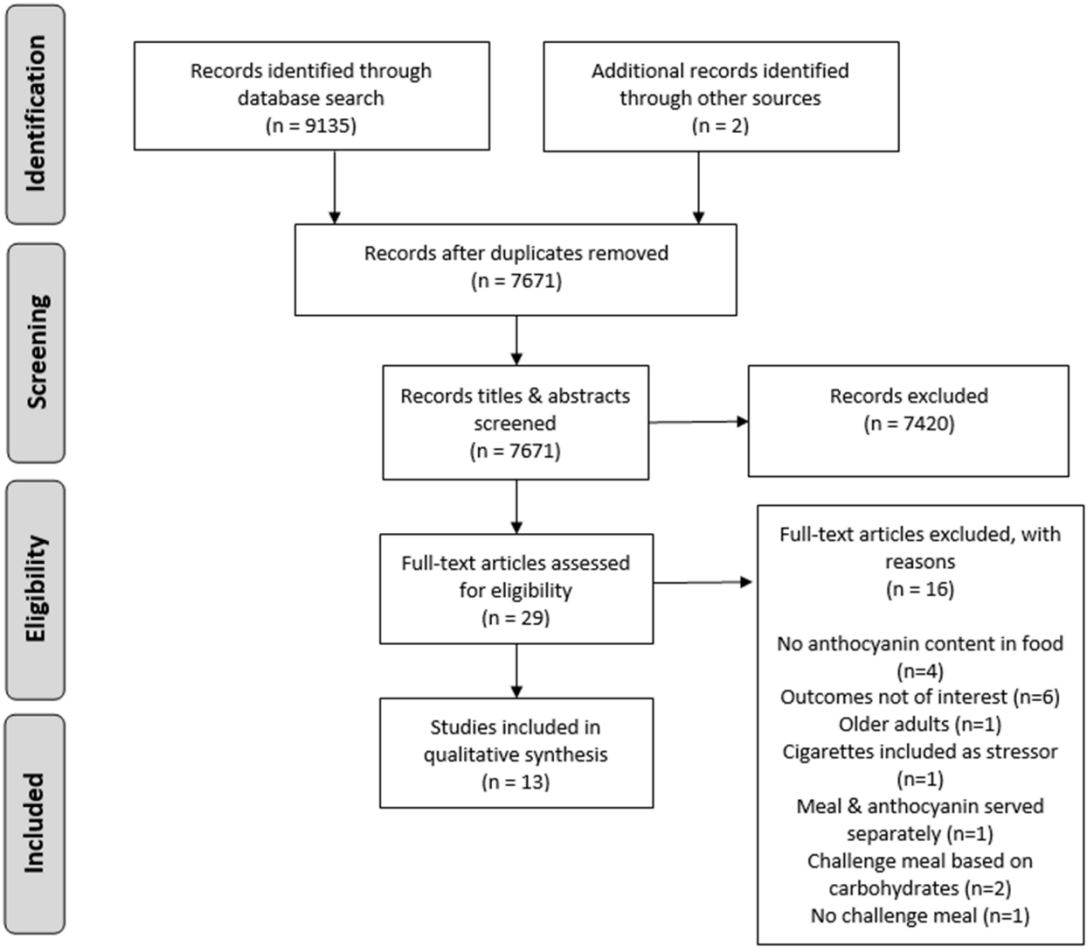


Figure 3-1: Flow diagram of the search and selection strategy.

C***haracteristics of studies included in review***

The characteristics of the 13 studies reviewed are summarised in Table 3-2. All studies included had a cross-over design with a wash-out period ranging from 4 to 28 days, of which four were double-blinded (229–232), seven were single-blinded (37,233–238) and two did not present blind strategies (239,240). The mean age of subjects involved in included studies ranged from 20.2 to 50.9 years and five studies were conducted only in male subjects (229,232,233,237,240), while all other recruited men and women. The majority of studies were conducted on healthy individuals, except for one study on subjects with an atherosclerosis-prone phenotype (233), one on subjects with obesity and insulin resistance phenotype (235) and another one with participants with at least one CVD risk factor (239). In relation to the dose of anthocyanins used in the intervention, six studies used a dose <100mg (37,230,231,233,236,239) and four studies used doses >100mg (229,232,237,238), while two studies used three different doses (235,240) and one study used two different doses (234) within both ranges. Overall, the dose ranged from 11.2 to 1530mg of anthocyanins. The fat content within meal challenges were <40g in four studies (37,230,234,235) and >40g in nine studies (229,231–233,236–240). Concerning fat content as % of energy, the meal challenges in six studies provided >50% of energy from fats(229,230,232,233,239,240), while seven studies provided <50% energy from fats(37,231,234–238). The main type of fats within the challenge meals were derived from animal products with high content of saturated fats such as cream, sausages, cheese, fried potatoes, bacon, eggs and butter. There were no relevant source of omega-3 fatty acids included in any of the challenge meals. All studies used a macronutrient matched placebo in the control arm. In eleven studies(126,129,186,229–234,241), either a beverage, yoghurt or smoothie was used, while two studies(238,240) provided meals without incorporating the freeze-dried source of anthocyanins. One study used water as an additional control treatment(239), while another also matched the content of vitamin C in the placebo(232)

Table 3-2. High-fat meal challenge studies analysing cardiovascular risk factors

Reference	Population sample size	Diet intervention(s)	Control arm(s)	Test meal	Outcomes
Alqurashi et al. 2015, UK(229)	Healthy male adults (n=23), mean age 46yr ± 1.9, BMI 27.6kg/m2 ± 0.4	Açai smoothie: 150g frozen açai pulp, 50g banana, 155.1Kcal, 8.5g fat, 2.4g PTN, 17.2g CHO, 7.2g fibre, **493mg anthocyanins**	Colour and macronutrient matched smoothie	Meal+beverage: 869.7 Kcal (60.6% from fat), 58.5g fat, 74.4g CHO, 11.4g PTN	**Blood pressure:** ↔ **FMD**: ↑ 1.4%, p=0.001 at 2h and ↑ 0.8%, p<0.001 at 4h. **Plasma total oxidant capacity** (peroxide concentrations): ↓ area under the curve over 7h, p=0.02
Cerletti et al. 2014, Italy(239)	Adults (n=18: 9 females, 9 males), 36.9 ± 10.5yr, BMI 26.8 ±4.0. At least one CVD risk factor (overweight, hypertension, smoking, high serum cholesterol or TG levels	Red orange juice: **53.1mg anthocyanins**	a) Blonde orange juice; b) water	890 Kcal (52.6% from fat), 52g fat, 25g PTN, 81g CHO	**Augmentation index (vascular stiffness):** ↓ 2.18 ± 19.26 to -6.11 ± 11.90, p=0.0030 **Reactive hyperaemia index (vascular reactivity):** ↔ **Blood pressure**: ↔ (diastolic blood pressure within group:↓ p=0.0045) **TAG:** ↔ (↓ within the intervention group, p=0.0131)
Edirisinghe et al. 2010, USA(37)	Healthy adults (n=24, 14 females, 10males) BMI 29.2 ±2.3, 50.9yrs ± 15	Milk based beverage + strawberry powder, **39.04mg anthocyanins**	Macronutrient matched placebo beverage	962.3 Kcal (28.1% from fat), 30g fat, 36g PTN, 135g CHO	**hsCRP:** ↓ 3.1 (SEM 0.1) vs 2.7 (SEM 0.5) mg/L, P=0.02 at 6h **IL-6:** ↓ 3.4 (SEM 0.5) vs 4.5 (SEM 0.5) pg/ml, P<0.05 at 6h. **TNF-α:** ↔ **IL-1β:** ↔
Miglio et al. 2012, Italy(230)	Healhy adults (n=14), 45yr ± 9, BMI 26.8kg/m^2 ± 2.2	a) Fruit juice (86% mix apple, grape, blueberry, pomegranate juices), **6.5mg anthocyanins** b) Fruit juice (63%	Energy and sugar matched placebo, 66g CHO	1344 Kcal (54.2% from fat), 81g fat, 104g CHO, 52g PTN, 3.1g fibre	**Urinary FRAP**: ↑ 35%, p<0.01 **Plasma TRAP**: ↑ p<0.05 at 1h, ↑ 8%, p<0.001 at 2h, ↑ p<0.01 at 4h. **Plasma FRAP:** ↔ **Plasma uric acid**: ↓ p<0.05 at 8h

		mix of pineapple, blackcurrant and plum juices), **16mg anthocyanins**			**Plasma Thiols:** ↓ $p<0.05$ at 0.5, 1, 4 and 8h, ↓ $p<0.01$ **Ascorbic acid:** ↔
Ono-Moore et al. 2016, USA(234)	Healthy adults (n=23, 18 females, 5 males), 30yr ± 3yr, BMI 21.9kg/m2 ± 0.4	Yoghurt + freeze dried blueberry powder: a) **87.9mg anthocyanins**; b) **154.5mg anthocyanins**	Macronutrient matched control yoghurt: 191 Kcal, 0.6g fat, 45.1g CHO, 1.2g PTN, 10g fibre	653 Kcal (40.0% from fat), 29g fat, 73.6g CHO, 24g PTN	**TAG:** ↔ **LDL-c:** ↔ **HDL-c:** ↔ **IL-8:** ↔ **IL-1β:** ↔ **TNF-α:** ↔
Park et al. 2016, USA(235)	Adults with obesity and insulin resistance phenotype (n=21, 16 females, 5 males), 39.8yr ± 13.8, BMI 40.2 ± 7.2	Freeze dried whole strawberry powder: a) **42.2mg anthocyanins**; b) **87.9mg**; c) **154.5mg**	Milk based colour and macronutrient matched beverage	967 Kcal (23.6% from fat), 25.4g fat, 146.2g CHO, 36.9g PTN, 12.3g fibre	**TAG:** ↔ **ORAC:** ↔ **IL-6:** ↔ **OxLDL-c**: ↓ –3.0 ± 0.8 U/L, $p<0.05$
Richter et al. 2017, USA(238)	Healthy adults (n=30, 13 females, 17 males), BMI 31 kg/m^2 ± 0.5, 28yr ± 2.0	Freeze dried strawberry powder: **163.41mg** anthocyanins (added to meal)	Strawberry flavoured powder (added to meal)	1004Kcal (44.8% from fat), 50g fat, 105g CHO, 32g PTN, 7g fibre	**Blood pressure:** ↔ **TAG:** ↔ **OxLDL-c:** ↔ **MDA:** ↔ **Augmentation index:** ↔ **Aortic stiffness (PWV):** ↔
Urquiaga et al. 2016, Chile(240)	Healthy adult males (n=9), 20.2yr (18.7-27.3), BMI 24.6 kg/m^2 (20.7-29.4)	Berry concentrate: a) added on beverage, **90mg anthocyanins**; b) added on burger and on beverage, **174.3mg anthocyanins**	Plain burger and water	527 Kcal (58.9% from fat), 48.7g PTN, 34.33g fat, 4.6g CHO	**TAG:** ↔ **MDA**: ↓ $p<0.05$ at all time points for intervention "b" and ↓ $p<0.05$ at 5 and 6h for intervention "a" **PTNcarbonyls:** ↓ $p<0.05$ at 2, 3, 4, 5 and 6h for intervention "b" and ↓ $p<0.05$ at 4 and 6h for intervention "a" **Plasma FRAP:** ↔ **Ascorbic acid:** ↔

Huang 2016, USA(236)	Healthy adults (n= 14, 9 males and 5 females), 25yrs ± 4, BMI 26kg/m^2 ± 2	Freeze dried strawberry added to a beverage, **49.02mg anthocyanins**	Macronutrients matched beverage: 41Kcal, 0.8g PTN, 0.1g fat, 9.1g CHO	841 Kcal (43.9% from fat), 41g fat, 96g CHO	**TAG:** ↔ **OxLDL-c:** ↔ **IL-6:** ↓ p=0.048 (intervention consumed before the meal) over 10h; ↔ **(**intervention consumed within and after the meal, trend p<0.1) over 10h
Kay & Holub 2002, Canada(237)	Healhy males(n=8), mean age 46.9 ±1.9), BMI 23.8kg/m^2 ± 0.8	Freeze dried wild-blueberry supplement: **1160mg anthocyanins**	Control supplement matched in CHO and energy	853 Kcal (49.3% from fat), 46.7 g fat, 75.2g CHO, 32.4g PTN, 4.5g fibre	**ORAC:** ↑ p<0.05 **Total antioxidant status**: ↑ 4.5%, p=0.05. **TAG:** ↔ **TC:** ↔ **LDL-c:** ↔ **HDL-c:** ↔
Peluso et al. 2011, Italy(231)	Healthy adults, (n=14, 12 males and 2 females), 45.1yrs ± 8.6, BMI 26.8 kg/m^2 ± 2.2	Blackcurrant, plum and pineapple beverage: **16mg anthocyanins**	Placebo beverage devoid of antioxidant activity	Meal+beverage: 1344 Kcal (30.0% from fat), 184g CHO, 44.8g fat, 49g PTN	**TAG:** ↔ **TC:** ↔ [prevented a significant increase (p<0.001) observed only at the control group over 8h] **IL-17:** ↓ p<0.05 at 4 and p<0.01 at 8h **IL-6:** ↓ 0.5h (p<0.01), 1h (P<0.05) and 2h (p<0.001) **TNF-a:** ↓ at multiple time points (p<0.01 at 1h, p<0.05 at 2 and 6h, p<0.001 at 4 and 8h)
Huebbe et al. 2011, Germany(233)	Adult with atherosclerosis prone phenotype (n=11), 37.4 yrs ±1.9, BMI 32.1 ±1.2	Blackcurrant based beverage (15% blackcurrant puree, 9% raspberry puree, 7% cherry puree, 39% red grape juice + banana puree): **11.2mg anthocyanins** (7.5mg delphinidin-3-glucoside, 7.5mg cyanidin-3-glucoside,	Macronutrient matched beverage: 1029Kcal, 63.8g fat, 6.0g PTN, 107.6g CHO, 1.8g fibre	Blackcurrant based beverage (added cream and sugar): 1029 Kcal (55.8% from fat), 63.8g fat, 6.0g PTN, 107.6g CHO, 1.8g fibre	**TAG:** ↔ (↓ trend, p=0.059) **LDL-c:** ↔ **HDL-c:** ↔ **TC:** ↔ **OxLDL-c:** ↔ **IL-1β:** ↔ **TNF-α:** ↔ **ORAC:** ↑ p<0.040 at 90min and p<0.02 at 120 min **Ascorbic acid:** ↑ 14 mmol/l, p<0.004

		0.8mg malvidin-3-glucoside)			
Polley et al. 2019, USA(232)	Healthy adults (22 yrs ± 3.0), BMI 25.5 ±3.4	Montmorency tart cherry concentrate: 1513.8mg anthocyanins	Macronutrient matched beverage +11mg of vitamin C: 166.5Kcal, CHO 39.4g; PTN 2.24g	Biscuit, sausage patty and butter: 920 Kcal (58.7% from fat), 60.0g fat, 22.2g PTN, 72g CHO,	**ORAC:** ↔ **FRAP:** ↔ **TG:** ↔

Abbreviations and symbols: CHO, carbohydrate; PTN, protein; FMD, flow-mediated dilatation; Kcal, kilocalories; TAG, triacylglycerol; TC, total cholesterol; HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol; OxLDL-c, oxidized low-density lipoprotein cholesterol; hsCRP, high sensitivity C-reactive protein; FRAP, ferric reducing ability of plasma; TRAP, total radical-trapping antioxidant parameter; ORAC: oxygen radical absorbance capacity; ↔, no significant changes; ↑, significant increase; ↓, significant decrease

Risk of bias

The overall risk of bias was judged as "low risk" for ten studies (37,229,230,232,234–236,238–240), while three studies (231,233,237) were judged as having "some concern" due to ranking as such in one or more risk domains (Table 3-3). Among the studies that were identified as having "some concern" regarding risk of bias, two studies (233,237) had missing information on domain 1 (risk of bias arising from the randomization process) and one study (231) had missing information on domain 3 (risk of bias due to missing outcome data) (228).

Table 3-3. Overall risk of bias of included studies

	Alqurashi et al, 2016	Cerletti et al., 2016	Edirisinghe et al., 2011	Miglio et al., 2014	Ono-Moore et al., 2016	Park et al., 2016	Richter et al., 2017	Urquiaga et al, 2017	Huang et al., 2016	Kay & Holub, 2002	Peluso et al. 2011	Huebbe et al., 2016	Polley et al., 2019
Domain 1: Risk of bias arising from the randomisation process	+	+	+	+	+	+	+	+	+	?	+	?	+
Domain 2: Risk of bias due to deviations from the intended interventions (effect of assignment to intervention)	+	+	+	+	+	+	+	+	+	+	+	+	+
Domain 3: Risk of bias due to missing outcome data	+	+	+	+	+	+	+	+	+	+	?	+	+
Domain 4: Risk of bias in measurement of the outcome	+	+	+	+	+	+	+	+	+	+	+	+	+
Domain 5: Risk of bias in selection of the reported result	+	+	+	+	+	+	+	+	+	+	+	+	+
Overall risk of bias	+	+	+	+	+	+	+	+	+	?	?	?	+

Symbols: + low risk; ? some concern

Outcomes

The outcomes analysed in the included studies were blood pressure, lipid profile, vascular and endothelial function, as well as biomarkers related to inflammation, oxidative stress and antioxidant status (Table 3-2). The postprandial response of such outcomes were analysed after the intake of the high-fat meal challenge, which means that a significant decrease may actually mean an attenuation of an increase pattern. Three studies measured blood pressure (229,238,239) before and after the HFMC, none of which found significant changes in systolic blood pressure. One study found a reduction in diastolic blood pressure in the anthocyanins intervention group ($p=0.0045$), while an absence of effect was observed in the other two arms administering water or a matched anthocyanins-free placebo (239). Vascular and endothelial function were evaluated by three studies (229,238,239). Alqurashi et al. (229) assessed endothelial function in the brachial artery by flow-mediated dilatation (FMD) with a postprandial increase of 1.4% versus 0.4% at 2h ($p=0.001$) and an increase of 0.8% versus a decrease of -0.3% at 6h ($p<0.001$), comparing the intervention with the placebo group, respectively. Cerletti et al. (239) assessed vascular stiffness through the augmentation index (AI) and vascular reactivity through the reactive hyperaemia index (RHI). The latter did not present significant changes, but there was a significant decrease (2.18 ± 19.26 to -6.11 ± 11.90, $p=0.0030$) in the AI 3h after the meal only in the anthocyanins intervention arm (239). Richter et al. (238) investigated AI and augmentation pressure through pulse wave analysis and the aortic stiffness assessed by carotid-femoral pulse wave velocity (PWV). None of the parameters were significantly different between intervention and control arms (238).

Regarding the lipid profile, ten studies evaluated triacylglycerol (TAG) (231–240), of which one study found a higher decrease ($p=0.0131$) in the intervention group (239) and other study found a trend($p=0.059$) of a decrease 60 minutes after ingestion of the HFMC (233). Total cholesterol (TC) was evaluated by five studies (231,233,234,237,239) and two studies found

significant changes. Cerltetti et al. (239) found a reduction (p=0.0339) only in the anthocyanin intervention arm (239), while Peluso et al. (231) found that the anthocyanins prevented an increase in the intervention group, therefore a significant increase was only observed in the control group over 8h (p<0.001) (231). The studies that measured HDL-c (233,234,237) and LDL-c (233,234,237) did not find any significant changes.

Among inflammatory markers, six studies evaluated IL-6 (37,231,233–236) concentrations, of which three (37,231,236) reported significant changes. In one study (37) the anthocyanin intervention was able to attenuate an increase in IL-6 response six hours after the HFMC compared to the placebo group [3.4 (SEM 0.5) versus 4.5 (SEM 0.5) pg/ml, P<0.05]. Similarly, Peluso et al. (231) found a significant attenuation in postprandial IL-6 concentration at 0.5 (p<0.01), 1 (P<0.05) and 2h (p<0.001) time points (231). Another study (236) found a trend to attenuate such response in two groups (consuming the anthocyanins intervention within or after the HFMC) and a significant decrease in the group consuming the anthocyanins intervention before the meal (p=0.048). The cytokine TNF-α was evaluated by four studies (37,231,233,234) and with one study (231) finding a significant result preventing a postprandial rise of TNF-α concentrations at multiple time points (p<0.01 at 1h, p<0.05 at 2 and 6h, p<0.001 at 4 and 8h).

There were no significant changes in IL-1β concentrations between treatments cross the three studies evaluated this cytokine (37,233,234). Only one study (37) evaluated hsCRP concentrations before and after the HFMC presenting a significant lower concentration compared to placebo [3.1 (SEM 0.1) versus 2.7 (SEM 0.5) mg/L, P=0.02]. Still, there was one study that measured IL-8 concentration finding not significant results (234) and one study that evaluate Il-17 which found a significant postprandial reduction at 4(p<0.05) and 8h(p<0.001) time points (231).

A large variety of oxidative stress and antioxidant status biomarkers were measured by nine studies (229,230,232,233,235–238,240) included in this review. Four studies (233,235,236,238) evaluated OxLDL-c concentrations; however, only one study (235) found a significant reduction (–3.0 ± 0.8 U/L, $p<0.05$) in the intervention arm compared to placebo. Plasma malondialdehyde (MDA) concentration was measured in two studies (238,240) in which one found that the anthocyanins interventions arms were able to prevent postprandial MDA accumulation ($p<0.05$ at multiple time points), and the mean value of the area under the curve of was reduced compared to placebo (240). This same study also found a reduction ($p<0.05$ at multiple time points for both anthocyanins interventions) in plasma protein carbonyls and an increase in the DPPH (2,2-diphenyl-1-picrylhydrazyl) plasma antioxidant capacity (240). The ferric reducing ability of plasma (FRAP) assay was used to examine antioxidant capacity in was evaluated by three studies (230,232,240), but no significant effects of anthocyanin intervention were detected. However, Miglio et al. (230) found a 35% increase in the urinary excretion of antioxidants as indicated by raised urinary FRAP ($P<0.01$). This same study found an increase ($p<0.05$ at 1h, $p<0.001$ at 2h and $p<0.01$ at 4h) in plasma total radical-trapping antioxidant parameter (TRAP), as well as a significant attenuation in the increase of endogenous antioxidants thiols and uric acid (UA) ($p<0.05$ at 8h). Plasma ascorbic acid (vitamin C) was measured in four studies (230,233,237,240), of which one study (233) found an increase in the anthocyanin intervention arm (+14 mmol/l, $p<0.004$) compared to the placebo. The oxygen radical absorbance capacity (ORAC) of plasma/serum was evaluated, before and after the HFMC, in five studies (232,233,235,237,240), of which two found significant changes. In one study (233), the intervention was able to prevent the postprandial decrease in the ORAC of plasma at 90 and 120 min following the HFM challenge ($p<0.040$ and $p<0.02$, respectively), while another study (237) found a significant increase in serum ORAC 1h after the HFMC in intervention group when compared to the control ($p<0.05$).

Furthermore, this same study found a significant increase in the total antioxidant status (TAS) assay (+4.5%, p=0.05). Lastly, one study (229) measured the total oxidant capacity in plasma by assessing the total peroxide concentrations, and found a significantly lower incremental area under the curve in the intervention group($p = 0.02$).

3.4 Discussion

The results of studies included in this systematic literature review indicate that the postprandial state, after exposure to a high fat meal, may provide a useful context to investigate acute metabolic changes, from well-known lipid responses to complex phenolic compound signalling pathways that contribute to the development of CVD. A wide range of risk factors such as blood pressure, lipid profile, vascular and endothelial function, as well as biomarkers related to inflammation, oxidative stress and antioxidant status were included as outcomes in the acute studies in the review.

Despite the lack of positive results in blood pressure, of which only study found a significant decrease in diastolic blood pressure (239), vascular and endothelial parameters showed more positive results. It is more likely that such parameters will respond in a higher magnitude than blood pressure in acute studies, due to the excess postprandial production of pro-oxidant molecules following the HFMC. This may in turn inactivate endothelial dependent factors, in particular nitric oxide, leading to impaired vasodilation and the onset of an inflammatory response, which further leads to the generation of more free radicals (226,242). In this matter, açai consumption was associated with improvements in endothelial vascular function, measured by FMD, in healthy overweight man, which was also followed by an increase in total oxidant capacity in plasma, assessed as a measure of total peroxide concentrations (229). The significant improvement in FMD found in this study(229) was 1% higher than in controls, a magnitude of effect that has been shown in a meta-analysis to be

associated with an overall 8 % reduction in CVD risk (RR= 0.92; 95%CI: 0.88; 0.95) for each percentage increase (92) . Flow-mediated dilatation is a non-invasive measurement of endothelium function that has been associated with CVD risk prediction(92,243). Another study found a decrease in arterial stiffness, measured by AI, after consumption of blood orange juice anthocyanins in individual with at least one CVD risk factor (overweight, hypertension, smoking, high serum cholesterol or triacylglycerol levels) (239).

A recent systematic review evaluated the postprandial inflammatory response to HFM challenge, in which IL-6 was stated as the inflammatory marker with the stronger response to this stress (244). IL-6 has a pleiotropic nature showing both anti and pro-inflammatory roles and regulating a plethora of immune and metabolic responses, however a high concentration of this cytokine has been associated with CVD and mortality (77,245). Still, IL-6 has a high expression on vascular endothelium and the pharmacological inhibition of IL-6 improves endothelial function (78). The inhibitory effect on postprandial IL-6 concentrations found within the studies added in this review demonstrates a potential therapeutic effect of anthocyanins for CVD (37,231,236). One possible mechanism that anthocyanins may exert this effect is through decreasing the activity of the NF-κB pathway, which is a transcription factor responsible for triggering and regulating inflammatory processes, leading to the expression of pro-inflammatory cytokines and enzymes (21,121). Concerning the clinical significance of the magnitude of effects reported in the studies that observed benefits associated with anthocyanin intake, the significant changes of between 0.34 and 0.90 pg/mL represent changes from baseline of >2 SD(37,236) and >3 SD(231). These results are clinically meaningful considering the predictive roles of IL-6 concentration in CVD risk. Hazard ratios of 1.80 are reported according to each 1-SD increase in IL-6 for risk of first-ever cerebrovascular events in individuals with vascular risk factors without any pre-existing cardiovascular disease(79). Further, a positive predictive value of 100% is reported for coronary artery disease when IL-6

concentrations exceed 1.0 pg/mL in patients who have an intermediate cardiovascular risk profile and chest pain(246). In a meta-analysis of 17 prospective studies investigating clinical coronary outcomes (i.e., myocardial infarction or coronary death), an odds ratio of 1.61 (95% CI 1.42–1.83) was found per 2 SD increase in baseline IL-6 (247). Another meta-analysis of 17 studies comprising 288,738 healthy individuals reported a significantly higher IL-6 concentration in CVD cases compared to non-CVD controls (standardized mean difference [95% CI]) of 0.14 [0.09, 0.20]/mean difference of 0.36 [0.28, 0.44] pg/mL) [52]. These significant changes in IL-6 concentration were found only in studies which the mean BMI of participants were >25kg/m^2. Overweight and obesity are an independent risk factor for CVD, as well as are associated with a low-grade continuous inflammation with high implications in the atherosclerotic process. The possibility of regulating this persistent not resolved inflammatory state can be crucial in attenuating the progress of the atherosclerosis disease, especially in the early stages. This is the same scenario, in which the only study (37) that evaluated CRP found a significant reduction of 0.4 (SD 0.1) mg/L in a study population with a mean BMI >29kg/m^2. This effect reduced the hsCRP concentration in the intervention arm to values <3.0 mg/L, which is considered a clinical threshold for many cardiovascular conditions, including a reduced hospitalisation rate for heart failure in subjects with stable coronary heart disease[53]. CRP is also associated with CVD and mortality (67,68), and has been implicated in endothelial dysfunction in *in vitro* and *in vivo* studies (65,66). On the other hand, the cytokines IL-1β and TNF-α not appear to transiently and/or robustly change in the postprandial period after a HFMC, thus a decrease in concentration of these molecules is not likely to occur with acute diet interventions (244). In the present review, the three studies that evaluate IL-1β were conducted in healthy normal weight (234), healthy overweight (37) and overweight/obese adults with atherosclerosis prone phenotype (233), however none of them found significant changes. TNF-α also plays a pleiotropic and major role in CVD, but high concentrations have

been associated with deleterious effects mainly through vascular dysfunction and atherogenesis by many mechanisms such as regulation of the vascular permeability, disruption of the endothelial barrier, degradation of glycocalyx, increased production of ROS and decreasing NO bioavailability and increase its removal (250,251). In line with the review by Emerson et al. (2017) (244), the studies included in our review found inconsistent changes of TNF-α concentrations after the HFMC, i.e. two studies found a decrease (233,234), one an increase (231) and other study reported no changes (37). The study that had a postprandial increase in TNF-α concentration was the only one that found a significant lowering effect with the diet intervention with anthocyanins (231). Another relevant finding in this same study was the inhibitory effect of on postprandial increase of IL-17, a cytokine with highly pro-inflammatory properties that are also associated with CVD, especially with cardiovascular events such as stroke and myocardial infarction (252). There is accumulating evidence that IL-17 it is involved in the pathogenesis of cardiovascular diseases by amplifying the inflammation induced by other cytokines in synergistic interactions (253). This cytokine is also positively correlated with OxLDL-c, a molecule with a crucial role in the oxidative stress mediated atherosclerosis development.

OxLDL-c is a key factor in the initiation and progression of atherosclerosis and contributes to endothelial dysfunction and plaque destabilization through various mechanisms (254). Among the four studies that measured postprandial concentration of Ox-LDL-c, the only study that found a significant reduction was conducted in participants with an obese and insulin resistant phenotype [35]. Several other oxidative stress and antioxidant status biomarkers were investigated within the added studies in this review, showing the most promising results in relation to the effects of anthocyanin intervention on parameters following the HFM challenge. MDA is an end product of lipid peroxidation, the radical-initiated oxidative decomposition of poly-unsaturated fatty acids (255). Urquiaga et al. (240) found a reduction in postprandial

concentration of MDA at multiple time points following two different anthocyanin interventions (i.e. added to food or beverage) using a berry concentrate. This same study also found a reduction in plasma protein carbonyls and an increase in the DPPH (2,2-diphenyl-1-picrylhydrazyl) plasma antioxidant capacity (240). Protein carbonylation is one of the most harmful irreversible oxidative protein modifications, and is considered a major hallmark of oxidative stress-related disorders (256). The DPPH assay is a method that is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors, and therefore can be used to evaluate antioxidant activity. Another anti-oxidant assay, the FRAP, (based on single electron transfer reaction to evaluate the antioxidant effect of nonenzymatic defense in biological fluids) was measured in three studies that reported no significant effect of the anthocyanin intervention in plasma (230,232,240). However, one of these studies found a 35% increase in the urinary excretion of antioxidants ($P<0.01$), which was followed by an increase TRAP and an attenuation in the increase of endogenous antioxidants thiols and UA. TRAP differs from FRAP as an assay that measures the ability of antioxidants to buffer a reaction probe against peroxidation, and it's determined by measuring the length of time that oxygen uptake is inhibited (257). Results from both FRAP and TRAP methods have to be carefully interpreted considering that FRAP has low specificity in measuring the antioxidant activity of many important antioxidants such as ascorbic acid, glutathione and albumin, and that TRAP does not necessarily provide a reliable or sensitive measure of the ability of plasma to interfere with lipid peroxidation[66]. UA levels are related to oxidative status, especially antioxidant capacity and it is well known that high plasma UA levels are strongly associated CVD (258). Taken together, the positive results of these three different oxidative stress biomarkers show a relevant anti-oxidant signalling effect of anthocyanins in these studies conducted in healthy adults (230,240). Another relevant biomarker of antioxidant status is vitamin C, which is a readily water-soluble and not storable in tissues. Therefore, vitamin C is a good biomarker for

short-term studies (259). One study added in this review found an increase in plasma vitamin C (233); however, even though the placebo was matched in macronutrients and energy, only the diet intervention had a relevant content of vitamin C (122.3 vs 0.3mg). Thus, it is unlikely that the plasma concentration of vitamin C was increased due to the anthocyanins. The ORAC of plasma/serum, which was evaluated in five studies in this review, appears to be a controversial method in regard to its application for *in vivo* studies, especially after the withdraw of the ORAC food database by the USDA in 2010. There is still debate regarding the absorbance and breakdown of polyphenols, such as flavonoids and subclasses, into smaller phenolics compounds with signalling, anti-inflammatory and anti-oxidant properties (242). Nevertheless, two studies found an increase in plasma ORAC following 60 (233), 90 and 120 (237) minutes after the HFMC, and one study also found a significant increase in TAS (237), another non-specific assay that assess the overall antioxidant status of a sample.

The post-prandial response in the lipid profile, when compared to fasting lipids, can represent a different and even independent risk factor for CVD (34,106). The transient lipid and lipoprotein accumulation that occurs in the circulation after a high-fat meal represents the individual capacity to metabolize an acute fat input (260) and has been associated as an important risk factor in atherosclerosis development (107). The most relevant postprandial lipid marker it is triacylglycerol. It is the lipid with the greatest post-prandial difference from fasting lipid markers (34), and has been associated as an independent risk factor for cardiovascular events (35). The results found in our review support these statements, as significant changes were only found in triacylglycerol (233,239) concentrations and total cholesterol (231,239), which has a composition of 20% triacylglycerol in its formula.

The choice of placebo in this type of acute study design is important in order to identify whether it is indeed the anthocyanin effect that is observed in the results, or whether there are other nutrients or food constituents that may be affecting the study outcomes. All studies

included in this review included a placebo that was matched in macronutrient and fibre content, however other nutrients such as vitamin C and other flavonoids that may also play a role in postprandial oxidative stress and inflammation are not commonly considered. It is complex, and often impossible, to provide a perfectly matched placebo in food studies of this type, therefore results from studies that use different placebos should be carefully scrutinized.

The most notable challenge and limitation of this study was to address a wide range of CVDs biomarkers assessed in this type of acute clinical trial. Overall, the comparison among studies had a relevant clinical importance of these metabolic, immune and physiological factors that has a synergistic impact on CVDs, however there was a variety of parameters addressed with different methods within each of such factors. This made the possibility of meta-analysis or other pooled analyses unfeasible, however it not impeded results to be compared narratively. Another source of heterogeneity in these studies may have resulted from variability in the HMF challenges regarding their macronutrient and energy content, particularly the types of fat, as well as the format in which the meals were delivered. Accumulating evidence suggests that the health effects of dietary fats vary(108). Substitution of saturated fatty acids from butterfat with omega-6 PUFA resulted in a decreased postprandial lipaemia, as well as reduced concentrations of IL-6, TNF-α, soluble TNF-α receptors, and soluble vascular cell adhesion molecule-1 in overweight men(110). However, all studies included in this review used animal products as the main source of fat, and there were two main types of meals: 1) beverages that were enriched with cream and/or milk; or 2) mixed meals including animal products with a high content of saturated fat such as sausages, cheese, butter, eggs and bacon. A standardized meal for these type of studies would be preferred but may be difficult to implement because of cultural diversity in cuisine and dietary patterns. Despite that all included studies were conducted in young and middle-aged adults (mean age ranging from 20.2 to 46.9y), there were slightly differences in mean BMI with two studies having participants with BMI<25kg/m^2

(234,240) six with BMI from 25-30kg/m^2 (37,229–231,236,239) and three with BMI>30kg/m^2 (233,235,238) .Only three studies were not conducted in healthy adults, of which participants were not considered healthy due to lipidaemia (233), insulin-resistance phenotype (235) or with at least one CVD risk factor (239). However, studies conducted in participants with major chronic diseases with implication on CVDs, such as diabetes and hypertension, were not included in this review. Another feature in the design of this type of study is that tests are conducted over multiple time following the HFM challenge, thereby raising a concern that the number of false positive findings may be inflated. For this reason, the interpretation of results found in only one or a few time points, and that are not sustained, has to be interpreted with caution. Still, parameters that have been used in the studies to investigate oxidative stress and antioxidant status represent a wide range of analytical methods, and there is no formal mechanism to establish consensus regarding the optimal biomarkers for such nutritional studies (259,261).

3.5 Conclusion

Despite some positive findings, there was heterogeneity for changes in some CVD risk factors between studies. The most promising results were for the attenuation of deleterious postprandial effects on oxidative stress and antioxidant status, triacylglycerol and total cholesterol concentrations, as well as for vascular endothelial function and inflammatory biomarkers. The post-prandial changes in blood pressure and lipoproteins were the parameters least affected by anthocyanin treatment. Further studies are required in order to advance in the knowledge of how post-prandial changes are associated with CVD incidence and progress, and to investigate how these imbalances can be attenuated by bioactive compounds such anthocyanins.

CHAPTER 4: Anthocyanins attenuate vascular and inflammatory responses to a high fat high energy meal challenge in overweight older adults: a cross-over, randomized, double-blind clinical trial.

The findings from the previous study '*The postprandial effect of anthocyanins on cardiovascular disease risk factors: a systematic literature review of high-fat meal challenge*' guided the original design and conduct of this clinical trial. Within this range of studies, another gap that was identified is the possibility of a more robust investigation of the vascular function in the postprandial state, which along with the immune response are important predictors of CVD. Therefore, we created a protocol to explore macro and microvascular parameters by combining classical and novel techniques with the latest imaging technologies, such as the flow-mediated dilatation (FMD) and the Laser Speckle Contrast Imaging (LSCI). To our knowledge, this was the first study to conduct this type of protocol in studies with nutritional interventions. Additionally, the present clinical trial also aim to determine if consumption of food anthocyanins has postprandial effects on inflammatory and oxidative stress biomarkers, and lipid profile following a high-fat high-energy meal in older adults with overweight or obesity

The results supported that fruit-based anthocyanins attenuated the potential postprandial detrimental effects of a HFHE challenge on parameters of vascular and microvascular function, and inflammatory biomarkers in overweight older adults.

The majority of this chapter forms the substantive content of a published article (Appendix B).

4.1 Introduction

Metabolic imbalances in the postprandial state, particularly following a high fat high energy (HFHE) meal, are associated with long term development of CVD(106,107). The underlying mechanisms involve a sharp increase in circulating triacylglycerol concentrations, along with a pro-inflammatory response and an aberrant production of pro-oxidant molecules, which together may impair vascular endothelial function(105,262,263). Endothelial dysfunction is associated with an impaired bioavailability of nitric oxide (NO) and an up-regulation of various other molecules involved in the adverse vascular function(6,264).

This is particularly evident in older adults, as several studies support a new immune-metabolic viewpoint for age-related diseases, termed "inflammaging" which is characterized by a chronic, low-grade inflammatory response in the absence of a pathogen(7). Additionally, excessive adiposity at all ages is associated with an up-regulation of a pro-inflammatory state, as the accumulation of adipose tissue mass promotes the secretion and release of inflammatory mediators, including high sensitive C-reactive protein (hs-CRP), interleukin-6 (IL-6), interleukin 1 beta (IL-1β) and tumour necrosis factor alpha (TNF-α)(63,64). This process also leads to chronic low-grade inflammation that is driven by a nutrient excess and/or overnutrition and has the same mechanisms as those underpinning "inflammaging"(7). A HFHE challenge is a method to investigate such imbalances that regularly occur in response to typical 'Western' dietary patterns(264,265). A review of 57 studies investigating the postprandial state has shown that a HFHE meal induces acute postprandial inflammation; however, heterogeneity was observed for cytokines and soluble adhesion molecules, while leukocyte surface markers, mRNA and proteins were elevated in almost all studies(266). Strategies that aim to attenuate these potential detrimental postprandial effects will be of clinical relevance.

Dietary factors play a major role in regulating the inflammatory state and mobilizing the endogenous antioxidant defences(9). Protective dietary components that are consumed

together with a HFHE meal may be beneficial in attenuating the potentially harmful postprandial responses(226). Anthocyanins, a subclass of flavonoids, are emerging as one such potential agent for ameliorating adverse CVD risk factors(21,222,267). Anthocyanins have been shown to elicit immunomodulatory(21,23) and antioxidant effects(23,268), thereby blunting the cooperative and synergistic deleterious effects of oxidative stress and inflammation(190) and may, therefore, provide protection against CVD risk factors. A number of *in vitro* and *in vivo* studies have shown that anthocyanins upregulate endothelial nitric oxide synthase (eNOS) mRNA and NO synthesis(126,144,269,270) via several signalling pathways, and prevent peroxynitrite-mediated ED(125,271). Anthocyanins can also prevent the expression of adhesion molecules and the adhesion of monocytes to endothelial cells challenged by pro-inflammatory agents, and have the ability to elicit cell adaptive responses involving the transcription factor Nrf2(272). However, there is still limited data regarding the effect of anthocyanins on vascular function(273). To our knowledge, no study to date has investigated such effects on the microvasculature and predictive values for CVD are not yet defined, as they are for vascular function measured by flow mediated dilatation(11), which is considered to be a well-recognized early biomarker of atherosclerosis and a key contributor to the onset and progression of CVD(243,274,275). Measurement of the systemic microcirculatory function may help to identify ED pathological processes, and better elucidate the mechanisms underlying structural changes among these two vascular beds(96,102,276).

The aim of this study was to investigate the postprandial effects of food anthocyanins on vascular and microvascular functions, inflammatory biomarkers and oxidative stress following a HFHE meal challenge in overweight older adults. A secondary aim was to evaluate the acute effects of the 4 day run-in period of food anthocyanins on such markers. Outcomes combined both vascular and microvascular function analyses, along with the evaluation of classic CVD risk factors and associated biomarkers(277,278), including blood pressure, serum

concentration of triacylglycerols (TG) and total cholesterol (TC), inflammatory markers including hs-CRP, IL-6, IL-1β, TNF-α, and serum derivatives of reactive oxidative metabolites (DROM).

4.2Methods

This study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the University of Wollongong Human Research Ethics Committee, New South Wales, Australia (HREC 2019/043). It was also registered with the Australian New Zealand Clinical Trials Registry (ACTRN12620000437965). Written informed consent was obtained from all subjects.

Study Subjects

Sixteen subjects (13 female and 3 male) were recruited from Wollongong, NSW, Australia through advertisements within the local community, and through the University of Wollongong and Illawarra Health and Medical Research Institute social media networks between June and August 2019. Inclusion criteria included males and females aged 55+ years with a body mass index (BMI) ≥25kg/m^2. Exclusion criteria were: current treatment or diagnosis of hypertension or diabetes; chronic liver or renal diseases; history of cardiovascular events; current administration of either anti-inflammatory medication, aspirin or warfarin; smoking; diagnosis or self-reported gastrointestinal disorders; allergy to stone fruits or food colorants, unwillingness to consume a full English style breakfast that included meat products (e.g. vegetarians) at the research facility.

Study Design and Treatments

The study was a crossover, randomized, controlled, double-blind design with a 4 day run-in period. The study design and procedures are outlined in **Figure 4-1**. Briefly, four days prior to the test days, participants were asked to consume either 250 mL of Queen Garnet plum juice (intervention) or apricot juice (control) per day. Participants underwent consultation with a dietitian that included education on avoiding foods that are rich in anthocyanins during the 4 day run-in period prior to testing. On the night prior to the testing day, a standardised low flavonoid frozen meal was consumed for dinner by all participants. Participants fasted (12h) prior to the testing day.

On the testing day, participants were required to consume a HFHE meal in conjunction with a 250mL dose of either the intervention or the control juice. Blood samples and blood pressure measures were collected at baseline (fasted state), and 2h and 4h following the consumption of the HFHE meal. Vascular function and microvascular blood flow was evaluated at baseline and 2h after the meal. Urine was collected for 24h following the HFHE meal.

After a wash-out period of 14 days, participants were allocated to the opposite treatment arm, starting with the 4 day run-in period. Randomization was conducted by a researcher independent to the data collection using a computer generated randomization sequence and, in order to minimize order effects, an across subjects counter-balancing process was used. Several blinding strategies were undertaken, including advertising the study to participants as a "fruit juice study" without providing information on which fruit was being investigated, as well as colouring of the control juice to match the intervention juice colour. The usual dietary intake of participants was collected before the beginning of the study using a 3-day food record, consisting of 2 weekdays and 1 weekend day, and analysed using Foodworks 10 (Xyris Software, Australia), which includes the Australian Food Composition Database 2019(279). (**Table 4-1**)

Table 4-1. Dietary intake of participants

Energy (kcal)	1724 (375.6)
Protein (g)	80.7 (26.0)
Fat – total (g)	71.4 (18.9)
Saturated fat (g)	29.2 (5.0)
Carbohydrates (g)	171.2 (42.3)
Dietary fibre (g)	20.7 (7.4)
Sodium (mg)	1870.5 (563.8)
Vitamin C (mg)	30.4 (22.9)
Vitamin E (mg)	3.7 (2.6)
Vitamin A* (µg)	362.6 (170.7)
Anthocyanins (mg)	19.1 (31.8)

Values are mean and SD. *Total Vitamin A equivalents.

Values were obtained from the Australian Food Composition Database 2019[26].

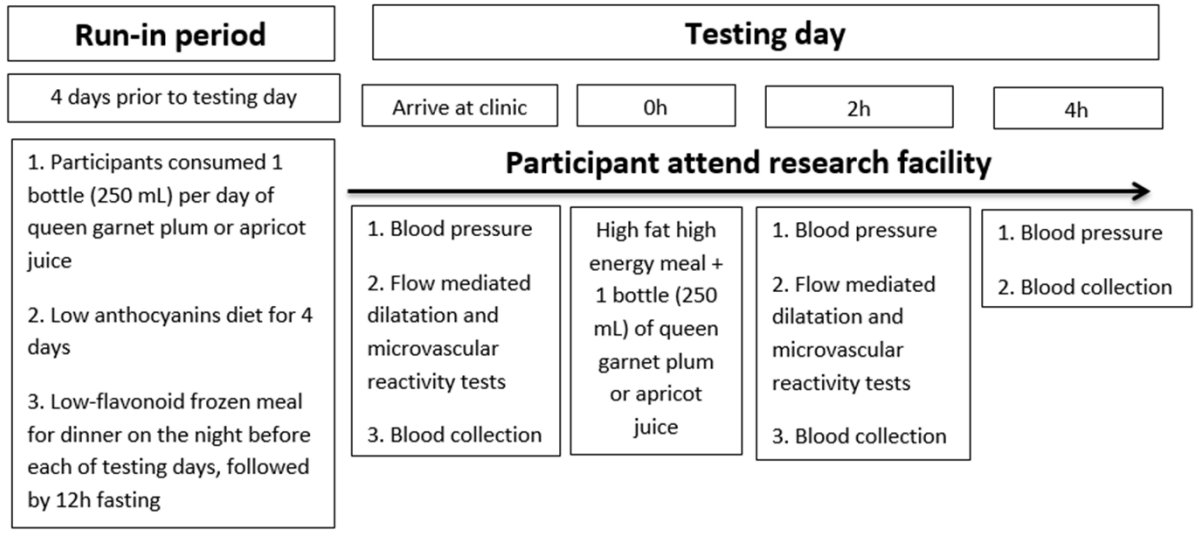


Figure 4-1. Study design and procedures repeated on each treatment arm

Test meals, and intervention and control juices

The test meal consisted of 1 hash brown (62g) 2 beef chipolatas (90g), 1 croissant (168g) served with unsalted butter (5g) and apricot jam (10g) and 2 scrambled eggs (2 x52g eggs, 30mL pure cream, 10g unsalted butter, 0.5g salt) (**Table 4-2**). One serving of Queen Garnet plum juice (intervention) was provided as 220g of plum puree with 30mL of water added. The anthocyanin content was analysed by the Queensland Department of Agriculture and Fisheries (DAF, Australia) by Performance Liquid Chromatography (HPLC) (91.3 mg of anthocyanins per 100g of Queen Garnet plum juice) and a pH differential method (94 mg/100g), following the standard AOAC 2005.02 protocol for total monomeric anthocyanin pigment(280). The 200 mg of anthocyanins provided per serve can be considered a high dose based on a systematic literature review of similar studies. In that review, only 2 of the included 13 studies used a higher dose, and a number of studies achieved significant vascular effects at doses lower than 100mg(273). Apricot juice was chosen as the control juice due to its similar consistency, nutritional content and total flavonoid concentration, but an overall lack of anthocyanins(281). Food dyes (red and blue) were added to the apricot juice to resemble the colour of the Queen Garnet plum juice.

Table 4-2. Nutrition information of the test meal and fruit juices

	Test meal[1]	Plum juice (250 mL)	Apricot juice (250 mL)
Energy (kcal)	856.9	98	96
Protein (g)	25.9	0.6	0.7
Fat – total (g)	65.3	< 0.1	< 0.1
Saturated fat (g)	32.9	< 0.1	< 0.1
Carbohydrates (g)	41.4	22.2	22.2
Dietary fibre (g)	1.7	4.4	4.0

Sodium (mg)	941	6	2
Vitamin C (mg)	0	0.3	1.2
Anthocyanins (mg)	0[2]	200.8[3]	0[2]
Anthocyanins (mg)	0[2]	206.8[4]	0[2]

[1]Whole meal including one hash brown (62g), two scrambled eggs (104g) with pure cream (30g), butter (10g) and salt (0.5g), two beef chipolatas (90g), one croissant (168g) with butter and apricot jam; [2]Phenol-explorer 3.0(282); [3]High Performance Liquid Chromatography; [4]pH differential method. Values were obtained from the Australian Food Composition Database 2019[26].

Blood pressure

BP was measured at baseline (fasted state), and 2h and 4h following the consumption of the HFHE meal using an using a Welch Allyn Connex 6700 Vital Signs Monitors (Welch Allyn, NSW, Australia). Participants were rested in supine position for 5 minutes, in a quiet room with a correctly fitting arm cuff. BP was measured on both arms, and a repeat measure taken on the arm with the higher reading, with the average of the two readings recorded(283).

Flow mediated dilation (FMD) and microvascular perfusion

Participants were rested in a quiet, temperature-controlled room (23 °C ±1), in a supine position for 20 minutes. In brief, the first procedure was the microvascular PORH test conducted on the left arm, followed by the FMD conducted on the right arm, and lastly iontophoresis of acetylcholine, combined with LSCI, conducted in the left forearm (with 30 minutes between tests in the left forearm).

Vascular function was measured using FMD following standard guidelines(284), by a trained researcher. The same blinded researcher administered the test for each participant at two time points (baseline and 2hrs post-meal consumption, **Figure 4-1**) on both treatment arms,

and was responsible for the analysis of the resulting images to prevent inter-rater error. FMD of the brachial artery was measured using an uSmart3300 Ultrasound system (Terason, Massachusetts, USA) in combination with a semi-automated computerized analysis system (FMD Studio, QUIPO, Pisa, Italy). The brachial artery was imaged longitudinally at 2-10cm proximal to the antecubital fossa. Video recording collected beat to beat measures of the diameter and velocity for 1 minute, and the average was used as the baseline. Then the blood pressure cuff placed around the forearm was inflated to 60mmHg above resting systolic blood pressure. Blood flow was restricted for 5 minutes, then the cuff was rapidly released, resulting in reactive hyperaemia. Video was recorded for the 5 minutes period following the cuff release. The FMD response was calculated as the relative diastolic diameter change from baseline compared to the peak diastolic diameter following hyperaemia and expressed as a percentage. This flow-mediated dilatation protocol is routinely performed in our laboratory using the methods outlined by Francois et al. (2016)(285); with intra-subject coefficients of variation of 7.1% for %FMD.

Microvascular cutaneous vascular reactivity was measured using an LSCI system with a laser wavelength of 785 nm (Pericam PSI System, Perimed AB, Järfälla, Sweden). The image acquisition rate was 21 images/s, and the distance between the laser head and the skin surface was fixed at 25 ±0.5 cm. The skin of the volar side of the left arm was gently cleaned with 70% isopropyl alcohol swabs. Three equidistant skin areas (region of interest) of approximately 80 mm^2 were selected in the central volar part for the left arm(286), avoiding any skin mark or bulge areas(95). Participants were instructed to avoid any movement, and not to speak or breathe deeply during the record(95). During the PORH test, the baseline perfusion was measured in the volar side of the left forearm for 2 minutes, followed by an arterial occlusion maintained for 3 minutes using a blood pressure cuff around the upper arm inflated to a pressure of 50-60 mmHg above systolic pressure reading(96,276). After the blood pressure cuff was

released, the PORH response was recorded for 3 minutes. The following parameters were extracted: baseline flow (BF), biological zero (BZ) and peak value (PV)(95). The PV was obtained with a 5 second "time of interest" starting from the highest value after deflation of the cuff(286). RF was calculated as BF – BZ. The maximum PORH perfusion (PORHmax) was calculated as PV – RF (**Supplementary material, Figure S1 and Table S1**). This PORH protocol is routinely performed in our laboratory; with intra-subject coefficients of variation of 7.7% for PV and 11.4% for PORHmax.

Additionally, a pharmacological reactivity test was conducted using iontophoresis of acetylcholine (2%, dissolved in deionized water) using a micropharmacology system (PF 751 PeriIont USB Power Supply; Perimed) with a single electrical current of 0.1mA for 30s(101). Microvascular blood flow was recorded for 2 minutes prior to the current and 8 minutes after the pharmacological stimulus. The maximum perfusion following the iontophoresis of acetylcholine (IONTmax) was calculated by using subtracting the RF from the PV using the same procedures used in the PORH test (**Supplementary material, Table S1**).

Blood and urine samples

Plasma and serum samples were stored at -80 °C, prior to analysis. IL-1β, IL-6 and TNF-α, were analysed in a Luminex 200 using a Human High sensitivity T cell magnetic bead panel kits (Merck Millipore, Billerica, MA, USA). hs-CRP, TC and TG were analysed on a BK400 automated chemistry analyser using a commercial immunoturbidometric assay (Biobase, Shandong, China). Derivatives of reactive oxidative metabolites were analysed in a Cobas Mira Plus (Roche, Washington, USA) using a commercially available colorimetric kit (Diacron, Grosseto, Italy).

Urine samples were collected between 0-4h, 4-12h and 12-24h and stored at -80 °C, prior to analysis. Aliquots of 3 mL from each timepoint were pooled and 1 mL of the pooled

sample was analysed using ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) to determine differences in excretion of total anthocyanins (expressed as cyanidin-3-O-glucoside equivalents equivalents), and phenolic acid metabolites related to anthocyanins (protocatechuic acid, hippuric acid and ferulic acid). Briefly, 1 ml urine was extracted by solid phase extraction using Discovery® DSC-18 (6 mL, 1 g) cartridges(287). The eluate was evaporated under nitrogen, reconstituted in 1 % formic acid in water and analysed by UPLC-MS/MS (Waters Acquity H-Class UPLC instrument coupled to a Waters Xevo TQ tandem mass spectrometer), operating in multiple reaction monitoring and positive electrospray ionization modes. The presence of each compound is expressed in ng/mL or µg/mL

Statistical Analyses

Data are presented as mean and standard deviation or median and interquartile range. Natural log transformation was used when non-normal data better fit the normal distribution (triacylglycerol, total cholesteterol, hs-CRP, PV and PORHmax). Two-way repeated measures ANOVAs were used to investigate the outcomes. Significant interaction effects were analysed by post-hoc comparisons with Bonferroni correction. Dependent t- and Wilcoxon signed-tank tests evaluated the difference in baseline measures to investigate the effects of the 4 day run-in period, as well as to investigate the differences in other time points among the treatments. Pearson and Spearman coefficients were calculated to evaluate potential baseline correlations between vascular parameters and inflammatory biomarkers. All LSCI parameters were transformed from arbitrary perfusion units (PU) to cutaneous vascular conductance (cvc) by dividing the obtained values by the mean arterial pressure to yield cvc in PU/mmHg. SPSS (version 25, IBM, Chicago, IL, USA 2019) was used for all statistical analyses. Significance

was accepted at alpha $p<0.05$, and non-significant p values between p=0.05-0.099 were considered a trend in the data.

The primary endpoint of change in FMD from baseline to 2 h was used for the power calculation. Based on a previous study that found a significant effect of anthocyanins on post-prandial changes in FMD(229), and aiming for a significant improvement of 1% in FMD, 13 participants were required to achieve a study power of 80% with an α of 0.05.

4.3 Results

All sixteen recruited subjects completed the study (n=16). The mean age and BMI for the study group were 65.9 years (SD 6.0) and 30.6 kg/m^2 (SD 3.9), respectively. Mean baseline body anthropometry and clinical measures (weight, height; total cholesterol, triacylglycerol, systolic and diastolic blood pressure) are presented in **Table 4-3**. No harms or unintended effects were observed through the study. The sample size of some vascular parameters was reduced due to experimental error (participant movement during the tests), resulting in n=15 for PORH and FMD, and n=13 for iontophoresis with acetylcholine. No significant correlations were observed between baseline vascular parameters and inflammatory biomarkers (P>0.05) (**Supplementary material, Table S2).**

Table 4-3. Baseline demographics

n	16
Female	13
Male	3
Age (years)	65.9 (SD 6.0)
Body weight (kg)	81.5 (SD 10.5)
Height (cm)	163 (SD 8.7)
BMI (kg/m^2)	30.6 (SD 3.9)
Total cholesterol (mmol/L)	6.28 (IQR 1.33)

Triacylglycerol (mmol/L)	0.94 (IQR 0.84)
SPB (mm Hg)	120.2 (SD 12.3)
DPB (mm Hg)	69.3 (SD 7.4)

Values are means and standard deviation or median and interquartile range. SD, standard deviation; IQR, interquartile range; DBP, diastolic blood pressure; SBP, systolic blood pressure.

Blood pressure, triacylgycerol and cholesterol

There were significant post-prandial changes on diastolic blood pressure, triacylglycerol and total cholesterol across the time points ($P<0.05$ for time effect), but no significant interaction or simple effect of treatment between the intervention and the control arms ($P>0.05$) (**Table 4-4**).

Table 4-4. Blood pressure, triacylglycerol, total cholesterol and triacylglycerol before and after a high fat high energy meal challenge in control and intervention groups.

Measures	Group	Baseline	2h	4h	Time[a]	Treatment[a]	Time x treatment[a]
SPB (mm hg)	Control	118.9 (SD 11.1)	116.3 (SD 12.6)	123.2 (SD 12.8)	0.001*	0.692	F(2,30)=0.278, P=0.759, ηp^2=0.018
	Anthocyanins	118.8 (SD 12.9)	117.7 (SD 10.6)	123.4 (SD 12.4)			
DBP (mm hg)	Control	70.1 (SD 6.9)	67.4 (SD 7.3)	70.5 (SD 7.6)	0.002*	0.148	F(2,30)=1.684, P=0.203, ηp^2=1.01
	Anthocyanins	68.0 (SD 6.7)	66.6 (SD 5.5)	70.8 (SD 6.6)			
Triacylglycerol (mmol/L)	Control	0.95 (IQR 0.79)	1.30 (IQR 0.96)	2.12 (IQR 1.45)	>0.001*	0.357	F(2,30)=0.083, P=0.921, ηp^2=0.006 [b]
	Anthocyanins	0.89 (IQR 0.89)	1.29 (IQR 1.35)	2.22 (IQR 2.35)			
Total cholesterol (mmol/L)	Control	6.16 (IQR 1.39)	6.02 (IQR 1.65)	6.09 (IQR 1.26)	>0.001*	0.756	F(2,30)=0.648, P=0.530, ηp^2=0.041 [b]
	Anthocyanins	6.28 (IQR 1.27)	6.40 (IQR 1.16)	6.27 (IQR 1.14)			

Values are mean and standard deviation or median and interquartile range (n=16). SD, standard deviation; IQR, interquartile range; ηp^2, partial eta-squared, DBP, diastolic blood pressure; SBP, systolic blood pressure. [a] P-values for two-factors repeated measures ANOVA; [b] results from log natural transformed dat

Vascular function

Postprandial changes (i.e. the difference between baseline and 2h after the HFHE meal) in mean FMD were +0.16% in the anthocyanins group and -0.47% in the control group (**Table 4-5**). There was a significant treatment effect (*P*=0.028); however, there was no main effect of time (*P*=0.474) or interaction between treatment and time (*P*=0.219) (**Table 4-5**). Dependent t-tests showed no significant difference between baseline in FMD results (*P*=0.212, +4.09% SD 1.12 intervention vs 3.58% SD 1.14 control arm) indicating no effect of the 4 day run-in period, and a significant difference (*P*=0.019) of 1.14% in post-prandial FMD among treatments (+4.25% SD 1.34 intervention vs 3.11% SD 1.00 control arm), (**Figure 4-2a**). There was no significant interaction between time and treatment nor a significant treatment effect for the peak shear rate; however, a trend towards a time effect was observed (*P*=0.091) (**Table 4-5**)

Table 4-5. Vascular and microvascular reactivity parameters before and after a high fat high energy meal challenge in control and intervention groups.

Parameter	Group	Baseline	2h	Time[a]	Treatment[a]	Time x treatment[a]
FMD (%)	Control	3.58 (SD 1.14)	3.11 (SD 1.00)	0.474	0.028	F(1,14)=1.653, P=0.219, ηp^2=0.106
	Anthocyanins	4.09 (SD 1.12)	4.25 (SD 1.34)			
Peak shear rate (sec^{-1})	Control	954.7 (SD 344.1)	881.9 (SD 219.6)	0.091	0.583	F(1,14)=1.293, P=0.275, ηp^2=0.085
	Anthocyanins	955.5 (SD 324.7)	811.5 (SD 300.5)			
PV (cvc)	Control	1.20 (IQR 0.37)	1.08 (IQR 0.28)	<0.001	0.125	F(1,14)=0.121 P=0.465, ηp^2=0.036 [b]
	Anthocyanins	1.21 (IQR 0.28)	1.16 (IQR 0.23)			
PORHmax (cvc)	Control	0.85 (IQR 0.28)	0.73 (IQR 0.23)	<0.001	0.062	F(1,14)=2.247 P=0.155, ηp^2=0.130 [b]
	Anthocyanins	0.88 (IQR 0.27)	0.83 (IQR 0.21)			
IONTmax (cvc)	Control	0.45(SD 0.20)	0.45 (SD 0.25)	0.748	0.620	F(1,12)=0.020, P=0.889, ηp^2=0.002
	Anthocyanins	0.43 (SD 0.22)	0.41 (SD 0.22)			

Values are mean and standard deviation or median and interquartile range. FMD, flow-mediated dilatation; SD, standard deviation; IQR, interquartile range; cvc, cutaneous vascular conductance in PU/mmHg; ηp^2, partial eta-squared; PV, peak value; PORHmax, post-occlusive reactive hyperaemia maximum perfusion; IONTmax, maximum perfusion following iontophoresis of acetylcholine. [a] *P*-values for two-factors repeated measure

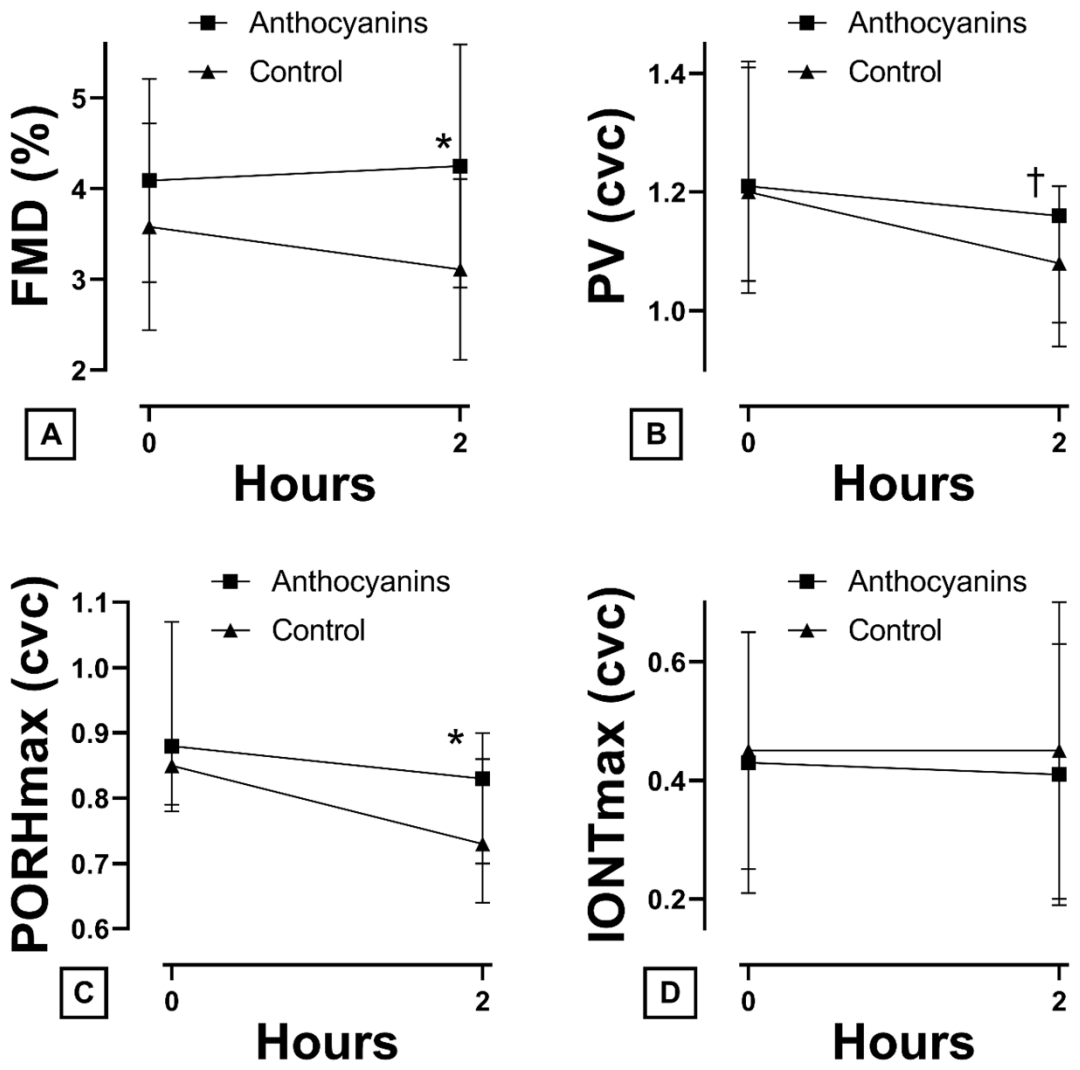


Figure 4-2. Flow mediated dilatation and microvascular reactivity parameters before and after a high fat high energy meal challenge in control and intervention groups (n=15 for panels A, B and C, and n=13 for panel D). Values are mean and error bars are standard deviation (A and D) or median and error bars are 1st and 3rd quartile (B and C). FMD, flow mediated dilatation; PORHmax, post occlusive reactive hyperaemia maximum perfusion; cvc, cutaneous vascular conductance in PU/mmHg *P<0.05 (dependent t-test at A and Wilcoxon signed-tank test at C); †P=0.088(Wilcoxon signed-tank test).

No significant interactions between time and treatment were evident for any parameter of the PORH test (**Table 4-5**). A significant time effect for PV and PORHmax ($p<0.001$ for both parameters) was found, indicating a post-prandial effect of the HFHE meal challenge independent of treatment. The treatment effect for PV was not significant ($P=0.125$) and dependent t-tests showed no significant difference resulting from the 4 day run-in period on fasting measures ($P=0.334$, Wilcoxon signed-tank test). There was a trend for a higher post-prandial PV in the anthocyanins group ($p=0.088$, Wilcoxon signed-tank test) (**Figure 4-2b**). The parameter PORHmax presented a trend towards a treatment effect ($P=0.062$) and no significant difference carried by the 4 day run-in period in fasting measures ($P=0.594$, Wilcoxon signed-tank test) (**Figure 4-2**). A significantly higher postprandial PORHmax of 0.10 PU/mmHg ($P=0.049$, Wilcoxon signed-tank test) was evident in the anthocyanins group compared to the control group (**Figure 4-2c**). The microvascular reactivity test induced by the iontophoresis of acetylcholine showed no significant effects for IONTmax (**Table 4-5**). There was no significant difference between the fasting ($P=0.582$, dependent t-test) and post-prandial ($P=0.684$, dependent t-test) IONTmax between the treatments (**Figure 4-2d**). The data used to calculate the microvascular parameters are presented in the supplementary material (**Table S3**).

Inflammatory markers and serum derivatives of reactive oxidative metabolites

A significant interaction effect was found for serum hs-CRP ($P=0.036$) (**Table 4-6)**. Corrected pairwise comparisons showed no significant differences between the baseline (fasted), 2h and 4h postprandial values for both treatments ($P>0.05$) (**Figure 4-3**). However, there was a significantly lower hs-CRP concentration 4h postprandially in the anthocyanins group when compared to the control arm ($P=0.026$, Wilcoxon signed-tank test), as well as trends in the baseline and in the 2h postprandial time point ($P=0.098$ and $P=0.083$, respectively,

Wilcoxon signed-tank test) (P=0.095 at baseline P=0.099 at 2h, and P=0.008 at 4h, Wilcoxon signed-tank tests) (**Figure 4-3**).

There were no significant effects for serum concentration of IL-6, TNF- α and IL-1β (**Table 4-6**). A trend in the treatment effect was evident for IL-6 concentrations (P=0.075). There was a significantly lower concentration of IL-6 at the 4h post-prandial time point in the anthocyanins group when compared to the control group (P=0.009, Wilcoxon signed-tank test) (**Figure 3**). There was no significant difference evident following the 4 day run-in period in fasting concentrations for any of the inflammatory biomarkers between groups (P>0.05, dependent t tests) (**Figure 4-3**).

There was a significant effect of time (P=0.002) in serum concentrations of DROM, however, no significant treatment or interaction effect was evident (P>0.05) (**Table 4-6**).

Urinary excretion of total anthocyanins and phenolic acids

The 24h concentration of total anthocyanins in the urine was higher in the intervention arm 1.86 ng/mL (IQR 3.23) than the control arm 0.015 ng/mL (IQR 0.12) following the HFHE meal challenge (P<0.001) (**Table S4, Supplementary material**). Concerning anthocyanins metabolites, there were significantly higher concentration of hippuric acid in the urine for participants within intervention arm versus the control (P=0.027), while no significant differences were observed for protocatechuic acid and ferulic acid.

Table 4-6. Serum concentration of inflammatory biomarkers and derivatives of reactive oxidative metabolites before and after a high fat high energy meal challenge in control and intervention groups.

Parameter	Group	Baseline	2h	4h	Time[a]	Treatment[a]	Time x treatment[a]
hs-CRP (mg/L)	Control	2.30 (IQR 1.95)	2.40 (IQR 2.05)	2.30 (IQR 1.95)	0.299	0.094	F(2,30)=3.763, P=0.036, ηp^2=0.212[b]
	Anthocyanins	1.70 (IQR 1.00)	1.70 (IQR 0.95)	1.80 (IQR 0.90)			
IL-6 (pg/mL)	Control	5.95 (IQR 4.20)	5.40 (IQR 3.70)	5.98 (IQR 4.73)	0.436[c]	0.075	F(2,30)=0.635, P=0.537, ηp^2=0.041
	Anthocyanins	6.00 (IQR 8.83)	5.85 (IQR 6.45)	5.55 (IQR 5.48)			
TNF-α (pg/mL)	Control	13.65 (IQR 10.65)	12.45 (IQR 9.25)	12.53 (IQR 9.89)	0.277	0.987	F(2,30)=0.868, P=0.430, ηp^2=0.055
	Anthocyanins	13.65 (IQR 8.88)	14.30 (IQR 7.95)	13.10 (IQR 7.33)			
IL-1β (pg/mL)	Control	1.60 (IQR 1.39)	1.55 (IQR 1.65)	1.55 (IQR 1.26)	0.896	0.868	F(2,30)=1.436, P=0.254, ηp^2=0.087
	Anthocyanins	1.20 (IQR 1.15)	1.40 (IQR 1.10)	1.45 (IQR 1.28)			
DROM (cu)	Control	487.3 (SD 77.6)	497.1 (SD 81.1)	502.6 (SD 68.3)	0.002	0.298	F(2,30)=0.752, P=0.480, ηp^2=0.048
	Anthocyanins	473.1 (SD 75.5)	496.4 (SD 77.0)	496.5 (SD 78.6)			

Values are mean and standard deviation or median and interquartile range. hs-CRP, high-sensitivity c-reactive protein; IQR, interquartile range; ηp^2, partial eta-squared, IL-6, interleukin-6; TNF-α. Tumour necrosis factor alpha; IL-1β, interleukin-1 beta; DROM, derivatives of reactive oxidative metabolites; cu, Carratelli units SD, standard deviation. a P-values for two-factors repeated measures ANOVA; b results from log natural transformed data; c Greenhouse-Geisser correction (Mauschly's test P=0.037, Epsilonb =0.727).

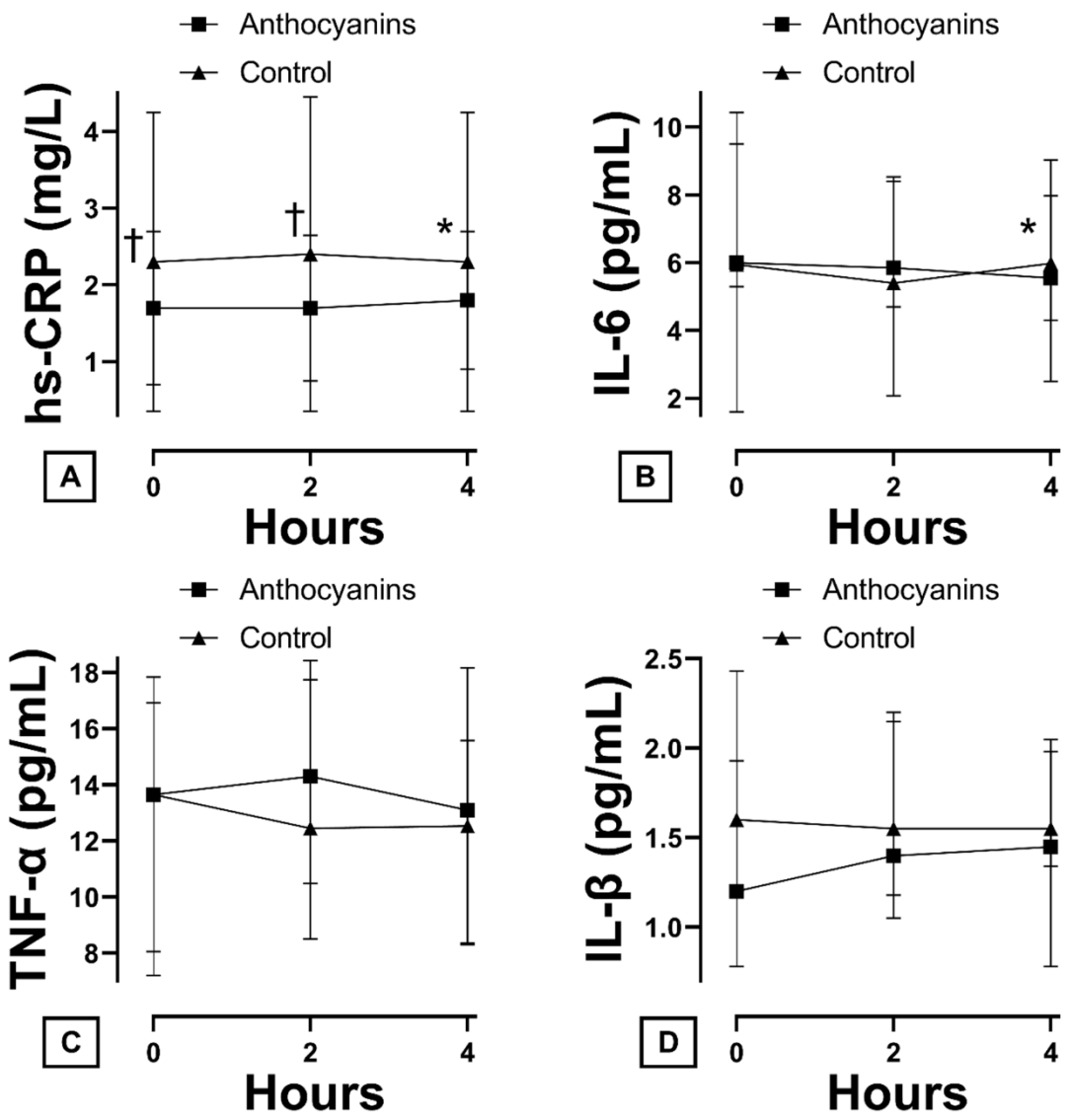


Figure 4-3. Serum concentration of inflammatory biomarkers before and after a HFHE meal challenge in control and intervention groups (n=16). Values are median and error bars are 1st and 3rd quartile. hs-CRP, high-sensitivity c-reactive protein, IL, interleukin, TNF, tumour necrosis factor. *P<0.05 (Wilcoxon signed-tank test); † P=0.095 at 0h and P=0.099 at 2h (Wilcoxon signed-tank tests).

4.4Discussion

The intake of food anthocyanins with a HFHE meal challenge was able to attenuate the potential detrimental effects on both vascular and microvascular function, and on the inflammatory response. Compared to the control arm, participants had a significant higher

postprandial FMD and some of the parameters of microvascular function, and significant lower concentration of postprandial hs-CRP, with a trend for lower IL-6 levels when allocated in the anthocyanins intervention arm. Several outcomes presented a significant time effect from the fasted state to the post-prandial measures, confirming that a single HFHE meal is a sufficient challenge to induce some negative vascular and inflammatory responses that are not apparent in the fasted state. Importantly, our findings indicate that food-based anthocyanins may confer protection against cardiovascular and inflammatory insults caused by a typical HFHE 'Western' diet in older overweight, though otherwise healthy, adults.

The endothelium plays a major role in vascular homeostasis and its implications in the development of atherosclerosis and CVD are well established(288,289). Flow-mediated dilatation is a non-invasive measurement of endothelium function that has been associated with CVD risk prediction(243,274). A meta-analysis of 23 studies, including 14,753 subjects, found an overall 8 % reduction in CVD risk (RR= 0.92; 95%CI: 0.88; 0.95) for each percentage increase in FMD(274). In the present study, food anthocyanins induced a slightly increase in the postprandial FMD, while a decline was observed in the control arm. Participants had a 1.14% higher post-prandial FMD when allocated in the anthocyanins intervention. The ability of anthocyanins to prevent the reduction in FMD caused by a HFHE meal challenge suggests that there are benefits for cardiovascular health in older adults that consume a high fat diet; however, the extrapolation of such results has to be cautious, considering the difference between FMD assessed in the fasted and in the postprandial state are still not elucidated. Although a 1.14% change may be considered small in healthy, normal weight young adults, this magnitude of effect is of higher clinical significance for individuals in the present study, who had a BMI >30kg/m^2 and were aged >65 years, two factors related to impaired brachial artery FMD(42,284,290). In the current study, the postprandial difference of 1.14% represents a 36.7% higher FMD in the anthocyanin compared to the control arm. Similar findings were

observed in a double-blind, randomized, crossover study (conducted in 23 male participants, aged 46y SD 1.9, BMI of 27.6 kg/m^2 SD 0.4) that investigated postprandial effects of an açaí smoothie following a HFHE meal challenge compared to a control smoothie matched by macronutrients and vitamin C(229). In that study, the intervention arm was provided more than a twofold (493mg) quantity of anthocyanins than that provided in the current trial but a similar improvement was observed in postprandial FMD after 2h (1.4% vs 0.4% for intervention vs control smoothie; P = 0.001)(229). The immunomodulatory(21,23) and antioxidant effects(23,125,268,271) of anthocyanins, as well as their capacity to upregulate eNOS mRNA and NO synthesis(126,144,269,270), are the proposed mechanisms to exert this vascular protective effect against the potential pro-inflammatory and oxidative stress state caused by the HFHE meal. However, NO is a highly reactive molecule with a short half-life, which complicates its direct measurement(291), and the evaluation and implication of its metabolites remains challenging in vascular biology research(292). Concerning the peak shear rate, there were no significant changes between treatment arms before and after the HFHE meal challenge. Participants had an identical peak shear rate before the HFHE meal, followed by a slightly postprandial decrease in both conditions.

In additional to effects on macrovascular function, we observed significant changes for microvascular reactivity tests. Flow-mediated dilatation and PORH both rely on measuring the transient increase in blood flow following the release of a brief occlusion; however, while FMD assesses changes in diameter of the brachial artery induced by shear stress in the artery wall(293), PORH assesses the cutaneous perfusion in the microvasculature, in this case in areas of the skin of the forearm(96). In the present trial, both the PV and PORHmax parameters were reduced after the high-fat meal challenge in both groups, but there was a significantly lower reduction when anthocyanins were consumed with the meal, indicating a preventative effect of anthocyanins in HFHE meal-induced changes in these parameters. Unlike FMD, the effects of

anthocyanin consumption on LSCI microvascular parameters and CVD outcomes have not yet been explored in larger studies. To our knowledge, this is the first study to report the results of LSCI microvascular parameters along with FMD following a dietary intervention. Our findings suggest that underlying mechanisms associated with the observed effects might be due to common mechanisms between different vascular beds. The mechanisms underlying the vascular reactivity in the PORH test are still being elucidated, but many mediators seems to contribute to vasodilation and although most of these responses are endothelial-dependent, NO and COX pathways do not appear to exert significant influences(102,103). The major contributors for peak and time course of this microvascular reactivity are the sensory nerves through an axon reflex response(95). The endothelium-derived hyperpolarizing factors (EDHF) are also involved, including large-conductance calcium-activated potassium channels (BKCa) stimulated by epoxyeicosatrienoic acids(93,104). Therefore, we also utilised LSCI technology with iontophoresis of acetylcholine to assess a more specific endothelial microvascular reactivity response related to NO availability(96). However, the cutaneous perfusion following iontophoresis of acetylcholine was not altered in either of the study arms. This may be in keeping with the finding that the NO response associated with endothelial function decreases with age, while other pathways are preserved(294–296).

The inflammatory response is a fundamental component of atherosclerosis and is related to the development of CVD,(190,277) with low grade continuous inflammation occurring in association with adiposity(297) and aging ("inflammaging")(298). Higher pro-inflammatory cytokine concentrations are also associated with decreased health-related quality of life in older adults(299). For these reasons, the target population for the current study was older adults who were overweight or obese. The ability to suppress a persistent inflammatory state is important to attenuate the progress of atherosclerotic disease(277,278). A large number of in vitro and animal studies(21,121,127,139) provide insight into these mechanisms related

to the anti-inflammatory actions of anthocyanins. These include: (1) modulation of arachidonic acid metabolism, in which lipid mediators that regulate inflammation (e.g. prostaglandins and leukotrienes) are modulated by anthocyanins through inhibition of the key enzymes cyclooxygenases and lipoxygenases; (2) decreased activity of the NF-κB pathway, which is a transcription factor responsible for triggering and regulating inflammatory processes that lead to the expression of pro-inflammatory cytokines and enzymes; (3) suppression of acute pro-inflammatory genes that regulate inducible nitric oxide synthase (iNOS), an enzyme responsible for moderating some of the production of nitric oxide (NO). hs-CRP has emerged as a major marker of vascular inflammation playing a direct role in promoting endothelial dysfunction(65,66) and clinical CVD events(67,68,297). In the present trial, we found a significant interaction effect between time and treatment for serum hs-CRP. Breaking down this simple effect, we showed no significant influence of time and a significantly lower hs-CRP concentration at the 4h post-prandial time point in the anthocyanin arm, as well as a trend for lower concentrations at the fasted state and at the 2h post-prandial time point. An ability of food-based anthocyanins to reduce hs-CRP concentrations in a population with risk factors such as advanced age and high BMI, could be of major clinical benefit regarding attenuation of the "inflammaging" process and chronic low-grade inflammation.

A systematic review of the literature examining the postprandial inflammatory response to a HFHE meal challenge reported consistent robust increases in IL-6 across studies, while IL-1β and TNF-α did not appear to transiently and/or robustly change in the postprandial period(244). Among the six studies(37,231,233–236) that evaluated the effect of food anthocyanins on post-prandial IL-6 concentrations, three studies(37,231,236) reported significant reductions in postprandial concentrations when compared to the control group. In the present trial, no significant main or simple effects were evident for serum concentration of IL-6; however, a trend was observed in the main effect of treatment, as well as significantly

lower IL-6 concentration at the 4h post-prandial time point (P=0.009). IL-6 has a high expression on vascular endothelium(64) and although IL-6 possess both pro- and anti-inflammatory properties(300), it is an independent contributor to variations in endothelium-dependent vasodilatation(301). Our results demonstrate changes associated with food-based anthocyanins on hs-CRP and IL-6 concentrations, which combined could indicate reduced vascular inflammation. Such findings may have positive implications in the progression of atherosclerosis and CVD events in this high risk group. Further studies are required to better elucidate the different clinical implications between fasted and post-prandial serum concentration of such biomarkers. Similar to other studies(244), serum concentrations of IL-1β and TNF-were not altered after the HFHE meal challenge by any treatment. Only a small number of studies (one(231) out of four(37,231,233,234)) that investigated TNF-α response to a meal challenge found that the anthocyanins prevented a postprandial rise of concentrations, while no significant effects were found for IL-1β in these studies(37,231,233,234).

Serum DROM was evaluated in the present trial as a marker of oxidative stress. DROM is increased in patients with coronary artery disease and is also associated with cardiovascular events, thus could provide clinical benefits for risk stratification of coronary artery disease where higher DROM place a person at greater risk(302). We found a significant time effect in serum concentrations of DROM, indicating postprandial oxidative stress induced by the HFHE meal; however, no significant difference between treatment arms was found. This same response was observed for systolic and diastolic blood pressure, as well for total cholesterol and triacylglycerol. Similar to our findings, no significant changes in postprandial systolic blood pressure were reported in 3 studies(229,238,239) following a food anthocyanin intervention, except for one study that found a reduction in diastolic blood pressure(239). A large number of studies(231–240) that evaluated postprandial total cholesterol and

triacylglycerol also reported no changes, while only two studies found a reduction in triacylglycerol(233,239) concentrations and total cholesterol(231,239).

The urinary analyses demonstrated higher concentrations of total anthocyanins and hippuric acid biomarkers in 24h urine following consumption of the intervention and control juices, confirming the uptake and metabolism of these compounds from the anthocyanin-rich plum juice. The absence of anthocyanins in the urine corresponding to the control arm confirms the anthocyanins dietary restriction during the 4-day run-in period, the lack of anthocyanins in the control juice, as well as a sufficient wash-out period. A large variation in the excretion of both anthocyanin and phenolic acid biomarkers reflects the large intra-individual variation in the metabolism of anthocyanins and flavonoids[46,85]. The lack of significant difference between the arms for protocatechuic acid and ferulic acid may reflect the potential presence of other flavonoids in the background diet and in the control (apricot) juice.

Limitations in the present study include the different numbers of females and males enrolled, thus precluding sub-analysis by gender; however, the small number of males included in the study had similar scores to females and the exclusion of them for sensitivity analyses did not alter the significance of any of the results. A further limitation was the impossibility to repeat the FMD and microvascular reactivity tests in case of experimental errors (participant movement during the tests) due to the protocol of measures being collected at specific time points. This resulted in a small sample size reduction for the FMD and PORH test (n=1) and the iontophoresis of acetylcholine (n=3). Concerning the evaluation of endothelium and non-endothelium mechanisms in vascular and microvascular reactivity, we were not able to conduct nitrate-mediated dilation nor iontophoresis of sodium nitroprusside due to temporal reasons, considering it would be not viable, if not impossible, in a postprandial study to conduct a protocol with all of these measures. Discussion of the results is therefore based on the tests that were conducted and the elucidated underlying mechanisms described in the literature. Another

limitation is that there were no measures before the start of the 4 day run-in period, and despite that there was no significant difference in baseline, the 4 day run in period might have modulated a few parameters in the anthocyanins group, and therefore facilitating the significant post-prandial difference between the treatment arms. On the other hand, this design simulates a daily diet routine, in which individuals could be constantly eating random sources of anthocyanins, or still consuming supplements or food items on a daily basis that continuously exposes them to many bioactive compounds. This allowed us to investigate the postprandial effect of a continuous anthocyanins stimulus, while at the same time, avoid an overestimated effect in a 'super controlled' setting.

An important strength and key feature in the design of this study should be considered for interpretation of the results, as well as for comparison with other post-prandial studies. It is challenging to investigate a specific bioactive compound, such as a specific flavonoid, in a clinical trial using foods as vehicles for delivery. A food item that is a rich source of a particular flavonoid may also contain other classes of flavonoids or still other bioactive compounds, such as other polyphenols, and/or inorganic compounds (such as nitrate) that might have similar or synergistic effects on the outcomes to those being assessed. In order to address this issue, the choice of the placebo food item for the control arm is an important consideration. A placebo that is only matched in macro, or even micronutrient content, can underestimate the effects of other bioactive compounds. In this study, we aimed to evaluate the acute effect of a high dose of anthocyanins using Queen Garnet plum juice as the food source. In order to isolate the effect of the anthocyanins, we chose apricot juice as the control due to its lack of anthocyanins, but its similarly matched macro- and micronutrient content, as well as its polyphenol profile(281), that includes flavanols (such as quercetin), catechins and epicatechins, all of which are potential mediators of cardiovascular health(200,303–305). The choice of this control juice is novel in

that the effects of the flavonoid subclass of anthocyanins can be isolated from other bioactive compounds.

4.5 Conclusion

The postprandial effects of food anthocyanins improved several CVD biomarkers in overweight older adults following a HFHE meal challenge. Potentially beneficial effects were observed in parameters of both macrovascular and microvascular function, as well as some inflammatory biomarkers. The HFHE meal challenge induced increases in postprandial blood pressure, DROM, triacylglycerol and total cholesterol; however, anthocyanin consumption did not attenuate these responses. Our findings may have a relevant role in the cardiovascular health of this high-risk group considering the major role of the vascular endothelium and the inflammatory response in the atherosclerosis disease and CVD events. Further studies are required to better elucidate the clinical implications of post-prandial biomarkers of CVD.

4.6 Supplementary Material

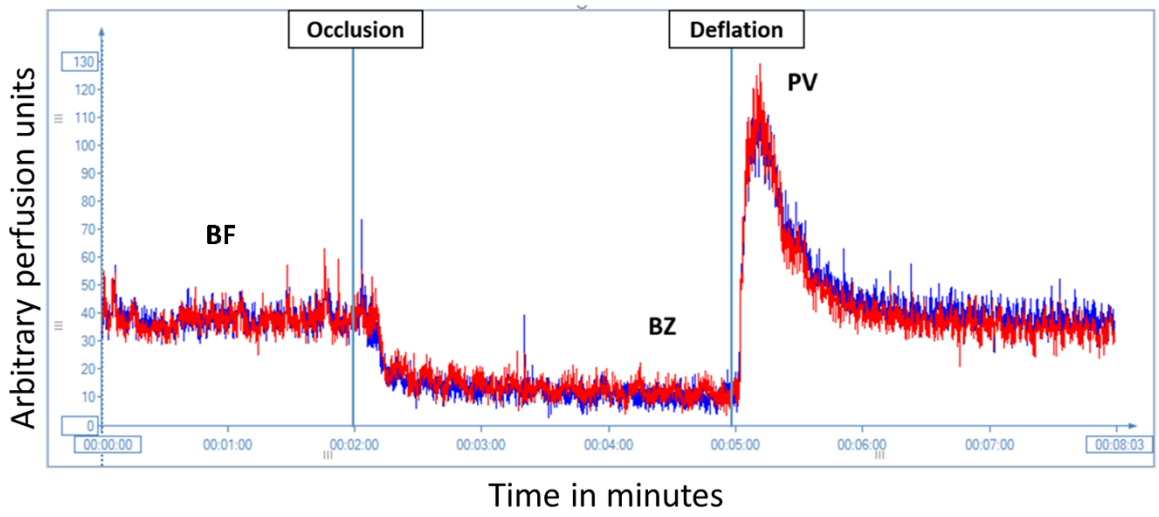


Figure 4-S1. Cutaneous perfusion recorded with laser speckle contrast imaging during post-occlusive reactive hyperaemia.

BF, baseline flow; BZ, biologic zero; PV, peak value

Table 4-S1. Description of Post-Occlusive Reactive Hyperaemia Microvascular Parameters

MICROVASCULAR PARAMETER	DESCRIPTION
Baseline flow (BF)	The most stable 15 seconds within the first 2 minutes of reading.
Biologic zero (BZ)	The most stable 3 seconds displaying the lower values before the deflation of the cuff.
Resting flow (RF)	BF-BZ
Peak perfusion value (PV)	Five seconds period starting from the highest value in the first rise (slope) after deflation of the cuff
PORHmax	PV – RF
Area under the curve (AUC)	Area under the curve comprising of 60 seconds after deflation of the cuff

PORHmax, post-occlusive reactive hyperaemia maximum perfusion.

Table 4-S2. Correlations between baseline vascular parameters and inflammatory biomarkers

		IL-1β	IL-6	TNF-α	hs-CRP	Peak value	PORH max	IONT max	FMD	Peak shear stress
IL-1β	Correlation Coefficient	1.000	.505	.594	-.265	-.052	-.032	-.480	.272	.324
	Sig. (2-tailed)	.	.046	.015	.341	.849	.905	.114	.326	.238
	N	16	16	16	15	16	16	12	15	15
IL-6	Correlation Coefficient	.505	1.000	.469	.039	-.043	.018	.161	.220	-.334
	Sig. (2-tailed)	.046	.	.067	.889	.875	.948	.618	.431	.223
	N	16	16	16	15	16	16	12	15	15
TNF-α	Correlation Coefficient	.594	.469	1.000	-.136	-.026	.021	-.469	.121	.007
	Sig. (2-tailed)	.015	.067	.	.629	.922	.940	.124	.666	.980
	N	16	16	16	15	16	16	12	15	15
hs-CRP	Correlation Coefficient	-.265	.039	-.136	1.000	.434	.209	.123	.064	-.007
	Sig. (2-tailed)	.341	.889	.629	.	.106	.454	.719	.829	.982
	N	15	15	15	15	15	15	11	14	14
Peak value	Correlation Coefficient	-.052	-.043	-.026	.434	1.000	.944	.336	.461	.346
	Sig. (2-tailed)	.849	.875	.922	.106	.	.000	.286	.084	.206
	N	16	16	16	15	16	16	12	15	15
PORH max	Correlation Coefficient	-.032	.018	.021	.209	.944	1.000	.413	.421	.211
	Sig. (2-tailed)	.905	.948	.940	.454	.000	.	.183	.118	.451
	N	16	16	16	15	16	16	12	15	15
IONT max	Correlation Coefficient	-.480	.161	-.469	.123	.336	.413	1.000	-.247 [a]	-.443 [a]
	Sig. (2-tailed)	.114	.618	.124	.719	.286	.183	.	.464	.172
	N	12	12	12	11	12	12	12	11	11
FMD	Correlation Coefficient	.272	.220	.121	.064	.461	.421	-.247 [a]	1.000	.286 [a]
	Sig. (2-tailed)	.326	.431	.666	.829	.084	.118	.464	.	.301

	N	15	15	15	14	15	15	11	15	15
Peak shear stress	Correlation Coefficient	.324	-.334	.007	-.007	.346	.211	-.443 [a]	.286 [a]	1.000
	Sig. (2-tailed)	.238	.223	.980	.982	.206	.451	.172	.301	.
	N	15	15	15	14	15	15	11	15	15

IL-1β, interleukin-1 beta; IL-6, interleukin-6; TNF-α. Tumour necrosis factor alpha; hs-CRP, high-sensitivity c-reactive protein; PORHmax, post-occlusive reactive hyperaemia maximum perfusion; IONTmax, maximum perfusion following iontophoresis of acetylcholine; FMD, flow-mediated dilatation.

All correlations derived from Spearman's rank correlation coefficient, unless described elsewhere.

[a] Correlations derived from Pearson correlation coefficient.

Table 4-S3. Additional data from microvascular parameters

Parameter	Group	Baseline	2h
PORH PV (PU) n=15	Control	103.7 (SD 14.1)	88.5 (SD 14.5)
	Anthocyanins	105.3 (SD 17.1)	91.3 (SD 12.6)
PORH BF (PU) n=15	Control	42.5 (SD 5.6)	42.5 (SD 6.7)
	Anthocyanins	42.6 (SD 7.5)	42.2 (SD 9.7)
PORH BZ (PU) n=15	Control	16.2 (SD 6.0)	15.9 (SD 5.3)
	Anthocyanins	15.0 (SD 4.6)	16.8 (SD 6.2)
PORH RF (cvc) n=15	Control	0.31(SD 0.07)	0.32 (SD 0.07)
	Anthocyanins	0.32 (SD 0.05)	0.31 (SD 0.08)
IONT BF (cvc) n=13	Control	0.35 (SD 0.08)	0.36(SD 0.11)
	Anthocyanins	0.33 (SD 0.07)	0.33 (SD 0.08)
IONT PV (cvc) n=13	Control	0.80(SD 0.23)	0.80(SD 0.28)
	Anthocyanins	0.75 (SD 0.26)	0.74 (SD 0.24)
AUC (PU) n=14	Control	3968.7 (SD 437.4)	3731.3 (SD 493.3)
	Anthocyanins	3941.5 (SD 591.9)	3783.9 (SD 542.5)
AUC (cvc) n=14	Control	0.0216 (SD 0.004)	0.0222 (SD 0.003)
	Anthocyanins	0.0218 (SD 0.003)	0.228 0.003)

Data are mean and standard deviation or median and interquartile range. PORH, post-occlusive reactive hyperaemia; PU, perfusion units, BF, baseline flow; RF, resting flow; IQR, interquartile range; cvc, cutaneous vascular conductance in PU/mmHG; BZ, biologic zero; SD, standard deviation; PV, peak value; AUC, area under the curve.

Table 4-S4. Urinary concentrations of total anthocyanins and phenolic acid biomarkers from a 24-hour pooled urine sample from the control and intervention arms.

Parameter	Intervention	Control	Within-subject contrasts [a]
Total Anthocyanins (ng/mL)	1.86 (IQR 3.23)	0.015 (IQR 0.12)	F(1,15)=27.44, $P<0.001$, $\eta p^2=0.647$
Protocatechuic acid (ng/mL)	86.78 (IQR 362.2)	20.08 (IQR 179.65)	F(1,15)=0.680, $P=0.423$, $\eta p^2=0.043$
Hippuric acid (μg/mL)	1565.70 (SD 912.89)	1022.36 (SD 322.21)	F(1,15)=6.05, $P=0.027$, $\eta p^2=0.287$
Ferulic Acid (ng/mL)	12.04 (IQR 72.22)	40.69 (IQR 65.09)	F(1,15)=0.752, $P=0.733$, $\eta p^2=0.008$

Values are mean and standard deviation (SD) or median and interquartile range (IQR)

[a] P-values from repeated measures ANOVA; ηp^2, partial eta-squared;

Total anthocyanins expressed as cyanidin-3-*O*-glucoside equivalents (CGE)

CHAPTER 5: Food anthocyanins decrease serum concentrations of TNF-α in older adults with mild cognitive impairment: a randomized, controlled, double blind clinical trial.

There are still several gaps to be addressed regarding the effects of anthocyanins on the vascular-inflammatory axis in older adults, especially among those with neurodegenerative diseases, such as mild cognitive impairment (MCI), a condition that shares pathological mechanisms with CVD. This is particularly evident in older adults, as several studies support a gradual loss of the vascular function, and a new immune-metabolic viewpoint for age-related diseases, termed 'inflammaging' which is characterized by a chronic low-grade inflammation. Therefore, the present trial aimed to evaluate the chronic effects of food anthocyanins (8 weeks) on the microvascular function, inflammatory biomarkers and 24 h ambulatory blood pressure (ABP) in older adults with diagnosis of MCI.

A daily high dose of fruit-based anthocyanins (201 mg) for 8 weeks reduced concentrations of TNF-α in older adults with MCI. Anthocyanins did not alter other inflammatory biomarkers, microvascular function or blood pressure parameters, while an overall lack of effects were observed in the low dose (47 mg) group.

Considering the major role of the inflammatory response and vascular function in CVD and cognitive decline, this novel data may present relevant clinical implications in this high-risk group; however, further studies with a larger sample size and longer period of follow-up are required to better elucidate whether these changes in inflammatory biomarkers will alter CVD risk and progression of cognitive decline.

The majority of this chapter forms the substantive content of a published article (Appendix D).

5.1 Introduction

Vascular function, blood pressure and inflammation are a triad of factors relevant to the pathogenesis of several major chronic diseases, including cardiovascular disease (CVD)(306,307), and neurodegenerative conditions, such as mild cognitive impairment (MCI)[3,4]. The endothelium can be considered an organ that regulates vascular homeostasis by maintaining an appropriate vascular tone, platelet activity, leukocyte adhesion, and angiogenesis. When one or more of these components is compromised due to impaired endothelial vascular signalling, endothelial dysfunction (ED) may occur(306). In addition to hypertension and ED, the inflammatory response mediates several CVD related underlying mechanisms[5,6]. The association between concentrations of pro-inflammatory cytokines, such as CRP, IL-6 and TNF-α, and the development of atherosclerosis and CVD is well established[5,7]. There is evidence to suggest that directly targeting inflammation may be beneficial for the secondary prevention of CVD, and that such benefits may be independent of traditional CVD risk factors, such as cholesterol[8,9]. The role of inflammatory cytokines provides a mechanism through which risk factors for atherosclerosis may alter arterial biology, and thereby result in a systemic pro-atherothrombotic milieu[8]. This is particularly evident in older adults; several studies support a new immune-metabolic viewpoint for age-related diseases termed 'inflammaging', characterized by a chronic, low-grade inflammatory response[10,11]. Furthermore, there is evidence that diseases of the cardiovascular system, such as stroke, atrial fibrillation, coronary heart disease (CHD), and heart failure are linked to cognitive decline[12]. A meta-analysis of 10 prospective cohort studies showed that CHD was associated with increased risk of cognitive impairment or dementia (OR = 1.45, 95%CI = 1.21–1.74, $p<0.001$)[13]. Several mechanisms underlying the association between CVD and cognitive decline have been proposed: [1] shared risk factors, which might alter clearance of brain toxins or otherwise increase neurodegeneration; [2] CVD might lead to clinical or subclinical strokes,

leading to cognitive impairment; and [3] CVD might directly alter cerebral perfusion[14]. In this context, interventions aiming to regulate the CVD risk factors are also relevant therapeutic targets to attenuate cognitive decline.

Potential nutritional therapeutic options are emerging, with anthocyanins, a subclass of flavonoids, showing promising benefits related mainly to immunomodulatory, vascular signalling and antioxidant effects(21), thereby blunting the cooperative and synergistic deleterious effects of oxidative stress and inflammation[5,16]. Anthocyanins are the largest class of water-soluble plant pigments that are responsible for the blue, purple and red colour of many fruits and vegetables, such as blueberries, blackberries, red grapes, plums and eggplants[17]. A large number of in vitro and animal studies(21,317–319) provide insight into these mechanisms related to the anti-inflammatory actions of anthocyanins. These studies also observed a potential positive effect on vascular function through the suppression of acute pro-inflammatory genes that regulate inducible nitric oxide synthase (iNOS), an enzyme responsible for moderating some of the production of nitric oxide (NO)[21], as well as attenuating endothelium-dependent dysfunction by anthocyanins metabolites[20].A meta-analyses of randomized clinical trials (RCT) observed an improvement in vascular reactivity measured by flow-mediated dilation (FMD) among 6 studies ranging from one week to six months of intervention, with anthocyanin daily doses of 12 to 320mg[22]. Concerning lipid profiles and blood pressure, a systematic review of 12 RCT (3-24 weeks of duration and dose range of 7.35–640 mg/day of anthocyanins) identified a significant effect only on lowering LDL-c among diseased individuals or those with elevated biomarkers[23]. Both reviews[22,23] did not find a dose-response relationship in the cited RCTs, nor evidence of adverse effects of anthocyanins within those concentrations. Therefore, the relevant concentration of anthocyanins required to produce physiologically relevant is still to be elucidated.

Among non-invasive techniques to measure endothelial function, Laser Speckle Contrast Imaging (LSCI) is a relatively new technique that provides a non-invasive index of blood flow in the microcirculation[24]. This technique, combined with dynamic tests such as post-occlusive reactive hyperaemia (PORH), is able to provide information on several different aspects of microvascular physiology[25]. Measuring 24-hour ambulatory blood pressure (ABP) has several benefits over traditional single assessment, such as minimising white-coat phenomena and masked hypertension, and demonstrating nocturnal hypertension and loss of normal dipping patterns[26]. ABP monitoring is a stronger predictor of cardiovascular morbidity and mortality than most traditional BP measuring techniques[26–28].

This paper provides secondary analysis of a clinical trial that investigated the effect of combining a cognitive training rehabilitation program with a dietary intervention focusing on increasing anthocyanin intake in older persons with MCI. The aim of the current analysis is to investigate the effects of a nutritional intervention consisting of 8 weeks of food-anthocyanins on inflammatory markers associated with CVD risk factors(277,278), along with analysis of microvascular function and 24-hour ABP in older adults with MCI.

5.2 Methods

The clinical trial was conducted according to the guidelines of the Declaration of Helsinki, and approved by the joint University of Wollongong and Local Health District Human Research Ethics Committee, New South Wales, Australia (HREC 2017/581). The study was registered with the Australian New Zealand Clinical Trials Registry (ACTRN12618001184268). This manuscript is reporting results from the secondary outcomes, while the primary outcome related to cognitive function will be reported elsewhere. Written informed consent was obtained from all subjects before beginning the study procedures.

Study Subjects

A total of 34 subjects enrolled in the study between April 2018 and November 2019. The inclusion criteria were: 55+ years; diagnosis of MCI-amnesic type with memory complaints, Mini Mental State Examination (MMSE) score of 24 or above, and estimated premorbid IQ of more than 80, confirmed by clinical psychologists (ZF, SB, AP). A diagnosis of MCI was given when an individual experienced cognitive change (typically 1 to 1.5 standard deviation below age and education matched peers on culturally appropriate normative data) in one of more cognitive areas that are insufficient to interfere with activities of daily living and without progressive deterioration. All subjects were participating in a 6 week 'Making the most of your Memory' rehabilitation group. Exclusion criteria were: a diagnosis of dementia; significant neurological history, untreated hypertension or diabetes; chronic liver or renal disease; smoking; diagnosis or self-reported gastrointestinal disorders; acute upper respiratory tract infection; allergy to stone fruits or food colorants.

Study Design and Treatments

The study was a randomized, controlled, double-blind clinical trial conducted at the Illawarra Health and Medical Research Institute and at the Port Kembla Hospital, NSW Australia. On test days, participants attended the research facility for the study procedures after a 12 hour overnight fast (Figure 5-1). Briefly, the PORH test (microvascular reactivity) and blood collection were completed before provision of a standardized breakfast (minimal anthocyanin-containing cereals, milk, bread, butter and anthocyanin-free fruit jam), followed by collection of data including socio-economic status, anthropometry, medication use and dietary intake.

The usual diet was assessed using a 3-day food record that participants were instructed by a dietitian to complete at home (Table 5-1) and analysed using Foodworks 10 (Xyris Software,

Australia), which includes the Australian Food Composition Database 2019(325). The anthocyanins content was calculated using the 'PhenolExplorer' polyphenol food composition database(206). Participants were instructed to maintain their usual diet throughout the course of the study.

Participants were fitted with a 24-hour ambulatory blood pressure monitor which was collected from their home the following day. Participants were randomly allocated to one of three dietary interventions (250 ml/day): a) low-anthocyanins Queen Garnet Plum (QGP) juice; b) high-anthocyanins QGP juice; c) apricot juice (control). Juice was supplied weekly to participants when they attended the memory clinic. The duration of the dietary intervention was 8 weeks, after which time participants returned to the research facility for the final data collection. Compliance was measured by requesting the empty bottles from participants.

Block randomization (3x3) was conducted by a researcher independent to the data collection or enrolment of participants using a computer generated randomization sequence. Blinding strategies included colouring of control juice, as well as advertising and consenting participants to a "fruit juice study" without providing information on which fruit was being investigated.

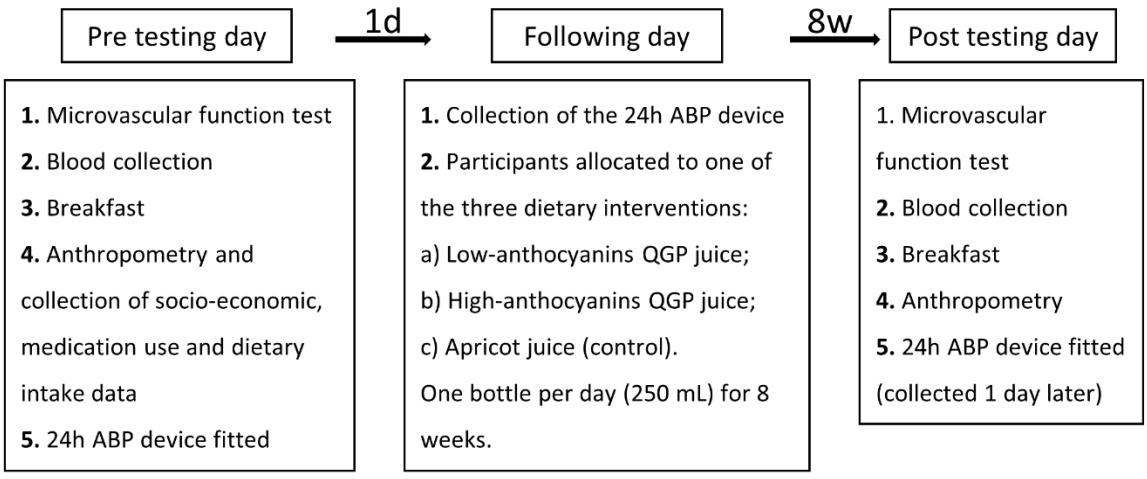


Figure 5-1. Study design and procedures. PORH, post-occlusive reactive hyperaemia; ABP, ambulatory blood pressure; QGP queen garnet plum.

Table 5-1. Dietary intake of participants at baseline, assessed using 3-day food records.

	Control	Low anthocyanins	High Anthocyanins	All	*P*-value[a]
n	14	9	6	29	
Energy (kcal)	2170.4 SD 745.1	2072.1 SD 495.8	2040.8 SD 177.2	2113.1 SD 580.4	0.880
Protein (g)	103.2 SD 38.6	95.0 SD 28.6	88.9 SD 10.5	97.7 SD 31.3	0.634
Fat – total (g)	86.7 SD 35.9	72.1 SD 26.4	72.8 SD 17.5	79.3 SD 30.1	0.453
Saturated fat (g)	35.9 SD 18.1	29.8 SD 13.0	31.0 SD 8.6	33.0 SD 14.9	0.608
Carbohydrates (g)	227.3 SD 79.9	239.4 SD 52.0	236.4 SD 32.3	233.0 SD 62.9	0.900
Dietary fibre (g)	28.2 SD 13.4	26.5 SD 10.0	28.1 SD 7.3	27.6 SD 11.1	0.934
Sodium (mg)	2619.5 SD 1434.4	2468.4 SD 567.2	2082.7 SD 397.2	2461.4 SD 1057.6	0.599
Vitamin C (mg)	90.4 IQR 99.9	76.1 IQR 59.7	101.7 IQR 122.5	86.7 IQR 85.6	0.546
Vitamin E (mg)	9.6 IQR 5.4	8.2 IQR 6.3	8.3 IQR 1.5	9.0 IQR 4.8	0.441
Vitamin A* (µg)	881.2 IQR 529.4	611.5 IQR 767.5	887.7 IQR 330.5	807.7 IQR 590.7	0.875
Anthocyanins (mg)	2.7 IQR 22.5; 13.9 SD 22.9	3.6 IQR 17.8; 68.1 SD 188.3	1.4 IQR 5.4; 73.3 SD 101.5	43.1 IQR 82.8; 43.1 SD 114.2	0.426

Values are mean and standard deviation or median and interquartile range. [a]*P*-value for one-way ANOVA among the three treatments; * Total Vitamin A equivalents. Values obtained from the Australian Food Composition Database 2019(325). Anthocyanins content values obtained from the 'PhenolExplorer' polyphenol food composition database(206).

Intervention and control juices

The low-anthocyanins QGP juice consisted of 99% fruit and 1% water that went through a high pressure-low temperature treatment. The high-anthocyanins QGP juice was a blend of 220g of frozen QGP with 30mL of water added. The anthocyanin content of both

juices were analysed by the Queensland Department of Agriculture and Fisheries (DAF, Australia) by Performance Liquid Chromatography (HPLC) and the pH differential method, following the AOAC 2005.02 protocol. An apricot juice was chosen as the control arm due to similar consistency, nutritional content and flavonoid content(282) to the QGP juice, but without anthocyanins. Food dyes (red and blue) were added to the apricot juice to closely approximate the colour of the QGP juice. The total anthocyanins content for the 250 mL bottle of low-anthocyanins QGP juice was 48 mg based on the HPLC method and 44 mg based on the pH differential method, while the total anthocyanins content for the 250 mL bottle of untreated QGP juice was 201 mg based on the HPLC method and 207 mg based on the pH differential method. Additional nutritional information of the fruit juices can be found in the supplementary material (Table 5-S1).

Blood samples

Pre and post (baseline and 8 weeks) fasted plasma and serum samples were collected in the morning, following the microvascular reactivity test, and then stored at -80 °C, prior to analysis. IL-1β, IL-6 and TNF-α were analysed in a Luminex 200 using a Human High sensitivity T cell magnetic bead panel kits (Merck Millipore, Billerica, MA, USA). High sensitivity CRP was analysed on a BK400 automated chemistry analyser using a immunoturbidometric assay (Biobase, Shandong, China).

24-hour ambulatory blood pressure

The 24-hour ambulatory blood pressure was measured using an automated Welch Allyn ABPM 7100 (Welch Allyn, NSW, Australia). Participants were fitted with the device and appropriate instructions were provided(322). Participants were asked to record any moderate or intense physical activity. Criteria for measurements errors include the exclusion of the

following measures: 70 mmHg ≤ SBP ≤ 250 mmHg and 30 mmHg ≤ DBP ≤ 130 mmHg. Daytime blood pressure was defined as 09-21h and night-time as 11-05h. The "dipping" pattern was calculated using a ratio of night-time/daytime blood pressure(322).

Microvascular function

Participants were rested in a quiet, controlled temperature room 23 ℃ ±1, in a supine position for 15 minutes. Blood pressure was measured before the PORH test procedure using a Welch Allyn Connex 6700 Vital Signs Monitors (Welch Allyn, NSW, Australia), according to clinical guidelines(283). Microvascular cutaneous vascular reactivity was measured by using LSCI (Pericam PSI System, Perimed AB, Järfälla, Sweden), The baseline perfusion was measured in the volar side of the left forearm for 2 minutes, followed by the PORH test: an arterial occlusion was maintained for 3 minutes using a BP cuff around the upper arm inflated to a pressure of 50-60 mmHg above systolic BP reading; after the BP cuff was released, the PORH response was recorded for 3 minutes. The following parameters were extracted: baseline flow (BF), biological zero (BZ) and peak value (PV). The PV was obtained with a 5 second "time of interest" starting from the highest value after deflation of the cuff(326). RF was calculated as BF – BZ. The maximum PORH perfusion (PORHmax) was calculated as PV – RF (Supplementary Figure 5-S1 and Table 5-S2. By international convention all parameters are expressed in arbitrary Perfusion Units (PU). Microvascular flow parameters were then transformed to cutaneous vascular conductance (CVC) by dividing mean arterial pressure to yield CVC in PU/mmHg. This PORH protocol is routinely performed in our laboratory; with intra-subject coefficients of variation of 7.7% for PV and 11.4% for PORHmax. Additional LSCI data combined with the PORH test are listed in the supplementary material (Supplementary Table 5-S3).

Statistical Analyses

Data are presented in mean and standard deviation (SD) or median and interquartile range according to data distribution. Natural log transformation was used to normalize the data, where appropriate. Differences in the baseline demographics among the treatment groups were investigated by one-way ANOVA and chi-squared tests. Outliers were graphically inspected and exclusions were conducted with Z-scores >2.5 or modified Z-scores >3.5. A two-way mixed [1 between-subject factor with 3 levels (treatment) and 1 within-subject with 2 levels (time)] ANOVA was used to investigate the outcomes and significant effects were followed by corrected post-hoc pairwise comparisons(327,328). Parameters with significant baseline between-group imbalances (IL-6 and IL-1β) were further inspected using ANCOVA(329,330). The Kruskal-Wallis test was used to inspect results from ANOVA tests conducted with data with normality concerns. SPSS (version 25 SPSS Statistic Subscription, IBM, Chicago, IL, USA 2019) was used for all statistical analyses. Significance was accepted at alpha $p<0.05$, and non-significant p values between p=0.05-0.099 were considered a trend in the data. Post-hoc power analysis were conducted for each parameter that had a significant result or trend.

5.3 Results

A total of 47 participants were assessed for eligibility and 31 participants (19 female and 12 male) out of 34 enrolled participants completed the trial (Figure 5-S2). Two withdrawals were related to the participants reporting that the study was too onerous and one withdrawal was related to an underlying gastrointestinal condition that made the juice consumption unviable. Baseline mean age and BMI of participants were 75.3 (SD 6.9) years and 26.1 (SD 3.3) kg/m^2, respectively. Additional baseline demographics are listed in Table 5-2.

Table 5-2. Baseline demographics

	Control	Low anthocyanins	High Anthocyanins	All	*P*-value[a]
n	14	10	7	31	
Gender (M/F)	5/9	6/4	1/6	12/19	0.043
Age (years)	74.9 (SD 7.8)	76.1 (SD 6.7)	75.1 (SD 6.1)	75.3 (SD 6.9)	0.875
BMI (kg/m^2)	25.2 (SD 3.1)	27.1 (SD 2.3)	26.6 (SD 4.7)	26.1 (SD 3.3)	0.377
Hypertension[b]	5 (35.7%)	4 (40%)	4 (57%)	13 (41.9%)	0.637
Day SPB (mm Hg)	127.9 (SD 17.9)	128.1 (SD 13.9)	128.5 (SD 16.9)	128.1 (SD 15.9)	0.997
Day DPB (mm Hg)	78.4 (SD 10.0)	78.6 (SD 8.7)	80.2 (SD 12.5)	78.9 (SD 9.9)	0.928
SPB night/day ratio	0.89 (SD 0.04)	0.90 (SD 0.09)	0.98 (SD 0.08)	0.92 (SD 0.08)	0.112
DPB night/day ratio	0.89 (SD 0.09)	0.88 (SD 0.09)	0.95 (SD 0.12)	0.91 (SD 0.10)	0.362

BMI, body mass index; SPB, systolic blood pressure; DPB, diastolic blood pressure. [a]*P*-value for one-way ANOVA or chi-square test among the three treatments; [b] Participants with controlled hypertension taking anti-hypertensive drugs.

Inflammatory biomarkers

A significant time and treatment interaction effect was found for serum concentrations of TNF-α [$F_{(2,27)}$=7.739, P=0.002, ηp2=0.364] with post-hoc analysis indicating a power of 99.2% The breakdown of this interaction showed a lower TNF-α in the high anthocyanins group (7.60 pg/mL SD 3.18) vs controls (11.00 pg/mL SD 3.51) after 8 weeks of intervention (P=0.047, independent t-test) (Figure 5-2a). Regarding serum concentrations of IL-6, there was a significant treatment effect (P=0.013), a trend towards an effect of time (P=0.096) and a trend for an interaction effect [$F_{(2,21)}$=2.933, P=0.075, ηp2=0.218]; however, post-hoc analysis indicated a power of 44.6%. Baseline IL-6 was higher in the high anthocyanins group when compared to the low anthocyanins group or controls (P<0.001 and P=0.009, respectively,

independent t tests), but between-group differences were not significant after 8 weeks of intervention (P>0.05) with a 36.6% decrease in IL-6 levels in the high anthocyanins group from baseline to 8 weeks (Figure 5-2b). An ANCOVA adjusting for baseline imbalances identified no significant treatment effect (P>0.05). The values of IL-1β were natural log transformed to approximate a normal distribution. There were significant time and treatment effects (P=0.022 and 0.038), and a trend for an interaction effect [$F_{(2,27)}$=2.972, P=0.068, ηp2=0.180], however, post-hoc analysis indicated a power of 42.3%. Similar to IL-6, baseline serum concentration of IL-1β was higher in the high anthocyanins group, compared to the low anthocyanins group or controls (P=0.025 and P=0.019, respectively, Mann-Whitney test), but not significant between groups after treatment (P>0.05), with a 23.3% decrease in IL-1β observed in the high anthocyanins group over time (Figure 5-2c). A Kruskal-Wallis test confirmed that differences between treatment groups at baseline (P=0.028) were no longer significant after 8 weeks of treatment (P=0.153). An ANCOVA adjusting for baseline imbalances identified no significant treatment effect (P>0.05). There was no interaction effect [$F_{(2,25)}$=1.146, P=0.334, ηp2=0.084], nor time or treatment effects (P>0.05) for serum concentration of CRP (Figure 5-2d). Analyses conducted with natural log transformed data or with the Kruskal-Wallis test for non-parametric raw data confirmed no effects on CRP serum concentrations (P>0.05). There were no gender differences in baseline concentrations of TNF-α (P=0.931), IL-6 (P=0.061), IL-1β (P=0.233) and hsCRP (P=0.895).

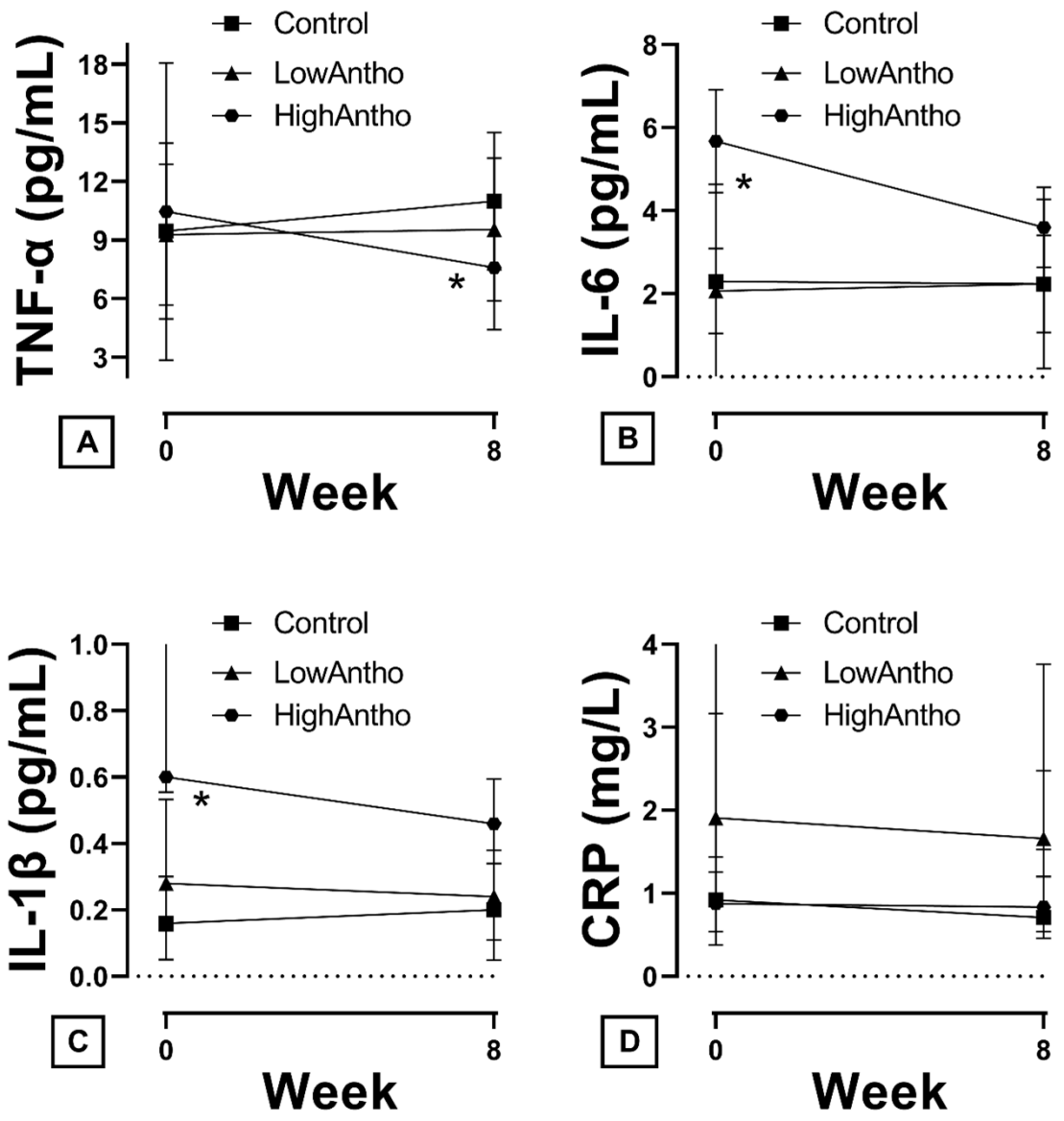


Figure 5-2. Serum concentration of inflammatory biomarkers before and after 8 weeks intervention. Values are mean and error bars are standard deviation (A and B) or median and 1st and 3rd quartile (C and D). TNF, tumor necrosis factor; IL, interleukin; CRP, c-reactive protein. A) time*treatment effect (P=0.002); *HighAntho vs Control (P=0.047, independent t-test). B) No significant treatment effect between groups (P>0.05); *HighAntho vs LowAntho (P<0.001, independent t-test); C) No significant treatment effect between groups (P>0.05); *HighAntho > Control (P=0.019, Mann-Whitney test) *HighAntho > LowAntho (P<0.025, Mann-Whitney test) D) No significant changes or differences between groups (P>0.05).

24-hour ambulatory blood pressure

There was no significant interaction, treatment or time effects on any of the 24-hour ambulatory systolic and diastolic BP parameters (daytime, nocturnal and 24-hour, and dipping patterns) (Table 5-3). Based on the classification of dipping ratios(322) of <0.9 and >0.8 for 'normal dipping', <1.0 and >0.9 for 'reduced dipping', and >1.00 for 'no dipping and rising', there were several changes between treatments. The mean systolic and diastolic BP dipping pattern of controls changed from 'normal dipping' at baseline to 'reduced dipping' after the intervention, and the systolic BP dipping pattern of the high anthocyanins group changed from 'reduced dipping' at baseline to 'no dipping and rising' after the intervention. There were no gender differences for any baseline 24-hour ambulatory systolic and diastolic BP parameters (all P's>0.05)

Table 5-3. 24-hour ambulatory blood pressure measures before and after treatment in control and intervention groups

Parameter	Group	*n*	PRE	POST	Time[a]	Treatment[a]	Time x treatment[a]
Day SPB	Control	9	126.3 (SD 14.6)	125.2 (SD 11.9)	0.595	0.911	F(2,21)=0.453, *P*=0.642, ηp^2=0.041
	LowAntho	8	127.7 (SD 14.8)	129.3 (SD 16.9)			
	HighAntho	7	127.4 (SD 14.7)	123.7 (SD 17.2)			
Day DPB	Control	9	79.9 (SD 10.9)	79.9 (SD 9.1)	0.962	0.970	F(2,21)=0.610, *P*=0.553, ηp^2=0.055
	LowAntho	8	78.5 (SD 9.3)	81.3 (SD 9.5)			
	HighAntho	7	80.2 (SD 12.5)	77.6 (SD 8.1)			
24h SPB	Control	9	121.4 (SD 12.9)	123.1 (SD 9.6)	0.818	0.745	F(2,21)=1.573, *P*=0.231, ηp^2=0.130
	LowAntho	8	123.2 (SD 11.0)	126.0 (SD 14.3)			
	HighAntho	7	129.3 (SD 19.9)	125.8 (SD 16.4)			
24h DPB	Control	9	75.9 (SD 9.3)	78.4 (SD 8.3)	0.529	0.928	F(2,21)=1.463, *P*=0.254, ηp^2=0.122
	LowAntho	8	74.6 (SD 8.4)	78.8 (SD 10.4)			
	HighAntho	7	80.0 (SD 13.8)	76.9 (SD 10.0)			
Nocturnal SBP	Control	9	113.6 (SD 10.5)	117.5 (SD 8.9)	0.510	0.284	F(2,21)=0.324, *P*=0.727, ηp^2=0.005
	LowAntho	8	114.9 (SD 11.9)	115.0 (SD 14.2)			
	HighAntho	7	126.4 (SD 27.1)	126.9 (SD 22.4)			
Nocturnal DBP	Control	9	71.0 (SD 8.4)	73.4 (SD 9.8)	0.623	0.651	

	LowAntho	8	68.6 (SD 9.9)	72.3 (SD 14.1)			F(2,21)=0.470, *P*=0.632, ηp^2=0.043
	HighAntho	7	71.9 (SD 19.1)	74.5 (SD 13.4)			
SPB night/day ratio	Control	8	0.89 (SD 0.04)	0.93 (SD 0.08)	0.308	0.103	F(2,20)=0.559, *P*=0.581, ηp^2=0.053
	LowAntho	8	0.90 (SD 0.09)	0.89 (SD 0.08)			
	HighAntho	7	0.98 (SD 0.08)	1.03 (SD 0.17)			
DPB night/day ratio	Control	9	0.89 (SD 0.09)	0.92 (SD 0.08)	0.555	0.236	F(2,21)=0.067, *P*=0.935, ηp^2=0.006
	LowAntho	8	0.88 (SD 0.09)	0.88 (SD 0.09)			
	HighAntho	7	0.95 (SD 0.12)	0.96 (SD 0.12)			

Values are means and standard deviation. SPB, systolic blood pressure; DPB, diastolic blood pressure; ηp2, partial eta-squared. [a] *P*-values for two-factor mixed ANOVA.

Microvascular function

No significant time or treatment effects were observed in the microvascular parameter 'peak value' in CVC. A trend towards an interaction effect was found [$F_{(2,24)}$=2.678, P=0.089, ηp^2=0.182]; however, post-hoc comparisons showed no significant effect of time nor treatment. There was no significant interaction effect [$F_{(2,24)}$=0.309, P=0.737, ηp2=0.025], nor time or treatment effects (P>0.05) in the maximum perfusion following the PORH test (PORHmax). There were no gender differences in baseline BF (P=0.549), BZ (P=0.858), RF (P=0.451), PV (P=0.675) and PORHmax (P=0.884) in CVC. A full description of all microvascular parameters in PU and CVC (PU/mmHg) are presented in the supplementary material (Table 5-S3).

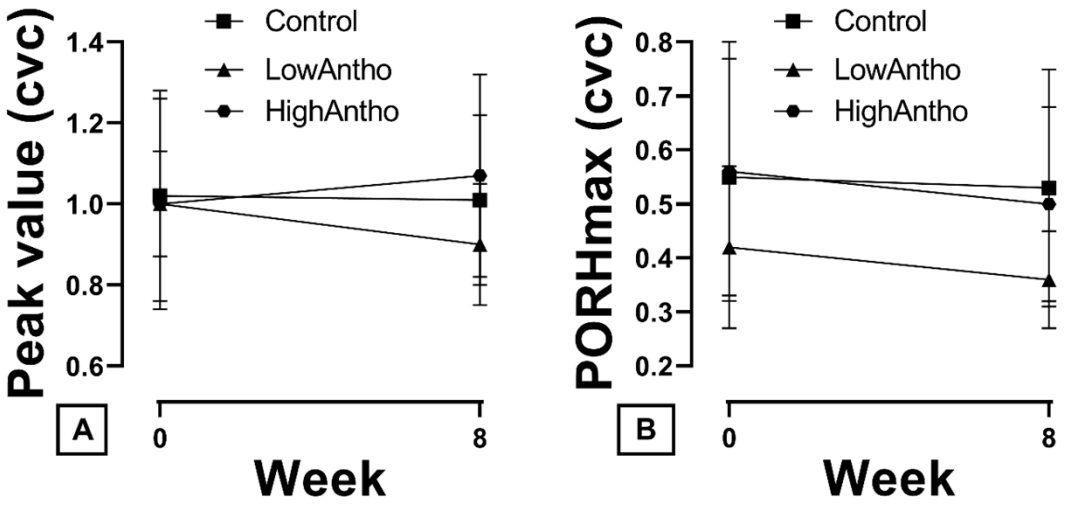


Figure 5-3. Microvascular reactivity parameters before and after 8 weeks intervention. Values are mean and error bars are standard deviation. CVC, cutaneous vascular conductance in PU/mmHg; PORHmax, post occlusive reactive hyperaemia maximum perfusion. No significant differences between groups (P>0.05).

5.4 Discussion

Consumption of anthocyanins provided in the QGP juice for 8 weeks decreased the serum concentrations of TNF-α in older adults diagnosed with MCI. This effect was only observed in the intervention group that received the higher dose (201 mg/day) of anthocyanins. Other outcomes, including other inflammatory biomarkers, parameters of microvascular function and 24-hour ABP measures, remained unchanged in both treatment groups compared to controls.

The major finding in the present trial was the significant decrease in serum concentration of TNF-α in the high-anthocyanins intervention group. TNF-α is a cytokine with a wide range of pro-inflammatory activities that is primarily produced by macrophages, endothelial cells, and smooth muscle cells of atherosclerotic arteries(64). TNF-α may influence the atherosclerotic process by causing metabolic perturbations and by increasing the expression of surface leukocyte adhesion molecules, chemokines and enhancing the production of other cytokines and growth factors. High concentrations of TNF-α have been associated with premature coronary artery disease, acute myocardial infarction, peripheral arterial disease, and congestive heart failure(277). The magnitude of reduction of TNF-α observed with the high dose of anthocyanins in this trial [2.86 pg/mL, from 10.46 (SD 2.84) to 7.60 (SD 3.18) pg/mL] may have important clinical implications considering the predictive role of TNF-α in CVD events supported by previous studies. For example, in a case-cohort study comprising 105 CAD cases and 638 individuals randomly selected from a cohort of 5,404 participants aged 35–74 years (mean follow-up of 6.1 years), TNF-α was significantly and independently associated with CAD (adjusted HRs=1.87;1.31–2.66)(331). Furthermore, there is evidence from large-scale prospective studies, such as a cohort of 2225 participants (70-79 years old) without baseline CVD that were assessed for incident coronary heart disease, stroke and congestive heart failure events during an average follow-up of 3.6 years. In that cohort, TNF-α was

significantly associated with coronary heart disease (per TNF-α SD increase: RR, 1.22; 95% CI, 1.04 to 1.43) and congestive heart failure (per TNF-α SD increase: RR, 1.59; 95% CI, 1.30 to 1.95) events(76). The beneficial outcomes reported in such studies were found for differences in TNF-α, which supports the clinical significance of the reductions observed in our study in older adults.

Despite a reduction in concentrations of IL-6 (36.6%) and Il-1β (23.3%) in the group receiving the high-anthocyanins treatment, these changes were no longer significant after adjusting for baseline imbalances between groups. These two interleukins are fundamental components of atherosclerosis, related to CVD(190,277). IL-6 is highly expressed by the vascular endothelium and the pharmacological inhibition of IL-6 can improve endothelial function(332). Concerning the clinical significance of the magnitude of effect found in the high anthocyanins intervention in the present trial, a significant reduction of 2.08 pg/mL represents a change from baseline of approximately 2 SD [5.68 (SD 1.24) pg/mL at baseline to 3.60 (SD 0.97) pg/mL after the treatment]. Other studies have reported hazard ratios of 1.80 according to each 1-SD increase in IL-6 for risk of first-ever cerebrovascular events in individuals with vascular risk factors, but without any pre-existing CVD(333). Furthermore, in a meta-analysis of 17 prospective studies investigating clinical coronary outcomes (i.e., myocardial infarction or coronary death), an odds ratio of 1.61 (95% CI 1.42–1.83) was found per 2 SD increase in baseline IL-6(334).

IL-1β synthesis is significantly upregulated after cardiovascular events such as myocardium infarction, as well as in advanced plaque formations in atherosclerosis disease; thus, it has been investigated as a therapeutic option in secondary and tertiary prevention of CVD(335). Local stimuli in the plaque induces the generation of active IL-1β through the action of a molecular assembly known as the inflammasome(336). The convincing links between IL-1β to pro-inflammatory diseases, such as atherosclerosis indicates this cytokine as

a potential therapeutic target to improve cardiovascular outcomes(336). In support of this, an anti-IL-1β therapy was investigated in a large randomized, double-blind, placebo-controlled trial of 10,061 patients (median follow-up of 3.7y) with a history of myocardial infarction and CRP ≥ 2 mg/L. Treatment with canakinumab (anti-IL-1β monoclonal antibody) led to 15% reduction in major adverse CVD events (p=0.007)(313). Furthermore, a meta-analysis including 6 cohort studies with 1,855 CVD cases and 18,745 non-cases with follow-up times between 5-16y investigated the role of Interleukin-1 receptor antagonist (IL-1RA), which counter-regulates IL-1β as an endogenous inhibitor in vivo by blocking the binding site for IL-1β, and incident CVD(337). A pooled standardized hazard ratio (95% CI) for incident CVD of 1.11 (1.06–1.17) was found after adjustment for age, sex, anthropometric, metabolic, and lifestyle factors (P<0.0001)(337). A lack of studies regarding the magnitude of change in IL-1β concentration and CVD prediction makes it complex to extrapolate the clinical significance of our findings. The significant treatment effect showed a reduction of 0.140 pg/mL [0.600 (IQR 1.065) pg/mL at baseline and 0.460 (IQR 0.215) pg/mL after treatment] in the high anthocyanins group, representing a 23.3% reduction from baseline. Overall, our results, combined with the strong evidence pinpointing a relationship between inflammatory markers and CVD suggest that food-derived anthocyanins may be an important potential therapeutic treatment for reducing inflammation and promoting subsequent health benefits. Another feature of the present trial is that all participants were diagnosed with MCI; therefore, such health benefits may have potential implications in attenuating the advance of cognitive decline, considering that a number of studies support the association between neuroinflammation and a decline in cognitive function(338–340). Several studies support that CVD are associated with increased risk of cognitive impairment and dementia(314–316,341,342).

The changes observed in concentrations of TNF-α were not accompanied by changes in vascular outcomes, either in parameters derived from the 24h ABP measurements or

microvascular assessment. The observed changes in mean systolic and diastolic BP dipping pattern are not clinically relevant considering that the ratios were borderline compared to the classification thresholds and had relatively high standard deviations. It has been suggested that the cutaneous microcirculation may mirror generalized systemic vascular dysfunction(343), and continuous high frame assessment of skin perfusion over wide areas measured through LSCI provides strong reproducibility for vascular challenges(344,345). Based on the ability of anthocyanins to exert beneficial physiological effects as antioxidant and anti-inflammatory compounds, we proposed that treatment might improve the vascular reactivity measured using the PORH test. Mechanisms underlying the vascular reactivity response in the PORH test are still being elucidated; however, the findings of the present study suggest that reduction in serum TNF-αmay not affect microvascular reactivity in older adults. The use of the LSCI in nutritional intervention studies is relatively new, and there are no published reference values for predicting any condition, nor values that have an established clinical significance in vascular biology literature. A recent study from our group found a positive acute effect (postprandial) of anthocyanins in attenuating vascular and inflammatory responses to a high fat high energy meal challenge in overweight older adults(346). An improvement for both microvascular and macrovascular function was observed in that study, as assessed by PORH test combined with LSCI and FMD, respectively.

Limitations of our study include the variation in sample size and gender between treatment groups. Forty two percent of participants were hypertensive; however, this is reflective of the overall prevalence of 45.2% in Australians aged 75 years and older(347) and values did not significantly differ between the treatment groups. The limited sample size hindered the ability to conduct sub-analysis by sex or prevalence of hypertension, and may have influenced the baseline between-group imbalances in IL-6 and IL-1β concentration despite randomization. It should be noted that RCTs conducted in participants with MCI have

a complex recruitment process, and that the sample size of the present study was larger than that in 44% of included studies in a systematic review of treatments for MCI(348). A key feature in the design of this study was the placebo chosen for the control group. Investigation of a specific bioactive compound, such as anthocyanins, in a clinical trial using foodstuffs as the vehicle of delivery is challenging. Foods that are rich in a particular flavonoid sub-class may also contain other classes of flavonoids or other bioactive compounds, such as other polyphenols, and/or inorganic compounds (such as nitrate) that might have similar or synergistic effects on the outcomes assessed. We aimed to evaluate the effect of two different doses of anthocyanins using differently processed juice as the food source. A potential limitation that may have led to the absence of effects in the low dose group is that the average mean intake of anthocyanins in the usual diet of participants was similar to the intervention *per se* (68mg compared to 48mg). In order to isolate the effect of anthocyanins from other bioactives, apricot juice was provided as the control due to its lack of anthocyanins, but its similarly matched macro- and micronutrient content, as well as its polyphenol profile(206), including flavanols (such as quercetin), catechins and epicatechins, all of which are potential mediators of cardiovascular health(200,303–305).

5.5 Conclusion

A daily high dose of fruit-derived anthocyanins for 8 weeks decreased serum concentrations of TNF-α in older adults diagnosed with MCI. This change was not accompanied by effects on other inflammatory biomarkers, 24h ABP or microcirculation function, and no effects were observed in the low anthocyanin dose treatment group. Considering the major role of the inflammatory response in CVD and cognitive decline, our finding suggest that a regular high intake of anthocyanins may have clinical implications in this high-risk group; however, further studies with a longer follow-up and larger sample size

are required to better elucidate if these changes in concentrations of TNF-α will alter CVD risk and progression of cognitive decline.

5.6 Supplementary Material

Table 5-S1. Description of Post-Occlusive Reactive Hyperaemia Microvascular Parameters

MICROVASCULAR PARAMETER	DESCRIPTION
Baseline flow (BF)	The most stable 30 seconds within the first 2 minutes of reading.
Biologic zero (BZ)	The most stable 3 seconds displaying the lower values before the deflation of the cuff.
Resting flow (RF)	BF-BZ
Peak perfusion value (PV)	Five seconds period starting from the highest value in the first rise (slope) after deflation of the cuff
PORHmax	PV - RF

PORHmax, post-occlusive reactive hyperaemia maximum perfusion

Table 5-S2. Nutrition information of the test meal and fruit juices (250 mL)

	Low-anthocyanins QGP juice	High-anthocyanins QGP juice	Apricot juice
Energy (kcal)	113	98	96
Protein (g)	0.7	0.6	0.7
Fat – total (g)	< 0.1	< 0.1	< 0.1
Saturated fat (g)	< 0.1	< 0.1	< 0.1
Carbohydrates (g)	23.1	22.2	22.2
Dietary fibre (g)	2.9	4.4	4.0
Sodium (mg)	7	6	2
Vitamin C (mg)	0.3	0.3	1.2
Anthocyanins[1] (mg)	46.7	200.8	0[3]
Anthocyanins[2] (mg)	43.7	206.8	0[3]

[1]High Performance Liquid Chromatography; [2]pH differential method; [3]Phenol-explorer[23]. Remaining values were obtained from the Australian Food Composition Database 2019[24].

Table 5-S3. Post-occlusive reactive hyperaemia parameters

Parameter	Group	*n*	PRE	POST	Time[a]	Treatment[a]	Time x treatment[a]
PV (pu)	Control	11	96.4 (SD 29.3)	93.5 (SD 22.0)	0.565	0.637	F(2,24)=0.418, *P*=0.663, ηp^2=0.034
	LowAntho	9	85.9 (SD 10.2)	82.2 (SD 12.6)			
	HighAntho	7	99.4 (SD 16.9)	101.4(SD 19.6)			
PV (cvc)	Control	11	1.02 (SD 0.26)	1.01 (SD 0.21)	0.598	0.637	F(2,24)=2.678, *P*=0.089, ηp^2=0.182
	LowAntho	9	1.00 (SD 0.13)	0.90 (SD 0.15)			
	HighAntho	7	1.00 (SD 0.26)	1.07 (SD 0.25)			
PORH (cvc)	Control	11	0.55 (SD 0.22)	0.53 (SD 0.22)	0.084	0.149	F(2,24)=0.309, *P*=0.737, ηp^2=0.025
	LowAntho	9	0.42 (SD 0.15)	0.36 (SD 0.09)			
	HighAntho	7	0.56 (SD 0.24)	0.50 (SD 0.18)			
RF (cvc)	Control	11	0.29 (SD 0.06)	0.31 (SD 0.07)	0.200	0.047	F(2,24)=1.403, *P*=0.265, ηp^2=0.105
	LowAntho	9	0.39 (SD 0.09)	0.38 (SD 0.07)			
	HighAntho	7	0.28 (SD 0.08)	0.37 (SD 0.18)			
BZ (pu)	Control	11	17.4 (SD 9.3)	15.7 (SD 6.9)	0.971	0.827	F(2,24)=3.369, *P*=0.051, ηp^2=0.219
	LowAntho	9	16.2 (SD 6.6)	14.4 (SD 3.9)			
	HighAntho	6	15.2 (SD 2.9)	18.9 (SD 4.9)			
BF (pu)	Control	11	44.8 (SD 12.3)	44.9 (SD 14.2)	0.134	0.564	

LowAntho	9	49.9 (SD 6.7)	49.0 (SD 7.4)	F(2,24)=2.751, P=0.084, ηp^2=0.187
HighAntho	6	44.0 (SD 8.3)	53.9 (SD 16.1)	

Data are mean and standard deviation. PV, peak value; ηp2, partial eta-squared ;PORH, post-occlusive reactive hyperaemia; RF, resting flow; BZ, biologic zero; pu, perfusion units; BF; baseline flow; cvc, cutaneous vascular conductance

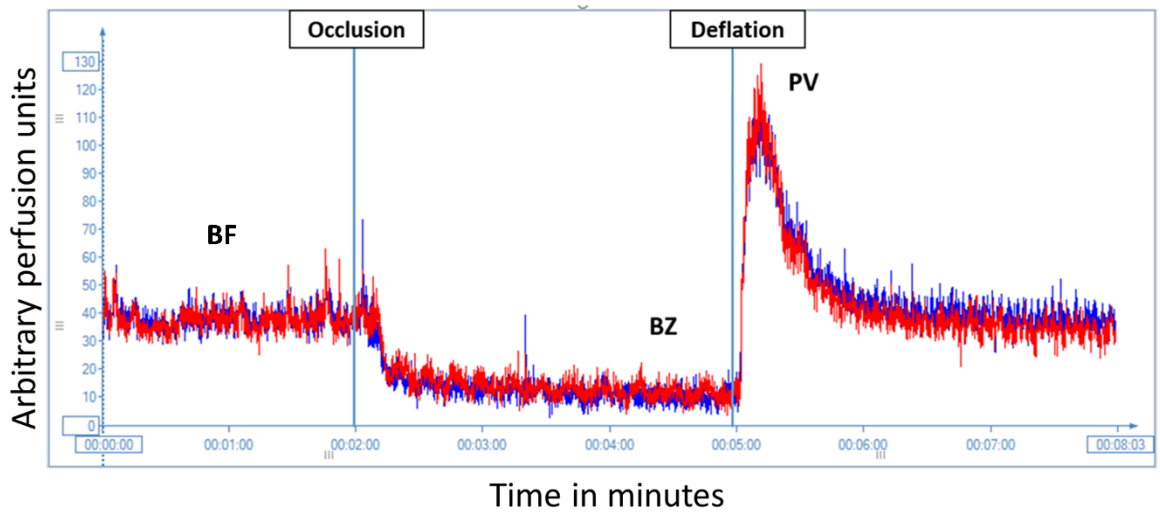


Figure 5-S1. Cutaneous perfusion recorded with laser speckle contrast imaging during post-occlusive reactive hyperaemia. BF, baseline perfusion; BZ, biologic zero; PV, peak value

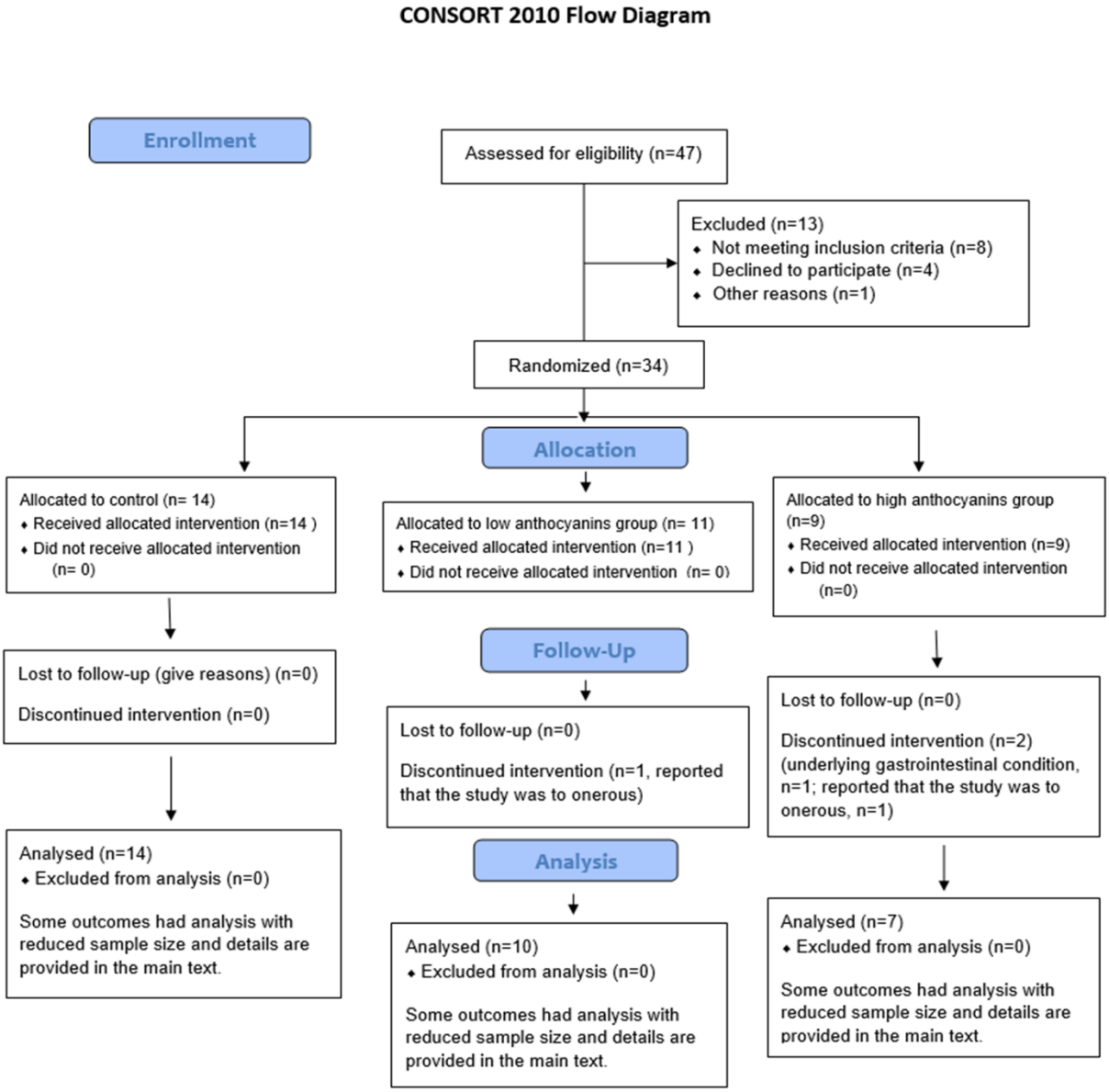


Figure 5-S2. Consort 2010 Flow diagram

CHAPTER 6: Conclusions and recommendations

6.1 Overview of core findings

The studies presented in this thesis utilised different study design methodologies to address the central research questions concerning the role of dietary intake of flavonoids and anthocyanins on vascular function, inflammation and other cardiovascular disease risk factors. Following an extensive exploration of the literature, gaps were identified that guided the design and conduct of all studies. The availability of resources, expertise of the research team collaborators and access to healthcare services in the local area were also considered in order to maximize efficiency of the body of work. This approach covered domains of: a) Epidemiological evidence: analytical work conducted in national representative cohort of Australian women: b) Knowledge syntheses: systematic literature review of randomized clinical trials; and c) Experimental evidence: conduct of two clinical trials to assess the acute and chronic effect of anthocyanin nutritional interventions in older adults.

The first gap identified was a lack of data from the Australian population regarding the association between the dietary intake of flavonoids and incidence of hypertension. Epidemiological evidence suggests that a higher dietary flavonoid intake is associated with a reduced risk of several chronic diseases, including hypertension. However, different dietary patterns and cuisines between populations affects the amounts and types of flavonoid-rich foods consumed, while differing lifestyle factors and life expectancy also influence the contribution of flavonoids to the incidence of hypertension and other chronic diseases. Moreover, the flavonoid content of foods is likely to be influenced by seasonality and differ between geographic areas. Therefore, a study was developed to investigate the association between intake of flavonoids and their subclasses, and incidence of hypertension among

Australian women in two age cohorts. Findings from this population-based prospective study showed an association between a higher dietary intake of flavonoids and lower incidence of hypertension. In the middle-aged cohort, higher intakes of the flavonoid subclasses, flavones, isoflavones and flavanones, found mainly in orange and orange juice, apples and soy milk, were associated with a reduced risk of hypertension among middle-aged women followed-up over 15 years. In the younger, reproductive-aged cohort, a higher intake of total flavonoids and flavanols, attributed mainly to orange juice, red wine, apples and onions, were associated with a reduced risk of hypertension over a 12 year follow-up period. These findings contribute to the current knowledge in his field, which may be used in nutritional messages and policies aiming to improve the cardiovascular health of women at these two different life stages. Interestingly, in two other similar studies (119,185) that found a reduction in incident hypertension in other countries, the benefits were attributed to other groups of flavonoids, such as flavonols and anthocyanins. This confirms a need to conduct studies in different regions because of variations in cultural and socio-economic characteristics that influence dietary patterns. Taken together, such findings suggest that nutritional messages and policies aiming to improve the cardiovascular health through increased consumption of dietary flavonoids may vary from population to population, as well as between different ages ranges.

The epidemiological study had a general approach regarding the classes of flavonoids, while the other studies that composed this Thesis had a scope focused in clinical nutrition, and advanced into a more specific investigation of one class of flavonoids, the anthocyanins. Additionally, although blood pressure and hypertension were addressed in all of these studies, a variety of parameters related to CVD were further investigated. Among studies that evaluate the effects of anthocyanins on CVD risk factors and associated biomarkers, a number of studies addressed the potential acute effects of anthocyanins in studies using a HFM challenge; however, such finding were not yet collated and synthetized. This gap led to the second study

of this Thesis, a systematic literature research that was conducted focusing in knowledge synthesis of the postprandial effects of anthocyanins on CVD risk factors in HFM studies. A total of 13 eligible studies were included and beneficial effects of anthocyanins were reported, with most promising results indicating attenuation of deleterious postprandial effects on oxidative stress and antioxidant status, triacylglycerol and total cholesterol concentrations, vascular endothelial function and inflammatory biomarkers. Post-prandial changes in blood pressure and lipoproteins were least affected by anthocyanins. The systematic literature review identified beneficial effects of acute dietary anthocyanin interventions on CVD risk factors following a HFM challenge; however, due to the heterogeneity in changes of some parameters identified between the studies, further studies are required in order to advance the current knowledge of the underlying mechanisms between post-prandial imbalances and CVD incidence and progression, and to investigate how these imbalances are attenuated by bioactive compounds such anthocyanins.

Based on the findings from the systematic literature review, another gap that was identified was a need for a more robust investigation of vascular function in the postprandial state, which along with the immune response, is an important predictor for CVD risk. This is particularly evident in older adults, as several studies support a gradual loss of the vascular function, and a new immune-metabolic viewpoint for age-related diseases, termed 'inflammaging' which is characterized by a chronic low-grade inflammation. Additionally, excessive adiposity at all ages is associated with an up-regulation of a pro-inflammatory state, as the accumulation of adipose tissue mass promotes the secretion and release of inflammatory mediators. This process also leads to chronic low-grade inflammation that is driven by a nutrient excess and/or overnutrition and has the same mechanisms as those underpinning "inflammaging". Therefore, a clinical trial was designed in the same setting of investigating the postprandial effects of anthocyanins following a HFHE meal challenge; however the

novelty was related to evaluation of both macro and microvascular parameters by combining classical and novel techniques with the latest imaging technologies, such as the flow-mediated dilatation (FMD) and the Laser Speckle Contrast Imaging (LSCI). In addition to vascular function assessments, classical CVD biomarkers such as lipid profile and blood pressure, and inflammatory biomarkers were also evaluated. To our knowledge, this is the first study to conduct this type of protocol in studies with nutritional interventions. The findings supported the postprandial effects of food anthocyanins in improving several CVD biomarkers in overweight older adults following a HFHE meal challenge. Potentially beneficial effects were observed in parameters of both macrovascular and microvascular function, as well as some inflammatory biomarkers. The HFHE meal challenge induced increases in postprandial blood pressure, diacron reactive oxygen metabolites, triacylglycerol and total cholesterol; however, anthocyanin consumption did not attenuate these responses. Such results corroborated with findings from the systematic literature review, and also advanced knowledge concerning the postprandial vascular regulation and how the effects exerted by anthocyanins can be observed in different vascular beds. Our findings contribute new evidence regarding the potential protective effect of dietary anthocyanins on vascular function and inflammatory responses, parameters that have a major role on atherosclerotic disease and CVD events.

Considering there were still several gaps to be addressed regarding the longer term effect of anthocyanins in this vascular-inflammatory axis in older adults, another clinical trial was conducted to investigate the chronic effects (8 weeks) of two different doses of food-anthocyanins on inflammatory markers associated with CVD risk factors, along with analysis of microvascular function and 24-hour ABP. This study was also conducted in older adults, but the aim was to investigate such parameters in individuals that had a diagnosis of Mild Cognitive Impairment, a neurodegenerative condition that shares pathological mechanisms with CVD. A daily high dose of fruit-derived anthocyanins for 8 weeks decreased serum concentrations of

TNF-α. These changes were not accompanied by effects on other inflammatory biomarkers (IL-6, hs-CRP and IL-1β), 24h ABP or microcirculation function, and no effects were observed in the low anthocyanin dose treatment group. Considering the major role of the inflammatory response in CVD and cognitive decline, our findings suggest that a regular high intake of anthocyanins may have clinical implications in this high-risk group; however, further studies with a longer follow-up are required to better elucidate whether this change on the i will alter overall CVD risk and/or progression of cognitive decline.

Overall, the findings from all four studies suggest that flavonoids and anthocyanins have an important role as a potential non-pharmacological treatment option to improve vascular health and reduce cardiovascular risk.

6.2 Strengths and limitations

The specific methodological strengths and limitations of each study have been addressed in-depth in each of the research chapters. However, more general aspects of the thesis will be addressed in this section.

Firstly, for the epidemiological aspect, the main strength of this study was the representative sample of Australian women across two age cohorts, the prospective design, and repeated measures over time. The statistical approach of using generalized estimated equations allowed for adjustments of key confounding factors. Potential confounders were extensively explored in our models, as there was access to a wide range of demographic, hypertension risk factor and dietary intake variables. The large sample size allowed for a stepwise regression with bidirectional elimination in order to create the models for such adjustments, therefore avoiding multicollinearity and overfitting of the model. Recruitment of participants was proportional to the population size in all Australian states, thereby enabling generalizability of the study findings to Australian women generally. However, a few limitations were present

such as the absence of men in this cohort and the use of self-reported data for hypertension. Moreover, the FFQ used may lack detail about some flavonoid-rich food sources due to it being developed over two decades ago, and it may not have included relevant high-flavonoid food items that have increased in popularity over this period, such as blackberries, cherries, blueberries and raspberries. This could have led to an underestimation of anthocyanin intake, as the mean intake in the present study was 4.7 mg/day in the middle-aged and 5.0 mg/day in the reproductive-aged cohort, compared to 24.2 mg/day reported in 2019 in the overall Australian population from a nationally representative sample that used two 24-hr recalls as the method of dietary assessment (222). This may have hindered a more robust overall message from the Thesis, considering that all other three studies focused in this specific flavonoid subclass.

The most notable challenge and limitation of conducting the systematic literature review was to address a wide range of CVD-related biomarkers assessed in this type of acute clinical trial. We had originally considered conducting a meta-analyses for the various outcomes included in the review, particularly for those outcomes that were most frequently reported. However, the major variation in reporting of the included meal challenge studies, presentation of data according to varying postprandial time points, and the use of different methodologies to measure outcome variables rendered the possibility of meta-analysis or other pooled analyses as unfeasible. However, the narrative analysis remains valuable. Another source of heterogeneity in the included studies may have resulted from the variability in the macronutrient and energy content of the HMF challenges, particularly regarding the types of fat, as well as the format in which the meals were delivered.

Despite these limitations, all included studies were conducted in young and middle-aged adults (mean age ranging from 20.2 to 46.9y), but there were differences in mean BMI between studies (two studies included participants with BMI<25kg/m2 [34,40], while six

targeted those with BMI between 25 and 30kg/m2 [26,29–31,36,39] and three included participants with BMI>30kg/m2 [33,35,38]). Another feature in the design of this type of study is that tests are conducted over multiple time periods following the HFM challenge, thereby raising a concern that the number of false positive findings may be inflated. For this reason, the interpretation of results found for only one or a few time points, and that were not sustained, had to be interpreted with caution. Still, parameters that have been used in included studies to investigate oxidative stress and antioxidant status represented a wide range of analytical methods, and there is no formal mechanism to establish consensus regarding the optimal biomarkers to use in such nutritional interventions (259,261). The main strength of the systematic literature review was to present the body of evidence to date in a clear and concise way, as well as discussing and suggesting which results need to be further investigated and identifying gaps that require further elucidation. The latter was what guided the design of the clinical trial included in Chapter 4.

A key feature of the two studies that comprised the experimental evidence part of this thesis was the ability to evaluate microcirculatory functioning using a cutting edge technology, the Laser Speckle Contrast Imaging (LSCI) method. Assessment of the microcirculation has been performed for decades, but the LSCI method is the first one to provide excellent reproducibility to assess skin microvascular reactivity (97). The main methodological strength of the acute study that evaluated postprandial effects of anthocyanins in older adults following a HFM challenge was the inclusion of a more robust evaluation of the vascular function, by exploring both macro and microvascular parameters by combining classical (FMD) and novel techniques (LSCI). To our knowledge, this is the first study to conduct this type of protocol in a nutritional intervention study. Although this innovative design allowed us to explore macrovascular and microvascular function, a few limitations need to be highlighted. It was not possible to repeat the FMD and microvascular reactivity tests in case of experimental errors,

such as a participant movement during testing, due to the protocol of measures being collected at specific time points. Concerning the evaluation of endothelial and non-endothelial mechanisms in vascular and microvascular reactivity, we were not able to conduct nitrate-mediated dilation nor iontophoresis of sodium nitroprusside due to the time limit between measures, considering it would be not viable, if not impossible, in a postprandial study to conduct a protocol with all of these measures. Another strength that can be attributed to both the acute and longer clinical trials was the choice of the control intervention. It is challenging to investigate a specific bioactive compound, such as a specific flavonoid, in a clinical trial using foods as vehicles for delivery. A food item that is a rich source of a particular flavonoid may also contain other classes of flavonoids, or still other bioactive compounds such as other polyphenols and/or inorganic compounds (such as nitrate), that might have similar or synergistic effects on the outcomes to those being assessed. In order to address this issue, the choice of the placebo food item for the control arm is an important consideration. A placebo that is only matched in macro, or even micronutrient content, can underestimate the effects of other bioactive compounds. In both studies, we aimed to evaluate the acute effect of anthocyanins using Queen Garnet plum juice as the intervention food source. In order to isolate the effect of anthocyanins, we chose apricot juice as the control due to its lack of anthocyanins, but its similarly matched macro- and micronutrient content, as well as its polyphenol profile (281), that includes flavanols (such as quercetin), catechins and epicatechins, all of which are potential mediators of cardiovascular health (20,303,305,349). The choice of this control juice allowed the effects of only anthocyanins to be isolated from other bioactive compounds. Concerning the second clinical trial that was conducted in older adults with a diagnosis of MCI, the small sample size was its main limitation. In addition to recruitment being slower than expected due to difficulties recruiting this patient group, and difficulties in ensuring a definite clinical diagnosis of MCI, the trial had to be cut short due to COVID-19 restrictions that

prevented face-to-face data collection. The smaller sample size of the final clinical trial has probably introduced issues of insufficient power and possibility of Type II error.

Overall, the main strength of this thesis is that all studies were highly connected to each other. The epidemiological study, answered a more general research question using data from a nationally representative cohort study, while the research questions were more narrowly focused in the experimental clinical trials. The systematic literature review and the first clinical trial addressed similar research outcomes. Both clinical trials shared several important features such as: food-based intervention of anthocyanins; microcirculation assessment using LSCI technology; evaluation of inflammatory biomarkers; and were conducted in older adults. The combination of studies presented in this thesis together helps address the main research questions in a complementary manner.

6.3 Future research and recommendations

Findings from this thesis have contributed novel findings to advance knowledge of this topic, but have also highlighted new areas for further research.

In the epidemiological approach of investigating the association of dietary intake of flavonoids in the Australian population, further studies with a similar methodology, but in different populations with varying outcomes are recommended. For example, a national representative study investigating the incidence of hypertension among Australian men is still lacking. Likewise, data on the incidence of other CVD and related conditions and their associations with the dietary intake of flavonoids are also needed. Considering that the dietary intake, in terms of sources of flavonoids, vary according to many factors over time, it is recommended that such analyses need to be conducted from time-to-time in different populations, using an appropriate and updated dietary intake assessment method for accurate estimation of dietary intake. Still, the possibility of evaluating blood, urine and faecal samples,

and measures of vascular function along with an epidemiological analyses, provide an important link between experimental and epidemiological evidence and would help elucidate the mechanisms underlying the dietary intake of flavonoids and incidence of various disease states.

At the clinical level, a number of key points and recommendations for future research are highlighted below:

- The choice of the intervention and placebo is an important consideration in order to compare and generalise conclusions across studies. As the evidence of the effects of anthocyanins to prevent and treat certain conditions is increasing, future studies will need to investigate specifically which types of anthocyanins exert these effects, as well as identify the synergistic effects of nutrients and food matrices that may be contributing to observed effects. There is also a need to focus on methodologies to stabilise or reduce degradation of these bioactive compounds in foods during processing and handling. In cases where the research question aims to explore the effects of a specific bioactive compound, a placebo choice that isolates this compound is recommended, in order to avoid non-specific effects of other polyphenols or nutrients such as dietary fibre and nitrate concentration.
- The assessment of postprandial parameters as CVD risk factors is still relatively new and less explored when compared to fasting measures. Although the findings from the systematic literature review and the clinical trial presented in Chapter 4 showed improvements in a variety of biomarkers associated with CVD, future studies should explore whether such changes will impact on CVD incidence and progression, and how these measures may differ from fasting measures. In other words, determine how the fasting and postprandial flow-mediated dilatation scores differ in prediction values for later CVD events.

- The use of the LSCI technology to assess microvascular function has increased in recent years; however, this remains an exploratory field, and further studies that include this outcome measure are required in the context of CVD research. There is a need to standardize the vascular reactivity tests protocols, in order to allow better comparison between studies and improve reproducibility of such tests within and between studies. Moreover, studies with larger sample sizes are required to investigate the association between these new microvascular parameters and other already established measures of vascular function, such as the gold-standard FMD, as well to investigate the association with other traditional and related CVD biomarkers.
- In studies that evaluate risk factors that are shared between CVD and neurodegenerative conditions such as in the clinical trial presented in Chapter 5, a longer nutritional intervention and follow-up period would be recommended in order to investigate if the improvements of such risk factors will reduce the overall incidence of CVD events and attenuate further cognitive decline over time.

6.4 Conclusion

The studies included in this thesis have addressed several gaps related to the role of dietary intake of flavonoids and anthocyanins on vascular function, inflammation and other cardiovascular disease risk factors. This body of research was conducted across three levels of evidence: Epidemiological evidence; Knowledge syntheses; and Experimental evidence. This was achieved by utilising a wide range of scientific methods in the medical sciences field, especially in nutritional epidemiology and clinical nutrition. From secondary data analysis, to the identification, selection and critical appraisal of relevant primary research, and the design and execution of original clinical trials, a vast array of interpersonal and laboratory skills, statistical methods and forms of presenting results were utilized.

Collectively, the four studies included in this thesis have resulted in novel findings that contributed to the advance of the knowledge in this field, as well as highlighting what is still have to be addressed, and identified new gaps to be explored in future studies. In a broader context, this research strengthen the concept that bioactive compounds, as part of diet or as a nutritional intervention, may have positive impact in health outcomes and help to attenuate the burden and incidence of chronic diseases.

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7 Appendices

7.1 Appendix A - Published Paper: Association between flavonoid intake and risk of hypertension in two cohorts of Australian women: a longitudinal study

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ORIGINAL CONTRIBUTION

Association between flavonoid intake and risk of hypertension in two cohorts of Australian women: a longitudinal study

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Abstract

Purpose Epidemiological evidence suggests higher dietary flavonoid intake is associated with lower risk of several chronic diseases. This study aimed to investigate the association between intake of flavonoids and their subclasses, and incidence of hypertension among Australian women in two age cohorts.

Methods This population-based study included 6599 middle-aged (52.5 ± 1.5 years) and 6099 reproductive-aged (27.5 ± 1.5 years) women from the Australian Longitudinal Study on Women's Health. Food frequency questionnaires were used to quantify intake of flavonoids by cross-referencing with the Phenol-Explorer food composition database. Generalised Estimating Equation analyses investigated associations with incident hypertension, adjusting for demographic and dietary variables and hypertension risk factors.

Results There were 1645 cases (24.9%) of hypertension during 15 years follow-up in the middle-aged cohort and 336 cases (5.5%) during 12 years follow-up in the reproductive-aged cohort. Higher intakes of flavones [adjusted relative risk (ARR) for quintile 5 vs. 1: 0.82, 95% CI 0.70–0.97], isoflavones (0.86, 0.75–0.99) and flavanones (0.83, 0.69–1.00) were associated with a lower risk of hypertension in the middle-aged cohort. In the reproductive-aged cohort, higher intakes of flavanols (0.70, 0.49–0.99) were associated with a lower risk of hypertension. Key foods that provided these flavonoids were oranges, orange juice, apples, red wine and soy milk.

Conclusion Higher intakes of total flavonoids and subclasses were associated with a lower risk of hypertension in Australian women. These findings can be used in nutrition messaging and policies for improved cardiovascular health of women.

Keywords Flavonoid · Hypertension · Blood pressure · Polyphenols · Cohort

Introduction

Cardiovascular disease (CVD) is a major cause of mortality globally and is the leading cause of death for Australian men and women. In 2018, CVD was responsible for over 41,800 deaths in Australia (26% of all deaths) [1]. Following

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7.2 Appendix B – Published Paper: The postprandial effect of anthocyanins on cardiovascular disease risk factors: a systematic literature review of high-fat meal challenge studies

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CARDIOVASCULAR DISEASE (JHY WU, SECTION EDITOR)

The Postprandial Effect of Anthocyanins on Cardiovascular Disease Risk Factors: a Systematic Literature Review of High-Fat Meal Challenge Studies

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Published online: 1 July 2020

Abstract

Purpose of review Recurrent post-prandial metabolic imbalances are important contributing factors to the development of cardiovascular disease (CVD). This study evaluated whether anthocyanin consumption attenuates the deleterious postprandial response of high-fat meals on CVD risk factors including blood pressure, vascular endothelial function, lipid profile and biomarkers related to oxidative stress, antioxidant status and immune response.

Recent findings Five electronic databases were searched up to the period of 1 February 2020, yielding 13 eligible studies, including randomised or cross-over clinical trials (18–59 years of age), using PRISMA guidelines (PROSPERO registration: CRD42019126265). Potential bias was assessed using the revised Cochrane risk-of-bias tool for randomised trials. Beneficial effects of anthocyanins were reported in biomarkers of oxidative stress and antioxidant status in 6 out of 9 studies, and in 3 out of 6 studies for inflammatory response. Two positive results were found concerning attenuation of post-prandial endothelial dysfunction, increased triacylglycerol and total cholesterol exerted by the high fat meal. Blood pressure and lipoproteins were the parameters with least beneficial results.

Summary Our systematic literature review revealed beneficial effects of dietary anthocyanin interventions on CVD risk factors following a HFM challenge; however, heterogeneity in results exists. The most promising results were for the attenuation of deleterious postprandial effects on oxidative stress and antioxidant status, triacylglycerol and total cholesterol concentrations, vascular endothelial function and inflammatory biomarkers. Post-prandial changes in blood pressure and lipoproteins were least affected by anthocyanins. Further studies are required in order to better elucidate the post-prandial effects of anthocyanins and CVD risk factors.

Keywords Anthocyanins · Cardiovascular disease · Postprandial · Inflammation · Oxidative stress

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Introduction

Cardiovascular diseases (CVD) are still the number one cause of death globally, representing 31% of deaths in 2016. Most CVDs can be prevented by addressing and managing behavioural risk factors such as diet [1]. Several CVD risk factors are associated with the atherosclerotic process and other vascular dysfunctions closely linked to nutrition [2].

There is emerging evidence that metabolic imbalances during the postprandial state, particularly after a high-energy meal rich in fat, that are important contributing factors to the development of CVD [••3, 4]. Overall, the underlying mechanism involves a sharp increase in triacylglycerol along with an aberrant production of pro-oxidant molecules leading to an oxidative stress state. Oxidative stress impairs vascular and endothelial functions and

7.3 Appendix C – Published Paper: Anthocyanins attenuate vascular and inflammatory responses to a high fat high energy meal challenge in overweight older adults: a cross-over, randomized, double-blind clinical trial.

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Randomized Control Trials

Anthocyanins attenuate vascular and inflammatory responses to a high fat high energy meal challenge in overweight older adults: A cross-over, randomized, double-blind clinical trial

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SUMMARY

Background & aims: Postprandial metabolic imbalances are important indicators of later developing cardiovascular disease (CVD). This study investigated the effects of food anthocyanins on vascular and microvascular function, and CVD associated biomarkers following a high fat high energy (HFHE) meal challenge in overweight older adults.

Methods: Sixteen subjects (13 female, 3 male, mean age 65.9 SD 6.0 and body mass index 30.6 kg/m^2 SD 3.9) participated in a crossover, randomized, controlled, double-blind clinical trial (registered under Australian New Zealand Clinical Trials Registry, identifier no. ACTRN12620000437965). Participants consumed a HFHE meal with a 250 mL dose of either intervention (anthocyanins-rich Queen Garnet Plum) or control (apricot) juice. Blood samples and blood pressure measures were collected at baseline, 2 h and 4 h following the HFHE meal. Vascular and microvascular function were evaluated at baseline and 2 h after the HFHE meal.

Results: Participants had a higher 2 h postprandial flow-mediated dilatation (+1.14%) and a higher microvascular post-occlusive reactive hyperaemia (+0.10 perfusion units per mmHg) when allocated to the anthocyanin compared to the control arm ($P = 0.019$ and $P = 0.049$, respectively). C-reactive protein was lower 4 h postprandially in the anthocyanins (1.80 mg/L, IQR 0.90) vs control arm (2.30 mg/L, IQR 1.95) ($P = 0.026$), accompanied by a trend for lower concentrations of interleukin-6 ($P = 0.075$). No significant postprandial differences were observed between treatments for blood pressure, triacylglycerol, total cholesterol, serum derivatives of reactive oxidative metabolites, tumor necrosis factor alpha, interleukin-1 beta, or maximum microvascular perfusion following iontophoresis of acetylcholine.

Conclusion: Fruit-based anthocyanins attenuated the potential postprandial detrimental effects of a HFHE challenge on parameters of vascular and microvascular function, and inflammatory biomarkers in overweight older adults. Anthocyanins may reduce cardiovascular risk associated with endothelial dysfunction and inflammatory responses to a typical high fat 'Western' meal. Further studies are required to better elucidate the clinical implications of postprandial biomarkers of CVD.

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7.4 Appendix D – Published Paper: Food anthocyanins decrease serum concentrations of TNF-α in older adults with mild cognitive impairment: a randomized, controlled, double blind clinical trial.

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Food anthocyanins decrease concentrations of TNF-α in older adults with mild cognitive impairment: A randomized, controlled, double blind clinical trial

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Abstract *Background & aims:* Vascular function, blood pressure and inflammation are involved in the pathogenesis of major chronic diseases, including both cardiovascular disease (CVD) and mild cognitive impairment (MCI). This study investigated the effects of food anthocyanins on microvascular function, 24-h ambulatory blood pressure (ABP) and inflammatory biomarkers in older adults with MCI.
Methods and results: Thirty-one participants with MCI [19 female, 12 male, mean age 75.3 (SD 6.9) years and body mass index 26.1 (SD 3.3) kg/m^2], participated in a randomized, controlled, double-blind clinical trial (Australian New Zealand Clinical Trials Registry: ACTRN12618001184268). Participants consumed 250 mL fruit juice daily for 8 weeks, allocated into three groups: a) high dose anthocyanins (201 mg); b) low dose anthocyanins (47 mg); c) control. Microvascular function (Laser Speckle Contrast Imaging combined with a post-occlusive reactive hyperaemia test), 24h ABP and serum inflammatory biomarkers were assessed before and after the nutritional intervention.
Results: Participants in the high anthocyanins group had a reduction in serum tumor necrosis factor alpha (TNF-α) ($P = 0.002$) compared to controls and the low anthocyanins group (all P's > 0.05). Serum IL-6, IL-1β, c-reactive protein, and parameters of microvascular function and 24h ABP were not altered by any treatment.
Conclusion: A daily high dose of fruit-based anthocyanins for 8 weeks reduced concentrations of TNF-α in older adults with MCI. Anthocyanins did not alter other inflammatory biomarkers, microvascular function or blood pressure parameters. Further studies with a larger sample size and longer period of follow-up are required to elucidate whether this change in the immune response will alter CVD risk and progression of cognitive decline.

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