Dietary anthocyanins and cardiovascular risk factors: the influence of tart Montmorency cherries on health indices in middle-aged adults

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No part of this thesis has been submitted in the past or is to be submitted for any degree at any other University.

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Abstract

Anthocyanins, the subclass of polyphenol flavonoids responsible for the red-blue-purple pigmentation of fruit and vegetables, have gained much research focus recently due to their propensity to preserve or even improve vascular function. There is some evidence that higher-intake of foods rich in these compounds can positively impact risk factors associated with (CVD) cardiovascular disease such as blood pressure, endothelial function and arterial stiffness; albeit ambiguity remains as to whether this translates to a reduced risk of CVD. One of the most studied anthocyanin-rich foods in recent years has been tart Montmorency cherries (*Prunus Cerasus*, cv Montmorency; MC), particularly in exercise and to a lesser extent in health paradigms. Indeed, cherries can contribute to a considerable dietary intake of anthocyanins, but they are also a rich source of other polyphenols, which might confer health benefits due to the synergistic and additive properties on antioxidant or anti-inflammatory actions.

Certainly, there is some promising evidence from *in vitro* and animal models that MC can influence pathways that might improve cardiovascular and metabolic health. Moreover, accumulating evidence has shown that acute postprandial exposure to MC, potentially due to its anthocyanin content, effects vascular and metabolic function in humans. Despite this there is a paucity of original research relating to the longer-term influence on cardiovascular function, and health in general. Moreover, existing studies are difficult to interpret due to limitations and the paradoxical findings, highlighting the need for well-designed clinical trials investigating MC on cardiovascular risk factors. Taken all together, the overarching aim of this thesis, was to further investigate the potential role of dietary anthocyanins in cardiovascular health. The reliability of a test battery of cardiovascular risk factors was also assessed, leading to three experimental studies (Chapter 3-5).

Firstly, a systematic literature review evaluated the current evidence for the association between dietary anthocyanin intake and risk of CVD. In the largest and most comprehensive meta-analysis of existing prospective cohort studies, it was found that a higher intake of anthocyanins reduced risk of both coronary heart disease (CHD) and CVD mortality. These data further support the notion that exposure to anthocyanins could have a putative role in cardiovascular health, but clinical trials are still needed to verify these findings. Nonetheless, as a

prelude to a randomised controlled trial, Chapter 4 investigated the repeatability of a battery of vascular function test measures. The repeatability of blood pressure, arterial stiffness and macro-vascular endothelial function was found to be adequate and this was used to inform the final study. Chapter 5 reports the influence of anthocyanin-rich MC on physiological or cognitive function in middle-aged adults. This randomised, double-blind, placebo-controlled trial had several strengths such that it was successfully blinded, relatively large sample size, longer-term supplementation period and well controlled compared to other studies of a similar nature. However, the results suggested no influence of MC juice on vascular function, metabolic health, cognitive function, or exercise capacity after three months in middle-aged adults. There was an effect on mental fatigue and alertness visual analogue scales (VAS) during the cognitive function test battery, suggesting an anti-fatiguing effect of MC. Moreover, Chapter 6 outlines important limitations and future research directions, that will help strengthen the evidence base regarding the intake of anthocyanin-rich foods and CVD. Collectively, these studies have contributed to knowledge on longer-term exposure to dietary anthocyanins and how they can impact on indices associated with health outcomes in humans.

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List of Abbreviations

Ach	acetylcholine chloride
AIx	augmentation index
AIx@75	AIx normalised for a standard heart rate of 75 bpm
AUC	area under the curve
BDNF	brain derived neurotrophic factor
BMI	body mass index
BP	blood pressure
C3G	cyanidin-3-glucoside
C3GR	cyanidin-3-glucosylrutinoside
C3R	cyanidin-3-rutinoside
CAD	coronary artery disease
CBF	cerebral blood flow
CHD	coronary heart disease
COX	Cyclooxygenase
CREB	cAMP response element-binding protein
CRP	C reactive protein
CV	coefficient of variation
CVD	cardiovascular disease
DBP	diastolic blood pressure
DV	digital vigilance
DVP	digital volume pulse
DXA	dual-energy X-ray absorptiometry
eNOS	endothelial nitric oxide synthase
ERK	extracellular signal-regulated kinases
ET-1	endothelin 1
F&V	fruit and vegetables
FFQ	food frequency questionnaire
FMD	flow mediated dilation
GLUT2	glucose transporter
HbO_2	oxygenated haemoglobin
HDL	high-density lipoproteins
hHb	deoxygenated haemoglobin
HOMA-IR	homeostatic model assessment of insulin resistance
HR	hazard ratio
Hr	heart rate
ICC	intra-class correlation coefficient
IL-6	interleukin-6
IQR	interquartile range
LDI	laser Doppler imaging with iontophoresis
LDL	low-density lipoproteins
LOX	Lipoxygenase
MAPK	mitogen-activated protein kinase
MC	tart Montmorency cherries
MET	metabolic equivalent
MI	myocardial infarction
MOOSE	meta-analysis of observational studies in epidemiology

NF-κβ	nuclear factor-kappa beta
NIRS	near infra-red spectroscopy
NO	nitric oxide
NOX	nicotinamide adenine dinucleotide phosphate-oxidase
PPAR	peroxisome proliferator-activated
PRISMA	preferred reporting items for systematic reviews and meta-analysis
PSQI	Pittsburgh Sleep Quality Inventory
PTT	pulse transit time
PU	perfusion units
PWA	pulse wave analysis
PWV	pulse wave velocity
RI	reflection index
RR	relative risk
RVIP	rapid visual information processing
SBP	systolic blood pressure
SD	standard deviation
SE	standard error
SF-36	short form quality-of-life survey
SGLT1	sodium-glucose linked transporter
SI	stiffness index
SMD	standardised mean difference
SNP	sodium nitroprusside
TAC	total anthocyanin content
TE	typical error
tHb	total haemoglobin
TNF-α	tumour necrosis factor-alpha
TPC	total phenolic content
VAS	visual analogue scale
VSMC	vascular smooth muscle cells
WMD	weighted mean difference

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Declaration

I declare that the work contained in this thesis has not been submitted for any other award and that it is all my own work. I also confirm that this work fully acknowledges opinions, ideas and contributions from the work of others.

Any ethical clearance for the research presented in this thesis has been approved. Approval has been sought and granted by the Faculty Ethics Committee at Northumbria University.

I declare that the Word Count of this Thesis is 34894 words.

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Signature:

Date: 06/11/2020

Chapter 1:

Introduction

1.1 Introduction

Cardiovascular disease (CVD) and type II diabetes combined are the primary cause of global mortality (Danaei et al., 2014). Advancing age is the strongest risk factor for these diseases, with ~90% of CVD occurring in adults aged 40-80 years or older (Ekoé, Punthakee, Ransom, Prebtani, & Goldenberg, 2013; Yazdanyar & Newman, 2009) and the number of middle-aged and older adults is rapidly increasing (ONS, 2017). However, amongst modifiable lifestyle factors of age-related diseases, diet can play an integral part (Afshin et al., 2019). For example, increased intake of fruit and vegetables (F&V) have also been shown to reduce risk of type II diabetes, premature death and CVD mortality (Aune et al., 2017; Forouzanfar et al., 2016). Fruit and vegetables are an important source of dietary phytochemicals, in particular polyphenols, that are thought to contribute to the observed inverse relationship between intake of F&V and chronic disease (Adriouch et al., 2018a; Kesse-Guyot et al., 2011). In recent years there has been an enormous research focus into anthocyanins, a specific subclass of polyphenols, and how foods which contain these compounds can be exploited to improve human health (Cassidy, 2017; Keane et al., 2016b; Rodriguez-Mateos et al., 2019a). Accumulating data suggests that these compounds might protect against diseases due to their antioxidant and anti-inflammatory actions and their ability to interact with cell signalling pathways involved in cell adhesion, cell migration, and cell differentiation (Krga, Milenkovic, Morand, & Monfoulet, 2016; Reis et al., 2016; Rodriguez-Mateos et al., 2019a).

One of the most studied anthocyanin-rich foods in recent years has been tart Montmorency cherries (*Prunus Cerasus*, cv Montmorency; MC) in health and exercise. Indeed, the physiological and biochemical effects that MC (and its constituents) might afford in both clinical and exercise domains has garnered much attention (Bell, McHugh, Stevenson, & Howatson, 2014c; Kelley, Adkins, & Laugero, 2018; McCune, Kubota, Stendell-Hollis, & Thomson, 2011). There is now emerging evidence that MC can positively influence risk factors for cardiometabolic and neurodegenerative diseases, effects that have been largely attributed to its anthocyanin content (Chai, Davis, Wright, Kuczmarski, & Zhang, 2018; Desai, Roberts, & Bottoms, 2019; Keane et al., 2016b). Given the global health issues associated with poor cardiovascular and cognitive function, foods rich in naturally occurring compounds such as anthocyanins that could be incorporated in to the diet to help reduce the severity and prevalence of these

diseases would not only have economic implications, but would also improve health and wellbeing. However, epidemiological studies (Ponzo et al., 2015; Tresserra-Rimbau et al., 2014a) supporting the putative role of anthocyanins in cardiovascular health, and indeed recent findings from longer-term MC supplementation on CVD risk factors, are inconsistent and equivocal (Chai et al., 2018; Desai, Roberts, & Bottoms, 2020; Johnson et al., 2020; Lynn et al., 2014). Moreover, due to the heterogeneity amongst those existing studies the current understanding of the potential application anthocyanin-rich foods in human health is limited. This area requires more attention due to the existing challenge for the primary prevention and management of non-communicable diseases in the globally aging population.

The overarching aim of this thesis is to provide novel insight into potential role of dietary anthocyanins in cardiovascular health.

This aim is broken down into the following specific aims which will be addressed in the subsequent experimental chapters:

1) To synthesize and evaluate the relationship between dietary intake of anthocyanins and the risk of CVD and related mortality from prospective cohort studies (Chapter 3)
2) As a prelude to investigating the influence of anthocyanins on cardiovascular risk factors – to determine the reliability of a battery of commonly used non-invasive measures of vascular function *in vivo* (Chapter 4)
3) To investigate the influence of longer-term (3 month) tart cherry (*Prunus Cerasus*, cv Montmorency) supplementation on vascular function, cognitive function, and exercise capacity in free-living middle-aged adults (Chapter 5)

Chapter 2:

Literature Review

2.1 Introduction

Given the diverse nature of this thesis which focuses on the application of dietary anthocyanins in the form of tart cherries in health and exercise paradigms, this review will examine the literature pertaining to the following areas:

- Phytochemical uses, structure and function with particular emphasis on those present in tart cherries.
- The pharmacokinetics and the factors that affect the bioavailability of phenolic compounds in tart cherries.
- The potential mechanisms of action of tart cherry polyphenols.
- The current research pertaining to effects of tart cherry supplementation on various aspects of health and exercise.

Many fruits grown in the Rosaceae plant family have significant economic importance, including; apples, apricots, blackberries, cherries, peaches, pears, plums, raspberries, and strawberries (Farinati, Rasori, Varotto, & Bonghi, 2017; Yue et al., 2017). Cherries (genera *Prunus*) ripen first among the stone fruits and are grown in two main species; sweet (*Prunus avium* L.) and tart (*Prunus cerasus* L.), also known as sour (Quero-García, Iezzoni, Pulawska, & Lang, 2017). In recent years, there has been an increased demand for tart cherry production, with the USA reportedly producing more than 140 thousand tonnes in 2016 alone (FAO, 2018; Thornsbury & Martinez, 2012). Unlike sweet cherries, which are normally consumed as fresh fruit, tart cherries are mostly processed, for example; powdered, frozen, dried, made into juice or juice concentrate (Chaovanalikit & Wrolstad, 2004). The sale of these and other fruit and vegetable derived products has burgeoned, as consumers have gained interest into their purported health and exercise related benefits (Lee, 2016; Wootton-Beard & Ryan, 2011). There is now a growing body of evidence to support that tart cherries, specifically from the Montmorency cultivar, elicit cardio- and neuro-protective properties (Keane et al., 2016b; Thangthaeng et al., 2016). Montmorency tart cherries (MC) have also been demonstrated to be effective in enhancing exercise performance and recovery (Bell, Stevenson, Davison, & Howatson, 2016; Howatson et al., 2010; Levers et al., 2016). Such benefits are thought to be attributable to the phytochemical, specifically anthocyanin, content of cherries that compare favourably against other fruits (Clifford, 2000). The high quantities of

phytochemicals and findings from recent studies suggested MC to be a salient functional food in both clinical and exercise domains; these will be discussed in the forthcoming sections (Keane, Haskell-Ramsay, Veasey, & Howatson, 2016c; Kirakosyan, Gutierrez, Ramos Solano, Seymour, & Bolling, 2018; Levers et al., 2016).

2.1.1 Historic and medicinal uses of phytochemicals

Phytochemical-rich plants have formed the basis of sophisticated traditional medicine systems that have been in existence for thousands of years. Ethnobotany has described a variety of medicinal plant and derivatives such as regional herbs, spices, teas, wines, fruits, barks, roots and leaves used to treat and cure a number of ailments. In the ancient Western world, the Greeks contributed significantly to the rational development of the use of medicinal plants, said to have originated with Hippocrates (460–377 BC) and Aristotle (384–322 BC), who were inspired by ancient beliefs from India and Egypt, some of the primary users of herbal medicines (Gurib-Fakim, 2006). As early as 668 BC the ancient Greeks were utilizing mushrooms, dried figs and various wine concoctions to enhance their sporting performance in the Olympics (Moffat, 2006). With regards to traditional herbal medicines; Indian Ayurveda, Islamic Unani and Chinese medicine represent good examples of philosophical systems that remain prominent despite the intellectual imperialism of Western medicine (Hoffmann, 2003). Two previous analyses of the phytochemistry of medicinal Chinese herbs demonstrated that some were particularly rich sources of melatonin and polyphenols, up to 3000 $ng \cdot g^{-1}$ dry mass and 280.46 mg gallic acid equivalents $(GAE) \cdot g^{-1}$, respectively (Chen et al., 2003; Liu, Qiu, Ding, & Yao, 2008). Interestingly, those herbs containing the higher levels of melatonin; *yinyanghuo* (1105 $ng \cdot g^{-1}$) and *sangbaipi* (1110 $ng \cdot g^{-1}$) were typically used to slow the aging process (Chen et al., 2003). On the other hand, the highest levels of total polyphenol content was observed in the Chinese white olive, which were predominantly used to cure pain and swelling in the throat, an action which might be related to their antioxidant capacity (Liu et al., 2008).

Historically, berries such as blackberries and raspberries, with more comparable phytochemical composition to cherries than the aforementioned herbals, have also been described as medicinal sources. These plants, from the *Rubus* genera, were traditionally used for preventing stomach-aches, excess fluxes of bodily

fluids and for treating droopy eyes, mouth sores, haemorrhoids, and snakebites (Hummer, 2010). Whereas, cherries were purportedly utilized in Unani medicine to treat various ancient diseases such as '*Usr al-Bawl* (Dysuria), *Qarha-e-Alate Baol*, *Hasah-al-kulya* (Nephrolithiasis), and (Cystolithiasis) *Hasah-al-Mathana* (Shamsi, 2017). Similarly, in Africa, their native cherries (*Prunus Africana*) were also widely exploited. The bark and leaves used to target illnesses (e.g. gonorrhoea) and symptoms such as fevers and pains (Stewart, 2003). More recently, there is accumulating evidence that tart cherries, particularly MC, could be a valuable medicinal plant in many age-related diseases, which is likely due to the unique phytochemical composition, including both melatonin and polyphenols (Bell et al., 2014c; Kelley et al., 2018; McCune et al., 2011).

2.1.2 Tart cherry phytochemical synthesis and classification

Tart cherries contain an array of bioactive phytochemicals; these include polyphenols, carotenoids and indolamine compounds (Ferretti, Bacchetti, Belleggia, & Neri, 2010). Notably, MC do not have the highest phytochemical content of any fruit, however, they contain more than sweet cherries and other tart cherry cultivars (Chaovanalikit & Wrolstad, 2004; Kirakosyan et al., 2010). Moreover, amongst the 20 most commonly consumed fruits, cherries appear to have the fifth highest total polyphenol content (Vinson et al., 2001). In addition their phytochemicals have previously been demonstrated to have synergistic and additive effects, thus any health effects associated with their intake could be enhanced by such interactions (Kirakosyan et al., 2010). The following section aims to summarise the phytochemicals specifically found in tart cherries an overview of which can be found in **Figure 1** below.

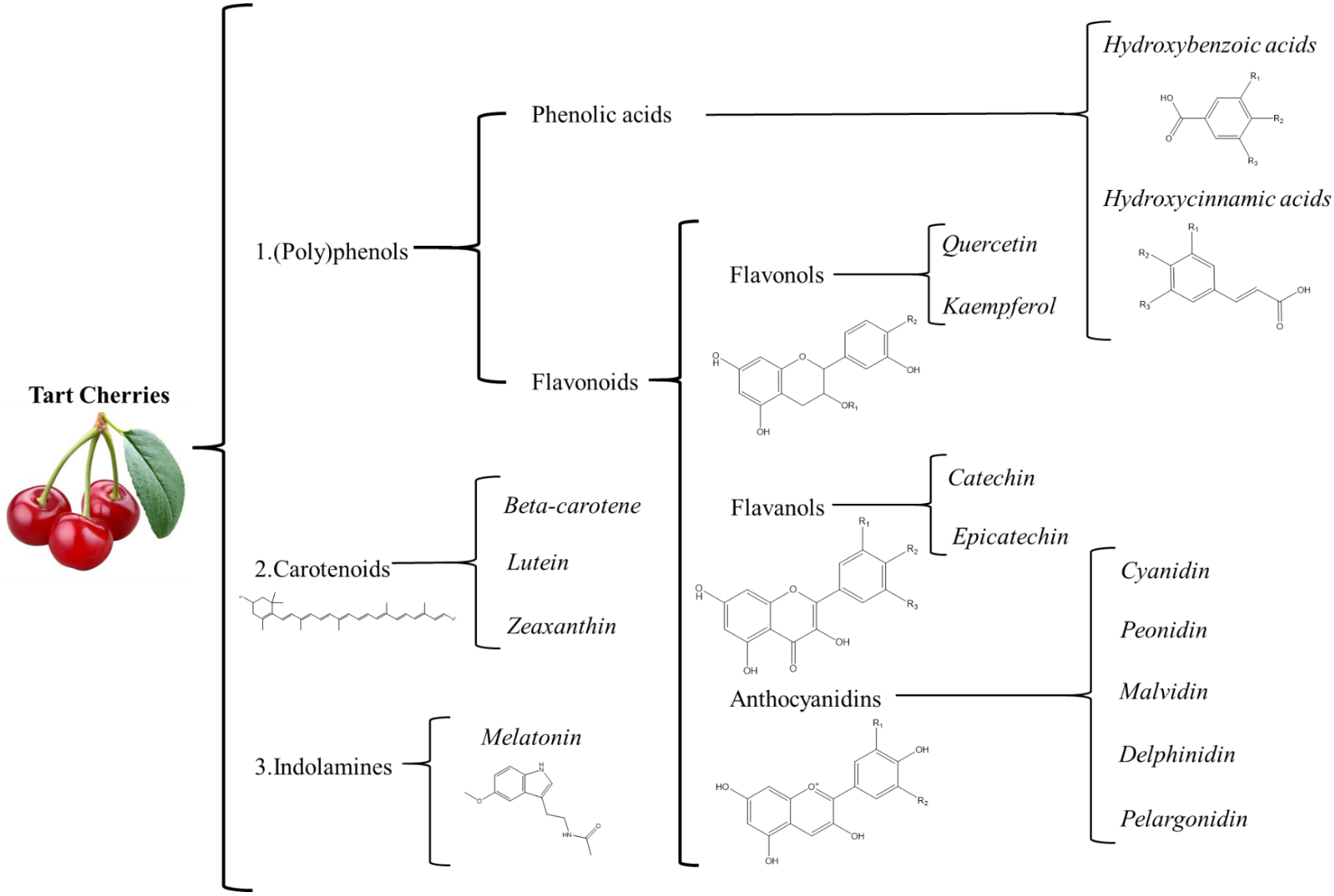

Figure 1. Breakdown of the bioactive phytochemical compounds found in tart cherries (Quero-García et al., 2017).

2.1.2.1 Tart cherry polyphenols

Polyphenols are naturally occurring compounds found largely in the fruits, vegetables, cereals and beverages. Used as an umbrella term, polyphenols describe phenolic compounds which are characterized by having at least one aromatic ring with one or more hydroxyl group attached. These can be further classified into four categories: phenolic acids, flavonoids, stilbenes and lignans (Pandey & Rizvi, 2009). These phenolics are not related to the plants primary metabolism of compounds which are vital for its immediate survival (e.g. in the same way as photosynthesis) but represent a secondary pathway that nonetheless, indirectly increases the survivability of the plant. As such, the term 'secondary metabolite' has been applied to polyphenolic compounds and the survivability-enhancing actions they confer are associated with three main areas. Firstly, they can act as deterrents to potentially damaging herbivores and competitors; secondly, they can attract potentially beneficial symbiotes; and finally they provide phytoalexin-mediated protection against potentially damaging stressors (Quideau, Deffieux, Douat-Casassus, & Pouysegu, 2011). Although long ignored, the function of these compounds is now attracting widespread attention because of their diverse range of bio-physicochemical properties (Crozier, Jaganath, & Clifford, 2006).

With regards to tart cherries, the total phenolic content (TPC) has previously been reported anywhere between 74-754 mg per 100 g of fresh weight (Quero-García et al., 2017). They have also been identified to be rich sources of phenolic acids, mainly hydroxybenzoic and hydroxycinnamic acids, and flavonoids. Flavonoids can be further sub-categorised, with MC and related products reportedly containing varying concentrations of flavonols (quercetin and kaempferol), flavanols (also known as flavon-3-ols; catechin and epicatechin) and anthocyanidins (cyanidin, malvidin, pelargonidin, peonidin, delphinidin and petunidin) the skeleton for which are shown in **Figure 1** (Bell et al., 2014a; Kirakosyan, Seymour, Llanes, Kaufman, & Bolling, 2009; Quero-García et al., 2017). Both flavonoids and phenolic acids are derived from the phenylpropanoid pathway and anthocyanins synthesized when the enzyme chalcone synthase produces tetrahydroxychalcone from 4-coumaroyl-CoA and malonyl-CoA (Starkevič et al., 2015).

Due to their distinct red colour, cherries are usually associated with high levels of anthocyanins, which are glycosides generated from anthocyanidins and are the pigments often responsible for the orange, red, and blue colours in fruits, vegetables, flowers, and other storage tissues in plants (Blando, Gerardi, & Nicoletti, 2004). As such, the highest proportion of anthocyanins in MC are found in the skin, with minimal amounts reported in the flesh and pits (Chaovanalikit & Wrolstad, 2004). The most abundant anthocyanins in tart cherry fruits are cyanidins and peonidins with glucose and rutinose sugar moieties; e.g. cyanidin 3-O-glucoside; C3G, cyanidin 3-O-glucosyl-rutinoside, cyanidin 3-O-rutinoside, peonidin 3-O-rutinoside and peonidin 3-O-glucoside (Rothwell et al., 2013). To date, interest in polyphenols, particularly anthocyanins (Rodriguez-Mateos et al., 2019a), has intensified because of their possible health-promoting properties (Blando et al., 2004; Pandey & Rizvi, 2009).

2.1.2.2 Tart cherry carotenoids

Carotenoids, a class of terpenoids (also known as isoprenoids), are another example of pigments and are responsible for red, orange, and yellow colours of fruits, vegetables and flowers. Carotenoids are classified by their chemical structure as: (1) carotenes that are constituted by carbon and hydrogen; (2) oxycarotenoids or xanthophylls that have carbon, hydrogen, and, additionally, oxygen. In plants, carotenoids primarily function as essential components required for photosynthesis (e.g. beta-carotene), photoprotection (e.g. zeaxanthin) and the production of phytohormones, hence can be either primary or secondary metabolites. These compounds are produced in fruit chromoplasts during the maturation process and are synthesized by the isoprenoid pathway, where isopentenyl pyrophosphate is the most common precursor (Delgado-Vargas, Jiménez, & Paredes-López, 2000).Tart cherries contain carotenoids, mainly beta-carotene (770 µg per 100 g fresh weight) and to a lesser extent lutein and zeaxanthin (85 µg per 100 g fresh weight) (Ferretti et al., 2010). Dietary sources of carotenoids, because of their potential role as antioxidants, have been suggested to be protective against chronic diseases (Tapiero, Townsend, & Tew, 2004). However, because of the limited evidence to support this concept (Krinsky & Johnson, 2005), carotenoids are not of particular concern in the current thesis.

2.1.2.3 Tart cherry indolamines

Indolamines, for example melatonin, are neurotransmitters, now widely accepted as important plant metabolites participating in diverse processes such as spanning reproduction, germination, vegetative growth, perception and morphogenesis (Erland, Shukla, Singh, Murch, & Saxena, 2017). Although the biosynthetic pathways in humans and mammals have been well established, the synthesis of indolamines in plants is less well understood. As with humans, the aromatic amino acid tryptophan is the metabolic precursor, which is converted to either serotonin (5-hydroxy-tryptamine) or normelatonin (*N*-acetyl-serotonin), and finally (*N*-acetyl-5-methoxy-tryptamine) melatonin (Arnao & Hernández-Ruiz, 2015). The melatonin content of MC has previously been reported as approximately 13 $ng \cdot g^{-1}$, more than that of sweet cherries (Burkhardt, Tan, Manchester, Hardeland, & Reiter, 2001; Kirakosyan et al., 2010). Exogenous intake of melatonin has been associated with improved regulation of circadian rhythms as well as reduced risk of various cancers, cardiovascular diseases, improved sleep quality, mood and cognitive function (Posadzki et al., 2018). Improved sleep in particular might reduce cardiovascular risk factors in older individuals (Carroll et al., 2015), and the dose of dietary melatonin currently recommended for dietary supplementation in this cohort is 0.3 to 2mg (Vural, Van Munster, & De Rooij, 2014). Although the levels of melatonin in tart cherries are much lower than those of melatonin given as a nutritional supplement, consumption (~85.2 мg per day) has been shown to significantly increases circulating melatonin levels and improve sleep efficiency (Howatson et al., 2012). Enhanced sleep following the consumption of tart cherries has been reported elsewhere (Losso et al., 2017; Pigeon, Carr, Gorman, & Perlis, 2010). While this has been speculated to be due to the melatonin content of tart cherries, it might also be due to the stimulation endogenous melatonin production due to the tryptophan content of tart cherries (Jiki, Lecour, & Nduhirabandi, 2018). However, another potential mechanism for these benefits has been attributed to the anti-inflammatory effects of tart cherry polyphenols (Losso et al., 2017), in that a number of inflammatory cytokines are intricately related to the modulation of sleep (Opp, 2004). Nonetheless, while the mechanisms are still impossible to discern, they are likely due to more than just melatonin content. Furthermore, previous studies have shown strong synergistic and additive activities for melatonin and tart cherry polyphenols (Kirakosyan et al., 2010), thus although the

focus of this thesis remains dietary anthocyanins it is important to take in to consideration that tart cherries contain other phytochemicals as summarised in **Figure 1**.

2.1.3 Bioavailability of tart cherry phytochemicals

The efficacy of any nutraceutical is reliant on the bioavailability *in vivo*, that is, following ingestion, the active compounds are absorbed through the gastro-intestinal tract and made available in the circulation, in sufficient quantities, to be utilized by cells (Rein et al., 2013). Consequently, it is not only important for tart cherries to contain sufficient quantities of phytochemicals, but it is also critical that those phytochemicals are absorbed and distributed in biologically effective doses. The former of which is influenced heavily by the cultivar and subject to a number of pre- and post-harvest factors.

Environmental factors such as rainfall, temperature, sun exposure and light intensity could all affect the synthesis and subsequently quantity of phytochemicals in tart cherries (Quero-García et al., 2017). Moreover, pre-harvest treatments is another important factor, as demonstrated in tart cherries harvested from ethephon-sprayed trees which had reduced anthocyanin content and antioxidant activity than non-sprayed controls (Khorshidi & Davarynejad, 2010). Furthermore, the degree of ripeness can also affect both the concentration and proportion of various phytochemicals, for example phenolic acid concentrations potentially decrease during ripening, whereas anthocyanin content increase (Gonçalves et al., 2007). Other post-harvest factors include, but are not limited to; food processing, transport, storage and cooking of tart cherries and related-products, all of which can impact the phytochemical, including polyphenol, content (Ferretti et al., 2010).

Bioavailability of tart cherry phytochemicals is further complicated by systemic and intestinal factors relating to the individual. Systemic factors include sex, age, and genetic variation. Notably, genetic variation in pathways affecting absorption, metabolism, and distribution of phytochemicals is likely to influence exposure at the tissue level. Likewise, genetic variation in cell signalling pathways, within which these compounds interact can alter biological response. Intestinal factors include variations in enzyme activity and colonic microflora (D'Archivio, Filesi, Varì, Scazzocchio, & Masella, 2010; Lampe & Chang, 2007). In recent years, an attempt has been made to establish the pharmacokinetics of tart cherry

phytochemicals (mainly anthocyanins;**Table 1**), the ensuing section will provide an overview of the disposition, through absorption, distribution, metabolism and excretion, of these and other tart cherry phytochemical compounds.

Table 1. Acute pharmacokinetics of tart cherry polyphenols

Study	Participants	***n***	**Delivery Mode and Timing**	**TPC** (~mg)	**TAC** (~mg)	**Metabolite of interest**	**C_{max}**	**T_{max}** (mins)
Bell *et al.* (2014a)	Healthy	12	30 ml MC concentrate 0-8 hours	-	273.5	C3G	0.05 ± 0.05 μg·L^{-1}	60
Bell *et al.* (2014a)	Healthy	12	60 ml MC concentrate 0-8 hours	-	547.0	C3G	1.28 ± 2.40 μg·L^{-1}	60
Seymour *et al*. (2014)	Healthy	12	45 IQF cherries (~187 g) -0.5-12 hours	13.0	-	a.C3GR b. C3R	a. 117,743 raw MS peak* b. 103,226 raw MS peak*	a. 120 b. 120
Seymour *et al.,* (2014)	Healthy	12	90 IQF cherries (~367 g) -0.5-12 hours	25.8	-	a.C3GR b. C3R	a. 183,873 raw MS peak* b. 122,582 raw MS peak*	a. 240 b. 120
Keane *et al.* (2016a)	Healthy males	12	30 ml MC concentrate 0-8 hours	71.4	31.2	a. PCA b. CHL c. VA	a. 2760 ± 100 μg·L^{-1} b. nd c. 300 ± 10 μg·L^{-1}	a. 60 b. nd c. 120
Keane *et al*. (2016a)	Healthy males	12	60 ml MC concentrate 0-8 hours	142.7	62.5	a. PCA b. CHL c. VA	a. 2750 ± 130 μg·L^{-1} b. nd c. 290 ± 30 μg·L^{-1}	a. 60 b. nd c. 60
Keane et al. (2016b)	Healthy males	16	60 ml MC concentrate 0-8 hours	178.8	73.5	a. PCA b. VA	a. 2350 ± 80 μg·L^{-1} b. 200 ± 10 μg·L^{-1}	a. 60 b. 60

Abbreviations; chlorogenic acid, CHL; cyanidin 3 glucoside, C3G; cyanidin-3-glucosylrutinoside, C3GR; cyanidin-3-rutinoside, C3R; individually quick frozen, IQF; mass spectrometry, MS; maximum concentration, C_{max}; Montmorency tart cherries, MC; Not detected, nd; protocatechuic acid, PCA; time to C_{max}, T_{max}; total anthocyanin content, TAC; total polyphenol content, TPC; vanillic acid, VA.
* = approximates (values not reported)

2.1.3.1 Absorption and distribution

The study of phytochemical absorption and distribution in the human body is crucial to determine those bioactive metabolites which might be responsible for any health benefits, however because polyphenols undergo extensive metabolism before they can be absorbed this remains poorly understood (Williamson, Kay, & Crozier, 2018). On the one hand, melatonin appears to be easily absorbed reaching relatively large concentrations in the serum, as per human bioavailability studies of melatonin-rich foods (Maldonado, Moreno, & Calvo, 2009; Sae-Teaw, Johns, Johns, & Subongkot, 2013). Whereas, although some MC anthocyanins are quickly absorbed from the stomach and small intestine, circulating in the plasma without undergoing metabolic changes and reaching maximum blood levels within one to two hours, these appear in small amounts which would suggest that tart cherry polyphenols exhibit poor bioavailability (**Table 1**). However, there are extensive differences between the absorption of polyphenols mainly because most are in the form of esters, glycosides, or polymers that cannot be absorbed in their intrinsic form. In fact, many compounds must be either deglycosylated in the mouth, hydrolysed in the stomach or enzymatically modified by intestinal enzymes (glycosidases, esterases, oxidases, and hydrolases; Phase I metabolism) before they can be absorbed into systemic circulation (Karaś, Jakubczyk, Szymanowska, Złotek, & Zielińska, 2017). Polyphenol aglycones may then enter the epithelial cells by passive diffusion as a result of its increased lipophilicity or alternatively polar glucosides are transported into the epithelial cells, possibly with the involvement of the active Na-dependent transporters, e.g. SGLT1 and GLUT2 (Teng & Chen, 2019).

A large proportion of dietary polyphenols, particularly those of large molecular mass, pass to the large intestine, where they are transformed by the gut microbes where they undergo microbial catabolism (Bohn et al., 2015). The level of biotransformations that polyphenols will undergo is dependent on the structure of: (a) the polyphenol and (b) intestinal microbial community. Subsequently, the gut microbiome (determined by the physiology of the host, host genotype and environmental factors) can potentially affect the metabolism of polyphenols and ultimately the distribution of any bioactive compounds (Zoetendal, Akkermans, Akkermans-van Vliet, de Visser, & de Vos, 2001). Ingested anthocyanins

predominately reach the large intestine, where they are catabolized by the microbiota, yielding an array of phenolic constituents that are absorbed, with some being metabolized to phase II conjugates as described in section 2.1.3.1 (Kay, Pereira-Caro, Ludwig, Clifford, & Crozier, 2017). *In vitro* studies have reported phenolic acids, such as protocatechuic (PCA), syringic, vanillic (VA), and p-hydroxybenzoic acids, to be main metabolites of anthocyanins after faecal fermentation (Aura et al., 2005; Keppler & Humpf, 2005). Both PCA and VA have been shown in the blood in larger concentrations than parent anthocyanins following ingestion of tart cherry concentrate (**Table 1**). However, only a limited number of metabolites have been studied to date, given the diverse nature of the phytochemicals previously described (**Figure 1**), larger more comprehensive studies of the absorption and distribution of tart cherry compounds are warranted, i.e. through the use of metabolomics.

Interestingly, because of their poor absorption in the upper gastrointestinal tract and anti-microbial actions, there is some evidence to suggest that some polyphenols, specifically anthocyanins, can act as prebiotics, modulating the microbiota (Coman et al., 2017). To what extent modulation of the gut microbial community is important in context of health benefits has yet to be fully elucidated. However, because microbiome dysbiosis has been associated with chronic diseases, such as cardiovascular disease, the importance of determining the relationship between diet and the gut bacteria is becoming more apparent (Moco, Martin, & Rezzi, 2012). Thus far, the interactions between human gut microbiome and tart cherry phytochemicals, likely important determinants of clinical efficacy, remains under-researched and human trials are equivocal with some evidence that modulation of microbiota may be specific to certain bacteria and is dependent on initial enterotype (Lear et al., 2019; Mayta-Apaza, Marasini, & Carbonero, 2017; Mayta-Apaza et al., 2018).

With regards to distribution, those studies summarised in **Table 1** demonstrate that a number of MC parent anthocyanins and their metabolites are absorbed and distributed, at least in the intravascular space. In rats, tissue bioavailability of certain cherry anthocyanin compounds (C3G, cyaniding-3-rutinoside, cyaniding-3-glucosylrutinoside, cyaniding-3-sophoroside, peonidin-3-glucoside and pelargonidin) has been investigated following 3 weeks of supplementation with either 1% or 10% individually quick frozen MC powder (Kirakosyan et al., 2015).

The authors demonstrated anthocyanin bioavailability in the bladder, kidney, liver, heart and brain, but not retroperitoneal fat. Moreover, there was a dose-response for the 1% and 10% group in the brain, 152.6 ± 9.7 vs. 337.8 ± 17.4 pg total anthocyanin content (TAC)·g^{-1} of tissue. Perhaps the most interesting finding of this study was that these compounds could cross the blood brain barrier and the preferential storage of certain phenolic compounds in these tissues. Collectively, there is some, but limited evidence that MC phytochemicals are absorbed and distributed into circulation and tissues, suggesting the ability of tart cherries to have biological effects. However, existing pharmacokinetic data is restricted to acute plasma studies, thus a gap in our understanding of the overall metabolism in these compounds following MC intake including excretion, and indeed the chronic exposure to these completely unexplored.

2.1.4.2 Metabolism and excretion

Other potential bioactive metabolites are those deriving from phase II metabolism of polyphenols in the liver. Compounds that are absorbed into the blood stream can undergo biotransformations such as sulphation, glucuronidation, methylation and glycine-conjugation, so that the resulting metabolites are nontoxic and become easier to excrete in urine or bile (Moco et al., 2012). Accordingly, previous human studies have demonstrated increases in urine anthocyanin metabolites, C3G equivalents and peonidin 3-glucoside equivalents following an acute dose of individually quick frozen (IQF) MC powder in 12 healthy humans (Seymour et al., 2014). Howatson and colleagues (2012) also demonstrated a significant increase in total urinary 6-sulphatoxymelatonin (a major melatonin metabolite) following a 7 day supplementation with MC concentrate (~85.2 μg·day^{-1} melatonin) compared with placebo. In addition, some compounds that are absorbed intact, without enzymatic modification, might still undergo metabolism in the liver, and those metabolites, can be recycled back into the intestine through the enterohepatic cycle, where further modifications may occur (Van Duynhoven et al., 2011). Therefore because of this potential enterohepatic as well as enteric recycling (in the small intestine) many polyphenols (Borges, Ottaviani, van der Hooft, Schroeter, & Crozier, 2018), including anthocyanins and their metabolites (Ferrars et al., 2014), exhibit a biphasic pharmacokinetic response (e.g. **Figure 2**). For example, serum and urine concentrations of anthocyanin and related conjugates was examined following a 500 mg bolus of

isotopically labelled cyanidin-3-glucoside ($^{13}C_5$-C3G) in 8 healthy males (Czank et al., 2013). The same authors reported large concentrations of some phase II conjugates in the serum and urine, up to 24 hours after supplementation, whereas previously described MC studies were limited to 8-12 hours.

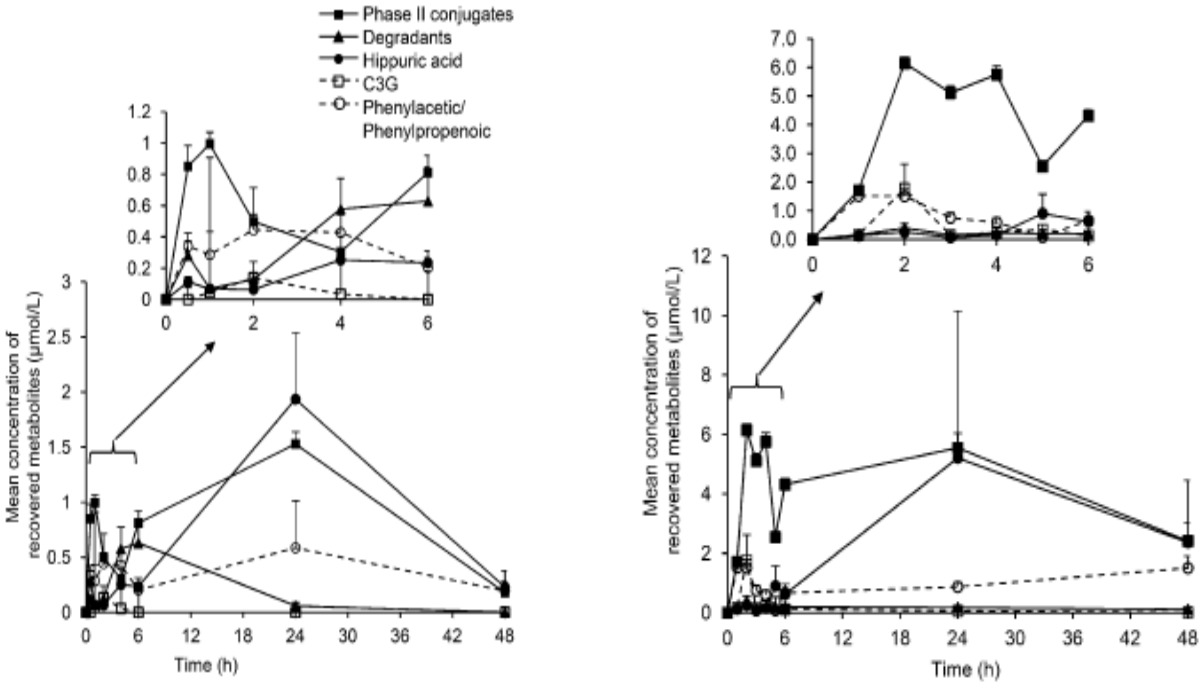

Figure 2. Serum (left) and urine (right) pharmacokinetics of cyanidin-3-glucoside (C3G) and phase II conjugates, after the consumption of a 500-mg bolus dose of isotopically labelled $^{13}C_5$-C3G by 8 healthy male participants over 48 h and expanded views of the first 6 h (Czank et al., 2013).

As of yet, there has been no attempt to determine the biliary excretion of phytochemical metabolites derived from the intake of tart cherries in humans, but based on previously described isotope studies, urinary excretion (of anthocyanins in particular) appears to be the primary route. Nonetheless, in a recent addition to the literature Mayta-Apaza and colleagues (2018) also established fermentation and degradation of other native tart cherry polyphenols through *in vitro* digestion. Bacterial degradation of tart cherry polyphenols resulted in high quantities of 4-hydroxyphenylpropionic and 3,4 and 4-hydroxybenzoic acids, which they speculated were derived from 5-caffeoylquinic acid and quercetin, respectively. However, these compounds have yet to be identified following ingestion of tart cherries in humans. Albeit, Mosele and colleagues (2015) demonstrated that a 4 week supplementation with 200 ml of polyphenol-rich pomegranate juice (968 mg of phenolic compounds) in 12 healthy volunteers

resulted in significant increases in faecal concentrations of phenolic metabolites; dihydroxy-urolithin, phenylpropionic acid, catechol, hydroxytyrosol and total phenolic metabolites ($mg \cdot g^{-1}$ dry faeces; $P < 0.05$). A limitation of this study is that no control group was used. In a more recent, randomized and controlled (33 case, 8 control) study of red wine, TPC: 1758 mg GAE$\cdot L^{-1}$, nontargeted metabolomics analysis after the 4-week intervention established microbial-derived red wine-metabolites in the faeces, but also high intra- and inter-individual variability of faecal metabolite content (Jiménez-Girón et al., 2014). Similar to tart cherries, both pomegranates and red wine are good sources of anthocyanins (Cassidy, 2017), suggesting that the intake of MC may result in faecal polyphenol catabolites, but human studies are needed to verify this.

The research summarised above demonstrates that phytochemicals are intensively modified and metabolised (**Figure 3**) and could be present in the body longer than 12 hours. To further illustrate this point the known metabolites from one anthocyanin (C3G) found in tart cherries are shown in **Table 2** below. subsequently, it is likely that the bioavailability of tart cherry phytochemicals has likely been underestimated in previous research. Similarly, another main limitation of the existing data regarding the bioavailability of tart cherry phytochemicals is they have often been restricted to single phytochemicals of interest, mainly anthocyanins (Keane et al., 2016a; Seymour et al., 2014). Although anthocyanins may be at least partly be having an effect there is need for a better understanding of a global perspective of exposure to MC. Since sustained exposure to anthocyanins and other phenolics may alter gene expression (Rodriguez-Mateos et al., 2019b) and metabolites represent endpoints of gene expression, the metabolome might better characterise global physiological effects following nutritional interventions. The use of metabolomics for example has become increasingly popular in nutritional research and showed that anthocyanin-rich foods increase circulating polyphenol metabolites that might be responsible for acute and chronic changes in health indices (Istas et al., 2019a; Jacobs et al., 2012). Once again highlighting the value of metabolomics in a study of MC intake, but to date this approach has not been utilised.

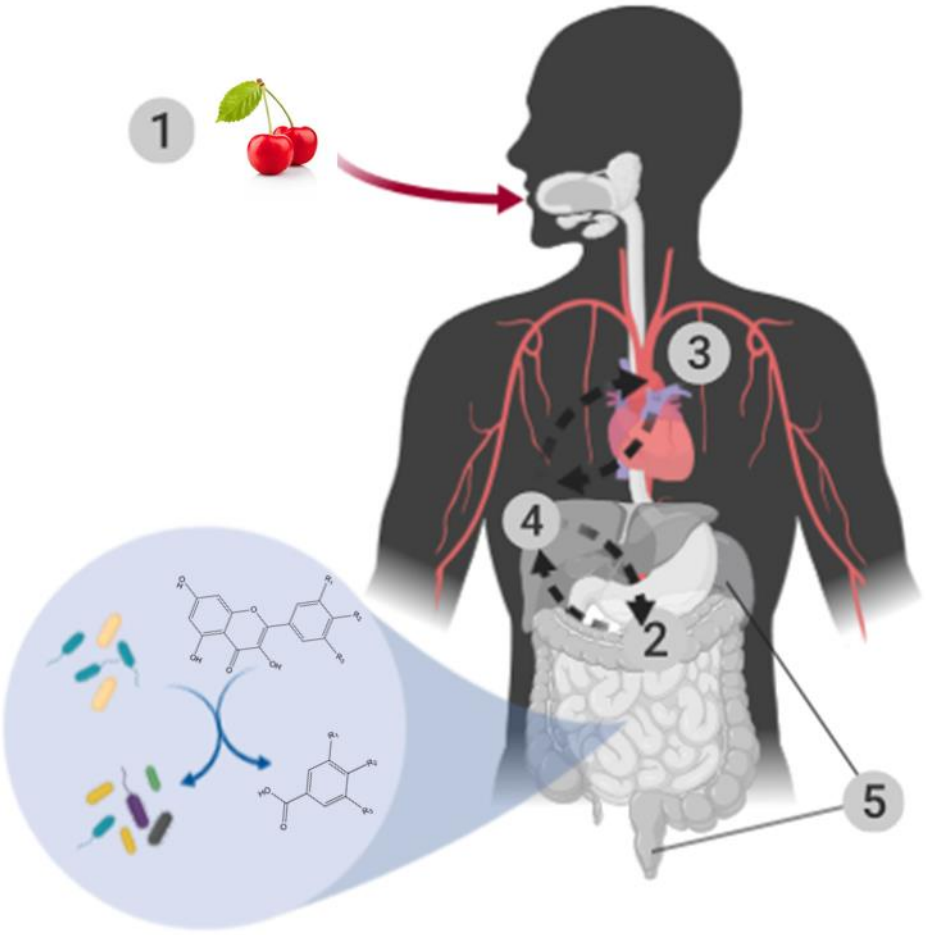

Figure 3. (Created with BioRender) Schematic representation of the fate of tart cherry phytochemicals: (**1**) ingested phytochemicals are liberated from food matrixes to become bioaccessible for absorption; (**2**) absorption, the movement of a compound from the site of administration through the gastrointestinal tract to the blood circulation, some compounds may undergo enzymatic modification in the mouth, stomach and small intestine (e.g. hydrolysis) or large intestine (host microbiome co-metabolism); (**3**) distribution, some compounds diffuse or are transferred from the intravascular (blood) to the extra-vascular space (body tissues) where they may elicit biological effect; (**4**) metabolism, the biochemical conversion or transformation of compounds into a form that is easier to eliminate (e.g. sulphation, glucuronidation, and methylation); and (**5**) excretion, unchanged compounds or metabolites are eliminated from the body, mainly via renal and to a lesser extent biliary pathways.

Table 2. Known metabolites from cyanidin 3-O-glucoside precursor

Metabolite	Biofluid identified in
3-Hydroxybenzoic acid	faeces, urine
4-Hydroxybenzoic acid	faeces, urine
Protocatechuic acid	faeces, serum, urine
Vanillic acid	faeces, serum, urine
3',4'-Dihydroxyphenylacetic acid	faeces, urine
4-Hydroxybenzaldehyde	faeces, urine
4'-Hydroxyphenylacetic acid	faeces, urine
Caffeic acid	Faeces
Ferulic acid	faeces, serum, urine
Hippuric acid	faeces, serum, urine
Peonidin 3-glucoside	liver, urine
2-Hydroxy-4-methoxybenzoic acid	faeces, urine
Protocatechualdehyde	faeces, urine
4-Methoxybenzaldehyde	Faeces
Cyanidin 3-glucoside glucuronide*	Urine
Cyanidin glucuronide	plasma, urine
Isopeonidin 3-glucoside*	Urine
Isopeonidin glucuronide*	Urine
Isovanillic acid	faeces, serum, urine
Isovanillic acid-3-O-glucuronide	faeces, serum, urine
Isovanillic acid-3-O-sulfate	faeces, serum, urine
Methyl-3,4-dihydroxybenzoate	faeces, serum, urine
Methylcyanidin 3-glucoside-glucuronide	Urine
Methylcyanidin glucuronide	Urine
Methylvanillate	Faeces
Peonidin 3-glucoside glucuronide*	Urine
Peonidin glucuronide*	Urine
Phloroglucinaldehyde	faeces, serum, urine
Protocatechuic acid-3-O-glucuronide	faeces, serum, urine
Protocatechuic acid-3-O-sulfate	faeces, serum, urine
Protocatechuic acid-4-O-glucuronide	faeces, serum, urine
Protocatechuic acid-4-O-sulfate	faeces, serum, urine
Vanillic acid 4-O-glucuronide	faeces, serum, urine
Vanillic acid-4-O-sulfate	faeces, serum, urine

Based on Phytohub database (Giacomoni et al., 2017)

*represents metabolites only identified in animal models

2.2 The role of tart cherries in human health and aging

Recent advances in technology and medicine have resulted in a universal aging population. For example, in the UK in the next 20 years it is estimated that almost a quarter of the population will be aged 65 or over (ONS, 2017). Although aging is an inevitable process, there are extensive inter-individual variability in health status and quality of life as humans grow older. This has originated the multidimensional concept of 'healthy aging', defined as not only longevity devoid of major chronic diseases, but also maintaining good cognitive, physical, and mental health (Rowe & Kahn, 1997). As the number of older adults is rapidly increasing, establishing strategies to facilitate healthy aging in these populations has become a research priority, with nutrition playing an integral role (Kiefte-de Jong, Mathers, & Franco, 2014).

One important challenge faced with aging is impaired vascular function (Thijssen, Carter, & Green, 2016). Poor vascular function is implicated in cognitive decline (Knopman et al., 2001), mood disorders (Paranthaman et al., 2010) and reduced physical and functional capacity that typically occur with advancing age (Poole, Behnke, & Musch, 2020). Moreover, endothelial dysfunction and arterial stiffening are independent risk factors of atherosclerosis and cardiovascular disease; CVD (Mudau, Genis, Lochner, & Strijdom, 2012; Niiranen et al., 2016), but are also associated with vascular aging. Additionally, vascular dysfunction might contribute to the genesis and progression of other age-related diseases such as type II diabetes and dementia (Dickstein et al., 2010; Wang et al., 2014a). However, the degree of deterioration in vascular function varies within older populations. Vascular aging is accelerated by other cardiovascular risk factors such as hyperglycaemia, hyperinsulinemia, hypertension, and dyslipidaemia (Rubin et al., 2012; Sowers et al., 1993; Terentes-Printzios et al., 2017) but is now considered a modifiable risk factor in itself (El Assar, Angulo, & Rodríguez-Mañas, 2013). Given, that vascular function appears to be a key mediator in human health and wellbeing, interventions that could improve these risk factors and help preserve vascular health, physical and cognitive function and reduce disease trajectory are of great interest.

Epidemiological data suggests that a greater anthocyanin intake is associated with reduced risk of type II diabetes (Rienks, Barbaresko, Oluwagbemigun, Schmid, & Nöthlings, 2018), all-cause mortality (Grosso et al., 2017) and greater

likelihood of better health and wellbeing in individuals surviving to older ages (Samieri, Sun, Townsend, Rimm, & Grodstein, 2014). As highlighted in the previous sections, MC are relatively rich in anthocyanins, which become bioavailable in the blood (see **Table 1**). More recently there has been emerging evidence that MC (possibly due to their anthocyanin content) can improve cardiovascular risk factors (Chai et al., 2018; Desai et al., 2019) and modulate vascular function (Desai et al., 2020; Keane et al., 2016b). This makes the expectation tenable that MC could have a putative role in cardiovascular, and subsequently overall health. Therefore, the ensuing sections will outline the current evidence pertaining to the influence of MC on human health, physical and cognitive performance, and disease.

2.2.1. Cardiovascular-protective properties of tart cherries

Cardiovascular diseases are comprised of a class of diseases that involve heart and systemic blood vessels. In coronary heart disease, cerebrovascular disease or peripheral arterial disease, impaired blood vessel function leads to an inadequate blood supply to organs (Mangge, Becker, Fuchs, & Gostner, 2014). Atherosclerosis is also the most common cause of these diseases and is preceded by endothelial dysfunction (Bonetti, Lerman, & Lerman, 2003). Under physiological conditions endothelial cells regulate vascular tone and structure, as well as vascular inflammation and thrombosis (Gonzalez & Selwyn, 2003). However, the aforementioned cardiovascular risk factors, as well as age, can alter endothelial function as a result of increased exposure and impaired ability for defence mechanisms to resist oxidative stress and inflammation (Thijssen et al., 2016). Initially reactive oxygen species (ROS) activate the endothelial cells stimulating expression of adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1), allowing mononuclear leukocytes, such as monocytes, to attach to the endothelium and infiltrate into the intima (Giannotti & Landmesser, 2007). Moreover, ROS can oxidise low-density lipoproteins (LDL) that have permeated the damaged endothelium. The monocytes differentiate into macrophages and internalize modified oxidised LDL (oxLDL) to become foam cells. These lipid-containing foam cells in the arterial wall can accumulate into atherosclerotic plaques or atheromas, which can cause hardening and narrowing of the vessels or rupture to cause adverse cardiovascular events such as thrombosis and even death (Gimbrone Jr & García-Cardeña, 2016).

Cardiovascular disease remains the most common cause of morbidity and mortality, increasing in prevalence with age (Townsend et al., 2016). Thus, improving cardiovascular health and delaying or preventing CVD would not only have important economic implications but would serve to promote healthy aging as described in the preceding section (Mooney & Mc Auley, 2016).

Although the exact biological mechanisms by which tart cherries might elicit cardiovascular-protection has yet to be fully elucidated it might relate to their antioxidant and anti-inflammatory actions, as well as the ability to interact with important cell-signalling pathways which will be summarised below (Kelley et al., 2018).

2.2.1.1 Tart cherry antioxidant and anti-inflammatory potential

Oxidative stress and inflammation, key mechanisms of endothelial dysfunction and arterial damage, link these risk factors to vascular aging and CVD (Guzik & Touyz, 2017). Moreover, there is inherent interplay between the two, as oxidative stress might be induced by inflammation, inflammation is also one of the most common outcomes of oxidative stress (Mukhopadhyay, Eid, Abdelmegeed, & Sen, 2018). As highlighted in section 2.2.1 vascular aging could be exacerbated by reduced resistance to ROS and inflammation, therefore natural antioxidant and anti-inflammatory agents might elicit cardiovascular protection. This section will outline the evidence for the antioxidant and anti-inflammatory potential of tart cherries.

Tart cherry phytochemicals, particularly flavonoids and melatonin, are potent antioxidants, due to their ability to scavenge oxygen free radicals and other reactive species. In particular, anthocyanins have been shown to act as more potent antioxidants compared to some other polyphenols (Pojer, Mattivi, Johnson, & Stockley, 2013). Subsequently, some but not all, species of tart cherry, including MC have been demonstrated to have high antioxidant activity (Wang, Nair, Strasburg, Booren, & Gray, 1999b; Wojdyło, Nowicka, Laskowski, & Oszmiański, 2014). Moreover, MC phytochemicals with the highest antioxidant capacity (e.g. kaempferol and melatonin) have been shown to have strong synergistic types of interactions (Kirakosyan et al., 2010) and importantly MC products; frozen and dried cherries, powders from individually quick frozen cherries and juice concentrate also had relatively high antioxidant capacity

despite industrial processing (Kirakosyan et al., 2009; Ou, Bosak, Brickner, Iezzoni, & Seymour, 2012a).

In murine models, intake of tart cherry juice was demonstrated to increase antioxidant enzymes, superoxide dismutase and glutathione peroxidase (GPx) compared to the controls (Sarić et al., 2009). In endothelial cells C3G has been shown to activate mitogen-activated protein kinase (MAPK), inducing Nuclear factor erythroid 2-related factor 2 (Nrf2), which in turn regulates a number of detoxification enzymes such as those described above (Speciale et al., 2013). Whereas, in humans longer-term supplementation with tart cherry juice compared to a control (with negligible phytochemicals) has been reported to increases in plasma antioxidant capacity in healthy middle-aged (Lynn et al., 2014) and older adults (Chai, Davis, Zhang, Zha, & Kirschner, 2019a). Moreover, these findings may be more pronounced in individuals exposed to increased oxidative stress, such as following; a high fat meal (Polley, Oswell, Pegg, & Cooper, 2019), ischemia-reperfusion (Traustadóttir et al., 2009) or exercise-induced muscle damage (Bell, Walshe, Davison, Stevenson, & Howatson, 2014b; Howatson et al., 2010), suggesting enhanced antioxidant capacity *in vivo.* As an aside, it should be acknowledged that there are extensive limitations with antioxidant assays because of the short half-life, low sensitivity, protein interference and that they are not inclusive of all antioxidant defence systems (Hollman et al., 2011). Therefore, the biological relevance of any direct antioxidant effects of tart cherry phytochemicals for human health based on the studies described above should be interpreted with a certain degree of caution. It should also be emphasised that phytochemicals are versatile bioactives rather than just mere antioxidants as will be described in the forthcoming sections.

Subclinical chronic low-grade inflammation has emerged as a cardiovascular risk factor and elevated pro-inflammatory biomarkers (such as interleukin-6 [IL-6], tumour necrosis factor-α[TNF-α] and C-reactive protein [CRP]) are associated with atherosclerosis and future cardiovascular events (Blake, Rifai, Buring, & Ridker, 2003; Haddy et al., 2003). Inflammatory cytokines, such as TNF-α and IL-6, promote adhesion of leukocytes to endothelial cells and cause an increase in vascular permeability (Maruo, Morita, Shirao, & Murota, 1992). Whereas CRP, secreted in response to IL-6, enhances clot formation, lipid oxidation and endothelial cell activation (Li et al., 2012). Inflammation is therefore fundamental

in atherogenesis through all stages of its development, from initiation, through progression to its thrombotic complication (Libby, 2012). Chronic inflammation is interrelated to vascular dysfunction as endothelial dysfunction stimulates inflammation and vice versa, chronic inflammation can promote endothelial dysfunction (Giannotti & Landmesser, 2007). However, chronic inflammation is a pervasive feature of aging (inflammaging) but is also aggravated by poor diet and other cardiovascular risk factors (Sanada et al., 2018). Moreover, inflammaging plays a critical role in the pathogenesis of age-related deterioration and diseases (Franceschi & Campisi, 2014). Hence anthocyanin-rich foods have become a focus of research due to their potential anti-inflammatory/immunomodulatory actions (Mena et al., 2014).

Tart cherries, potentially due to their anthocyanin content (Wang et al., 1999a), might elicit anti-inflammatory properties at both an enzymatic and transcriptional level. For example, MC powder has recently been shown to inhibit inflammatory enzymes, cyclooxygenase (COX)-1, COX-2 and lipoxygenase (LOX), *in vitro* (Kirakosyan et al., 2018). Interestingly, LOX inhibition was significantly higher than red raspberry, blueberry and grape extracts, despite reports that these fruits are richer in anthocyanins (Cassidy, 2017), once again suggesting possible synergy between MC phytochemicals. A dose-response inhibition of COX-2 has also been reported in mice fed with 10% and 50% tart cherry juice when compared with commercial pellet (Sarić et al., 2009). Moreover, MC derivatives have been shown to inhibit COX-1 activity *in vitro* with the MC concentrate exhibiting the greatest inhibitory effect, compared to frozen dried and dried cherries and our laboratory has previously reported higher anthocyanin content of the concentrate than the other analogues (Keane et al., 2016a; Ou, Bosak, Brickner, Iezzoni, & Seymour, 2012b). In addition to COX inhibitory activities, C3G and its metabolites have been shown to inhibit inducible nitric oxide synthase (iNOS) in macrophages, with PCA demonstrating the most potent inhibition compared to C3G and its aglycon (Min, Ryu, & Kim, 2010). These enzymes play a role in the pathophysiology of atherosclerosis by propagating the inflammatory process (Bishop-Bailey, Mitchell, & Warner, 2006; Poeckel & Funk, 2010). On a transcriptional level, Balstad and colleagues (2010) found significant suppression of the transcriptional factor, nuclear factor-kappa beta (NF-κβ), which regulates the expression of pro-inflammatory cytokines, in transgenic mice

fed tart cherry powder compared to those fed an isocaloric control. In male Zucker rats, 90 day supplementation with 1% tart cherry powder was also demonstrated to significantly reduce NF-κβ activity and plasma inflammatory markers, interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-α), compared to a calorie- and macronutrient-matched control diet (Seymour et al., 2009). In the pathophysiology of atherosclerosis, NF-κB is involved in the crosstalk between cytokines, adhesion molecules and growth factors, leading to atherosclerotic plaque formation, growth and eventual rupture and it has been identified as a potential therapeutic target for CVD (Dąbek, Kułach, & Gąsior, 2010). Nonetheless, despite some promising pre-clinical indications for the beneficial anti-inflammatory effects of tart cherries, the effects of this on humans, from a health perspective, has not received much attention until quite recently.

Collectively there is some evidence that MC influence inflammation in humans. This has predominantly been studied in the context of exercise recovery in which MC concentrate has repeatedly been demonstrated to reduce circulating IL-6 and CRP in response to strenuous exercise (Hill, Keane, Quinlan, & Howatson, 2020). Whereas, in health domains there have been limited studies investigating the effect of MC on inflammation, with the majority being published in the last few years. Moreover, despite the pre-clinical evidence for an anti-inflammatory effect the data from clinical trials remains inconsistent. For example, Lynn et al. (2014) reported no influence of 6-week supplementation with 30 ml MC (273.5 mg anthocyanins) on hs-CRP in healthy middle-aged adults. Similarly, 4-week supplementation with MC concentrate (296 mg anthocyanins) or MC powder (226 mg anthocyanins) consumed bi-daily had no influence on IL-6 or CRP in inactive middle-aged adults (Aboo-Bakkar et al., 2018; Lear et al., 2019). However, tart cherry juice was demonstrated to reduce TNF-α, but not IL-6 or CRP, in overweight and obese individuals in a 4 week randomised, placebo-controlled crossover study (Martin, Burrell, & Bopp, 2018). Furthermore, MC concentrate has been shown to reduce plasma CRP after 12 weeks in older adults (Chai et al., 2019a). In addition, a randomised double-blind crossover study, also reported significant reductions in serum high sensitivity CRP (hs-CRP) in osteoarthritic participants, following 6-week supplementation with MC juice (containing at least 30 mg of anthocyanins) compared to a placebo (Schumacher et al., 2013). Therefore, there is some evidence that tart cherries can reduce inflammation, but

it appears the most promising data comes from those in a pro-inflammatory state. Given the role of oxidative stress and inflammation in vascular aging and CVD, tart cherries through their antioxidant and/or anti-inflammatory actions could elicit cardioprotection.

2.2.1.2 Other tart cherry cardiovascular bioactivities

While earlier studies have focused on the antioxidant activities of purified anthocyanins it has become increasingly apparent in recent years that the cardiovascular activities associated with their intake are likely the result of circulating metabolites formed by chemical and microbial metabolism (see Section 2.1.3). For example, previous work from our research group has demonstrated that 60 ml of MC concentrate resulted in increases in simple anthocyanin metabolites, VA and PCA (Keane et al., 2016a). Moreover, when these two phenolic acids were administered to vascular smooth muscle cells (VSMC) in concert in physiologically relevant concentrations found in the blood, they increased cell migration, but not proliferation. This was speculated to be beneficial in blood vessel remodelling, however this was not specifically investigated. In addition, other studies have shown that C3G (a major tart cherry anthocyanin) increased endothelial nitric oxide synthase (eNOS) in human umbilical vein endothelial cells *in vitro* (Edwards, Czank, Woodward, Cassidy, & Kay, 2015). The same authors also demonstrated that physiologically relevant amounts of C3G phenolic metabolites; VA, PCA and syringic acid significantly reduced superoxide production, which might be attributable to modulation of nicotinamide adenine dinucleotide phosphate-oxidase (NOX-2). Upregulation of eNOS and reduced NOX-2 activity would favour increased nitric oxide (NO) bioavailability, by increasing production and reducing degradation by superoxide species. This is important because vascular NO released by eNOS is one of the major determinants of vascular tone and thus vasodilation. Additionally, NO released towards the vascular wall can protect against thrombosis by inhibiting platelet aggregation and adhesion to the blood vessels which is an early phase of atherogenesis (Jin & Loscalzo, 2010). Furthermore, decreased NO bioavailability has been associated with aging and pathological conditions including type II diabetes (Tessari et al., 2010), hypertension and CVD (Desjardins & Balligand, 2006). Conversely, increasing NO bioavailability has been proposed as a physiological target for nutritional approaches aiming to

mitigate age-related cardiometabolic and neurodegenerative diseases (Shannon, Stephan, Minihane, Mathers, & Siervo, 2018). Thus, the potential for MC anthocyanins to potentiate NO production and decrease NO degradation through their antioxidant potential is another potential mechanism by which they could elicit cardiovascular protective properties. Additionally, in a recent addition to the literature Rodriguez-Mateos and colleagues (2019b) reported that 4-week supplementation of blueberries resulted in an increase in 63 plasma metabolites and that 21 of them correlated with improved endothelial function and NO bioavailability following supplementation (as measured by flow mediated dilation; FMD). After nutrigenomic analysis it was shown that some of these anthocyanin metabolites influenced genes involved in regulation of cell adhesion, cell migration, inflammation, and cell differentiation processes (Rodriguez-Mateos et al., 2019a). Thus, it would appear that any cardiovascular activities associated with the intake of tart cherries are likely to involve multiple cell-signalling pathways such as those summarised above.

2.2.2. Modulation of vascular function in vivo after intake of tart cherries in humans

In line with evidence that tart cherry phytochemicals might have vasculo-protective properties, supplementation with MC has also been shown to improve vascular function *in vivo,* such as acute blood pressure (BP) modulation (Desai et al., 2019; Keane, Bailey, Vanhatalo, Jones, & Howatson, 2018; Keane et al., 2016b; Keane et al., 2016c). The most promising data comes from acute studies which suggests that 30-60 ml of MC concentrate can reduce systolic blood pressure (SBP) by ~7 mmHg approximately 2 hours after consumption as summarised in **Figure 4**. More recent evidence suggests a similar time frame may see the same transient improvements in augmentation index (AIx), a proxy measure of arterial stiffness (Desai et al., 2019). These measurements seemingly coincide with peak plasma levels of anthocyanins and their metabolites which suggest these compounds could be, at least partly, responsible (**Table 1**). However, the aforementioned studies have only shown transient reductions in SBP which return to baseline within 3-4 hours (Desai et al., 2019; Keane et al., 2016b) and evidence on whether these persist following sustained exposure is inconsistent.

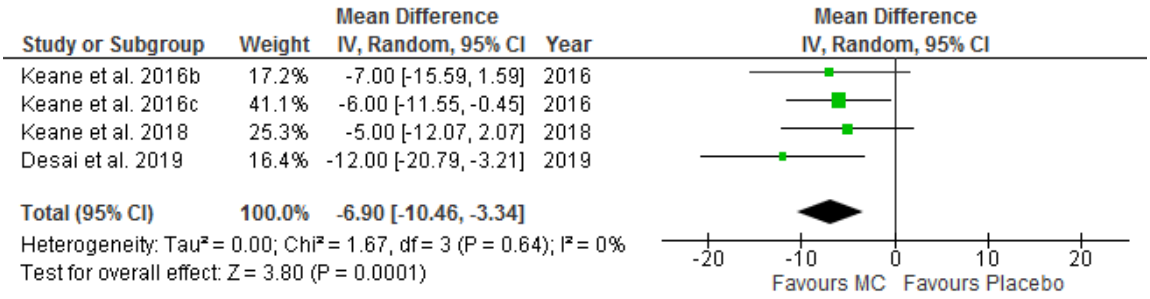

Figure 4. Random effects meta-analysis of peaks systolic blood pressure reductions following acute ingestion of Montmorency tart cherry concentrate or an isocaloric placebo. Studies included healthy participants (Keane et al., 2018; Keane et al., 2016c), males with early hypertension (Keane et al., 2016b) and participants with metabolic syndrome (Desai et al., 2019).

Whereas those studies above suggest an acute effect of MC on vascular function, mainly SBP, the research pertaining to the longer-term effects is inconsistent. The findings from existing studies are somewhat equivocal, as summarised in **Table 3**. There is tantamount evidence that tart cherries reduce SBP with longer-term (6-12 weeks) supplementation (Ataie-Jafari, Hosseini, Karimi, & Pajouhi, 2008; Chai et al., 2018), with other studies suggesting no effect (Johnson et al., 2020; Lynn et al., 2014). There are several factors that could influence the efficacy of tart cherry supplementation. For example, the baseline characteristics of the cohort as it is likely that those with higher SBP are more likely to be more amenable to anthocyanin interventions such as MC (Vendrame & Klimis-Zacas, 2019). In a recent addition to the literature Desai and colleagues (2020) found no effect of 6-day supplementation with 30 ml MC concentrate (270 mg anthocyanins) on SBP. However, the same authors reported that following 7 days ambulatory 24-hour readings of SBP, diastolic BP (DBP) and mean arterial pressure (MAP) were lower when compared to a placebo. These data could also call in to question the sensitivity of conventional BP measurements to detect changes that would undoubtably be small, particularly in individuals with measurements within the normal ranges. Alternatively, the findings could be conflicted by the shorter-duration and lower dose of MC concentrate used (30 ml). Similarly, there have only been few studies to investigate the influence of longer-term MC exposure on other markers of vascular function, such as arterial stiffness and endothelial function (Johnson et al., 2020; Lynn et al., 2014). As opposed to possible influences on AIx as previously mentioned, there have been no reported longer-term effects on these measures. Nonetheless, it is

inconclusive as to whether MC could influence endothelial function or arterial stiffness as previous studies have either been short of duration (Aboo-Bakkar et al., 2018; Desai et al., 2020) or used lower dosing strategies (Johnson et al., 2020; Lynn et al., 2014) than our research group have previously shown to be physiologically relevant. Certainly, there is some evidence that longer study periods (12 weeks) have shown an effect of foods abundant in anthocyanins on these markers (Istas et al., 2019b; Jeong et al., 2016; Zhu et al., 2011). Thus, further research is warranted given the major role of preserving vascular function in human health and disease.

Table 3. Summary of studies investigating the effects of longer-term tart cherry supplementation on vascular function

Study	Participants	*n*	Study design	Dosing	Anthocyanins (mg)	Baseline values		Key findings
						Cherry	Control	
Aitaie-Jafari et al. (2008)	Type II diabetes F	20	Quasi-experimental	40 g/day x 6 weeks	720	SBP: 129 ± 16 DBP:82 ± 8	–	↓SBP ↓DBP
Lynn et al. (2014)	Healthy middle-aged M/F	47	Parallel open-labelled controlled	30ml/day x 6 weeks	274	bkPWV: 8.2 ± 1.7 SBP: 111 ± 14 DBP: 70 ± 10	bkPWV: 8.0 ± 1.2 SBP: 110 ± 12 DBP: 67 ± 8	↔bkPWV ↔SBP ↔DBP
Desai et al. (2018)	Healthy younger M/F	11	Single-blind placebo-controlled, crossover	60ml/day x 20 days	540	SBP: 118 ±12 DBP: 74 ±10 HR: 65 ±11	SBP: 117 ±16 DBP: 73 ±10 HR: 65 ± 8	↔SBP ↔DBP ↔HR
Chai et al. (2018)	Older M/F	34	Parallel, double-blind, placebo-controlled	480ml/day x 12 weeks	NR	SBP: 141 ± 27 DBP:80 ± 17	SBP: 133 ± 15 DBP:78 ± 5	↓SBP ↔DBP
Aboo Bakkar et al. (2018)	Inactive middle-aged M	12	Double-blind placebo-controlled, crossover	1.7g/day x 4 weeks	226	FMD: 5.8 ± 1.0	FMD: 6.2 ± 0.8	↔FMD*
Martin et al. (2019)	Overweight/obese M/F	13	Placebo-controlled, crossover	240 ml/day x 4weeks	15.6	SBP: 123 ± 10 DBP: 77 ± 7	SBP: 128 ± 11 DBP: 84 ± 10	↔SBP ↔DBP
Johnson et al. (2020)	Metabolic syndrome M/F	19	Parallel, single-blind, placebo-controlled	480ml/day x 6 weeks	176	SBP: 124 ± 12 DBP: 77± 9 MAP: 94 ± 12 Hr: 63 ± 9 C-AP: 7 ± 3 C-SBP: 113 ± 12 C-DBP: 78 ± 9 C-MAP: 94 ± 12 AIx: 20 ± 6 AIx@75: 15 ± 9 cfPWC: 10.2 ± 1.5 baPWV: 12.8 ± 1.2	SBP: 128 ± 13 DBP: 78 ± 13 MAP: 96 ± 13 Hr: 65 ± 9 C-AP: 10 ± 6 C-SBP 118 ± 16 C-DBP: 79 ± 13 C-MAP: 96 ± 13 AIx: 24 ± 13 AIx@75: 19 ± 13 cfPWC: 11.0 ± 3 baPWV: 12.9 ± 1.9	↔SBP ↔DBP ↔MAP ↔HR ↔C-AP ↔C-SBP ↔C-DBP ↔C-MAP ↔AIx ↔AIx@75 ↔cfPWC ↔baPWV

						faPWV: 9.97 ± 0.9	faPWV: 9.3 ± 1.3	↔faPWV
Johnson et al. (2020)	Metabolic syndrome M/F	19	Parallel, single-blind, placebo-controlled	480ml/day x 12 weeks	176	SBP: 124 ± 12 DBP: 77± 9 MAP: 94± 12 Hr: 63 ± 9 C-AP: 7 ± 3 C-SBP: 113 ± 12 C-DBP: 78 ± 9 C-MAP: 94 ± 12 AIx: 20 ± 6 AIx@75: 15 ± 9 cfPWC: 10.2 ± 1.5 baPWV: 12.8 ± 1.2 faPWV: 9.97 ± 0.9	SBP: 128 ± 13 DBP: 78 ± 13 MAP: 96 ± 13 Hr: 65 ± 9 C-AP: 10 ± 6 C-SBP 118 ± 16 C-DBP: 79 ± 13 C-MAP: 96 ± 13 AIx: 24 ± 13 AIx@75: 19 ± 13 cfPWC: 11.0 ± 3 baPWV: 12.9 ± 1.9 faPWV: 9.3 ± 1.3	↔SBP ↔DBP ↔MAP ↔Hr ↔C-AP ↔C-SBP ↔C-DBP ↔C-MAP ↔AIx ↔AIx@75 ↔cfPWC ↔baPWV ↔faPWV
Desai et al. (2020)	Metabolic syndrome M/F	12	Single-blind placebo-controlled, crossover	30ml/day x 6 days	270	SBP: 134 ± 17 DBP: 75 ± 10 MAP: 98 ± 12 A-SBP: 124 ± 15 A-DBP: 81 ± 9 AP: 12 ± 7 AIx: 25 ± 14 AIx@75: 21 ± 12	SBP: 127 ± 16 DBP: 75 ± 10 MAP: 98 ± 12 A-SBP: 124 ± 12 A-DBP: 80 ± 7 AP: 11 ± 6 AIx:26 ± 11 AIx@75: 22 ± 10	↔SBP ↔DBP ↔MAP ↔A-SBP ↔A-DBP ↔AP ↔AIx ↔AIx@75
Desai et al. (2020)	Metabolic syndrome M/F	12	Single-blind placebo-controlled, crossover	30ml/day x 7 days	270	24h-SBP: 128 ± 10 24h-DBP: 76 ± 7 24h-MAP: 93 ± 9	24h-SBP: 128 ± 9 24h-DBP: 77 ± 8 24h-MAP: 94 ± 7	↓24h-SBP ↓24h-DBP ↓24h-MAP

Data is mean ± SD; **45 min after prolonged (20 min) occlusion, the FMD response was better preserved after MC vs. placebo*

Abbreviations: 24 hour ambulatory, 24h-; AIx normalised for Hr of 75 beats, AIx@75; aortic, A-; arterial pressure, AP; central, C-; augmentation index, AIx; brachial knee, bk; brachial-ankle, ba; carotid-femoral, cf; diastolic blood pressure, DBP; females, F; femoral-ankle, fa; flow mediated dilation, FMD; heart rate, Hr; males, M; mean arterial pressure, MAP; not reported, NR; pulse wave velocity, PWV; systolic blood pressure, SBP.

2.2.3 Effects of Montmorency tart cherries on metabolic health

Metabolic dysfunctions such as obesity, dyslipidaemia, insulin resistance, and hyperglycaemia are well established risk factors for both type II diabetes and CVD (Taube, Schlich, Sell, Eckardt, & Eckel, 2012). Moreover, aging *per se* is associated with significant metabolic changes, resulting in age-dependent increases in body weight, reduced insulin sensitivity, glucose tolerance and altered lipid metabolism (Liu & Li, 2015; Ryan, 2000). Thus, targeting metabolic health with respect to preventing or reversing CVD progression is an important aspect of a healthy aging phenotype.

Adverse lifestyle factors such as excess calorie intake and insufficient physical activity can accelerate these metabolic perturbations, whereas anthocyanin-rich foods might improve metabolic function (Yang et al., 2017). Moreover, epidemiological evidence suggests higher intakes of polyphenols, including anthocyanins, has been associated with lower risk of type II diabetes (Reis et al., 2016). This is paradoxical given the high sugar content of many anthocyanin-rich foods, e.g. 60 g per 100 ml and 50 g per 100 g for MC concentrate and freeze-dried blueberries, respectively. There is some pre-clinical evidence that MC polyphenols might influence metabolic health and body composition (Lachin, 2014; Seymour et al., 2009; Seymour et al., 2008). Most recently MC have been shown to increase the lifespan in *Caenorhabditis elegans* by altering metabolic pathways (Jayarathne, Ramalingam, Edwards, Vanapalli, & Moustaid-Moussa, 2020; van de Klashorst et al., 2020). These studies have suggested that tart cherries act as a calorie restriction mimetic and enhance mitochondrial function and reduce oxidative stress. Furthermore, tart cherries were shown to interact with the Peroxisome Proliferator-Activated Receptor (PPAR) signalling pathways (van de Klashorst et al., 2020). This has been shown in rodent models, for example Seymour et al. (2008) reported an increase in both PPAR -alpha (α) and -gamma (γ) mRNA in male Dahl rats supplemented with tart cherry extract for 90 days compared to the control. Upregulation of PPARs might indirectly provide cardioprotection because of their reported anti-inflammatory, anti-atherosclerotic and antidiabetic properties (Duan, Usher, & Mortensen, 2008). Thus, tart cherries and other anthocyanin rich foods might protect against and ameliorate the symptoms of cardiometabolic diseases associated with aging (Figure 5).

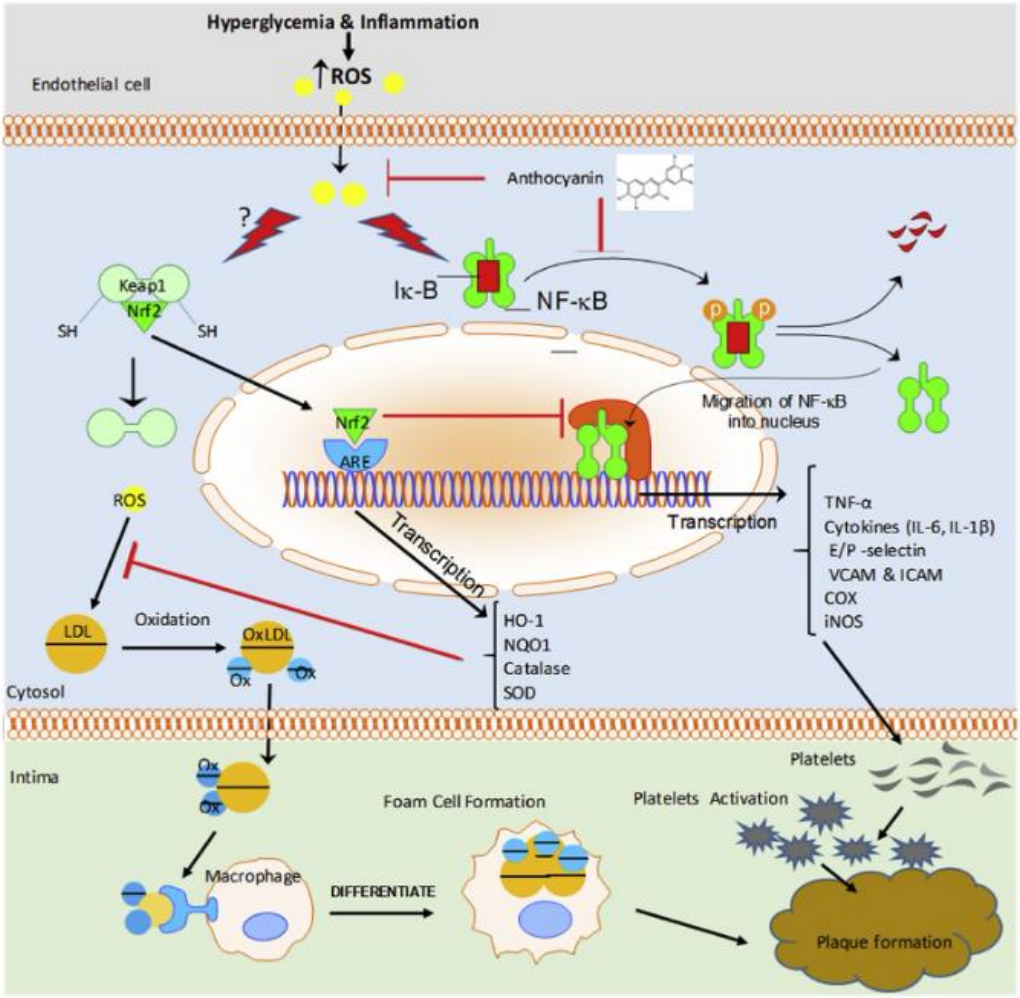

Figure 5. Potential anti-atherogenic properties of tart cherries in relation to other metabolic risk factors; taken from (Aboonabi & Aboonabi, 2020). Abbreviations: HO-1, heme oxygenase-1; NQO1, NAD(P)H, quinone oxidoreductase 1; SOD, superoxide dismutase; ROS, reactive oxygen species; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; IκBα, NF-κB inhibitor – alpha; IKK, IκB kinase; Nrf2, nuclear factor erythroid 2-related factor 2; ARE, antioxidant response element; Keep, Kelch-like ECH protein; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule 1; COX-2, cyclooxygenase-2.

Furthermore, through their potential prebiotic actions as demonstrated with *in vitro* and mouse models (Garcia-Mazcorro et al., 2018; Mayta-Apaza et al., 2018), tart cherry phytochemicals could also improve metabolic parameters. The observed changes in gut bacteria could be important because the microbiome in itself is a metabolic organ, but also through a myriad of poorly understood mechanisms might also influence insulin sensitivity, glucose tolerance and lipid profiles (Hansen, Gøbel, Hansen, & Pedersen, 2015). As described in earlier section, tart cherry phytochemicals could modulate the gut microbiota. For instance, in a recent meta-analysis, anthocyanins were shown to increase the microbial population of *Bifidobacterium* spp. and *Lactobacillus–Enterococcus*

spp. demonstrating their prebiotic actions (Igwe, Charlton, Probst, Kent, & Netzel, 2018). *Lactobacillus* strains of bacteria have been reported to alleviate aging-induced metabolic disorders in aged rats (Hor et al., 2019). Nonetheless, whether changes in microbial communities as a result of MC supplementation could translate to improved metabolic health remains unknown.

There have been few human trials determining the effects of MC on metabolic health. Desai et al. (2019) previously reported that a 30 ml of MC juice or capsules containing the same amount of anthocyanins (270 mg) acutely reduced serum insulin levels compared to an isocaloric placebo in individuals with metabolic syndrome. Interestingly, after consumption of the juice insulin was significantly lowered up to 3 hours post-prandially compared to a placebo. Moreover, the difference between the capsule and the juice was not significantly different refuting any superiority of capsules despite the limited sugar content (17.9 g vs. 0.1 g) and suggestions that inducing a glycaemic stress might outweigh any benefits (Desai et al., 2019). Nonetheless, as with the effects on vascular function the influence of longer-term tart cherry supplementation on indices of metabolic health are limited. The same authors reported no effects of 20 day supplementation with MC juice on glucose, triglycerides, total cholesterol in healthy individuals (Desai, Bottoms, & Roberts, 2018). Whereas in individuals with metabolic syndrome both short-term and long-term supplementation seem to improve some parameters of metabolic health (Desai et al., 2020; Johnson et al., 2020). For example, glucose, total cholesterol, LDL concentrations, total cholesterol:HDL ratio were reduced and there was a tendency for lower insulin levels following 6-day consumption of 30 ml MC concentrate (Desai et al., 2020). Following 12-week supplementation with 480 ml of MC juice participants had less oxidised LDL and a tendency for lower total cholesterol (Johnson et al., 2020). Moreover, Chai and colleagues (2018) reported reduced LDL cholesterol in older individuals using a similar dosing strategy. However, Martin and Coles (2019) reported no effect of 4 week MC supplementation (240 ml; 15.6 mg anthocyanins) on lipid profiles, glucose or insulin in overweight or obese individuals. Again, the limitations described in the previous section make it difficult to make any firm conclusions. However, because there is some evidence that tart cherries could improve indices of metabolic health, and because of the interplay between these markers and CVD (**Figure 5**), this area requires further attention.

2.2.4 The role of tart cherries on cardiovascular risk factors and cognitive function

As previously described maintaining good cognitive function and mental health is a key concept in healthy aging. However, gradual diminishment of cognitive functions commences in early adulthood and starts progressing more rapidly during mid-life (Harada, Love, & Triebel, 2013). Moreover, declining cognitive function and neurodegenerative diseases associated with aging has been linked to those previously mentioned cardiovascular risk factors (Dickstein et al., 2010; Grodstein, 2007). In particular, metabolic and vascular function dysfunction are well implicated in the pathophysiology of dementia and mood disorders (Ogle, Speisman, & Ormerod, 2013). Thus, interventions that could improve these factors, such as MC, could also have the propensity to improve cognition and mood and area of research that is now gaining research traction (Travica et al., 2020).

2.2.4.1 The influence of tart cherries on cerebral haemodynamics and cognitive function

Cerebral blood flow (CBF) and velocity regulates delivery of blood borne neural fuels, oxygen and glucose to the brain and thus is an important factor in optimal cerebral functioning (Ajmani et al., 2000). As such, compromised CBF due to vascular dysfunction and the resultant hypoperfusion to the brain has been suggested as a key contributor to cognitive function decline and a neurodegenerative diseases observed with advancing age (de la Torre, 2012). Thus, it is likely that dietary components such as polyphenols which have the potential to modulate of cerebral heamodynamics might influence cognition. As previously described in the preceding sections, MC polyphenols might elicit vasomodulatory effects, demonstrated by reduced SBP following consumption (Chai et al., 2018; Keane et al., 2016b) and this may translate into increased CBF. In accordance, previous work from this laboratory demonstrated that acute supplementation with the same dose of MC concentrate modulated CBF, measured indirectly by (NIRS) near-infrared spectroscopy (Keane et al., 2016c). This study found that a single bolus of MC concentrate resulted in increased blood volume (i.e. CBF; represented by total haemoglobin) and oxygen delivery (represented by oxygenated haemoglobin) in the prefrontal cortex toward the end of the 60-min resting/absorption period and during the cognitive assessment 1 hour post consumption. Acutely, this modulation in blood flow did not result in an

increase in cognitive function. However, our research group speculate that the population could have conflicted these results, as the participants were healthy with no apparent issues pertaining to cerebral blood flow or cognitive ability, therefore might not have benefited because CBF is already sufficient. This is further supported by the fact as opposed to the other parameters there was no observed difference deoxygenated haemoglobin, which essentially represents oxygen utilisation. To date this is the only study in tart cherries to try and match the CBF mechanism to cognitive outcomes.

This is surprising given that Kent and colleagues (2017a) who reported significant increase in verbal fluency, short term memory and long term memory, following 12 week supplementation with Bing sweet cherry juice, in participants with moderate dementia. Moreover, in a recent addition to the literature reported MC juice improved some aspects of cognitive performance, specifically visual sustained attention and spatial working memory, in healthy older adults without cognitive impairment after the same supplementation duration (Chai, Jerusik, Davis, Wright, & Zhang, 2019b). Importantly, both aforementioned studies found that post-supplementation SBP was reduced once again suggesting that the vaso-relaxatory properties might be at least partly driving this response, but the effects on cerebral haemodynamics were not measured making this only speculative. In contrast, has previously been demonstrated that regardless of dosing strategy cherry juice (presumably sweet; 55.8 mg anthocyanins) had no acute impact on cognitive function in young people or older people or dementia patients, despite influencing BP (Caldwell, Charlton, Roodenrys, & Jenner, 2016). Importantly, although to date there has been only one investigation following longer-term supplementation of MC on cognitive outcomes, the reported improvements in both vascular and cognitive function following longer-term anthocyanin supplementation are not in isolation (Ahles et al., 2020; Bowtell, Aboo-Bakkar, Conway, Adlam, & Fulford, 2017). In a recent addition to the literature Krikorian and colleagues (2020) reported improvements in semantic access and non-verbal memory cognitive performance following consumption of blueberries and this was correlated with urinary levels of blueberry anthocyanins suggesting these benefits are as a result of recurrent exposure to anthocyanins. It would therefore seem that the cerebrovascular response required to elicit measurable changes in cognitive function can be achieved with longer-term

anthocyanin dosing strategies that have the potential to induce sustained modifications in cognition but there is still ambiguity to whether these chronic changes are a result of enhanced CBF or some of the mechanisms as will be described in the ensuing section.

2.2.4.2 Other potential neuroprotective properties of tart cherries

Limited pre-clinical studies have shown tart cherry phytochemicals exert anti-neuro-inflammatory properties and to suppress neuronal apoptosis and stimulate pro-survival signalling cascades - mechanisms that might protect against cognitive aging (Kim, Heo, Kim, Yang, & Lee, 2005; Shukitt-Hale, Kelly, Bielinski, & Fisher, 2016; Thangthaeng et al., 2016). Recently, Thangthaeng and colleagues (2016) reported improvements in working memory, markers of inflammation (GFAP, NOX-2, and COX-2) and autophagy (phosphorylated mTOR, Beclin 1, and p62/SQSTM) in aged Fischer rats following 6 week supplementation with MC powder compared to the control. Moreover, pre-treatment with MC powder has recently been shown to reduce lipopolysaccharide-induced inflammation in HAPI rat microglial cells, as demonstrated by suppression of TNF-α and COX-2 *in vitro* (Shukitt-Hale et al., 2016). As previously described, tart cherry anthocyanins have also been demonstrated to cross the blood-brain-barrier where they might elicit protection on neuronal cells (Kirakosyan et al., 2015).

Besides their potential antioxidant and anti-inflammatory actions, tart cherries could stimulate cell signalling pathways involved in neuronal plasticity, and survival brain regions susceptible to degeneration. Notably, blueberries have also been reported to upregulate the MAPK-CREB-brain derived neurotrophic factor (BDNF) pathway in aged Lister-hooded rats (Williams et al., 2008). Williams et al. (2008) reported improved special working memory in the blueberry-supplemented rats following 12 weeks. The same authors speculated that anthocyanin stimulation of this cell signalling pathway in the brain was responsible for observed improvements in memory. Moreover, in rats fed blueberry powder or purified anthocyanins both resulted in improvements in spatial working memory to a similar extent (Rendeiro et al., 2013). These behavioural changes were paralleled by increases in hippocampal BDNF, suggesting a common mechanism for the enhancement of memory in accordance with the aforementioned study. However, unlike protein levels of

BDNF, the regional enhancement of BDNF mRNA expression in the hippocampus appeared to be predominantly enhanced by anthocyanins, suggesting a causal link between dietary anthocyanin intake and improved cognition. Although the polyphenol composition of blueberries and cherries differ, blueberries utilised in both studies contained both cyanidins and peonidins the major tart cherry anthocyanins (Rendeiro et al., 2013; Williams et al., 2008). This section is a brief synopsis of how tart cherries and their isolated components could influence cognitive function from existing *in vitro* and animal studies. These studies once again suggest interactions between dietary anthocyanins and multiple cell-signalling pathways might account for their potential neuroprotection, however, research involving humans is not as straight-forward. Given the role of good cognitive functioning in healthy aging future studies are needed.

2.2.5 The effects of tart cherries on exercise

Exercise capacity declines progressively with advancing age even in the absence of disease (Houghton et al., 2016). Moreover, risk of CVD is reduced by relatively small increases in exercise capacity (Kokkinos et al., 2009). Importantly, vascular function is a major determinant of exercise capacity in healthy individuals and vascular dysfunction predicates exercise intolerance and disease (Poole et al., 2020). Accordingly, foods rich in anthocyanins that could improve exercise capacity through improved vascular function have recently become a topic of interest (Cook, Sandu, & Joyce, 2020; Cook & Willems, 2019). Notably, increasing the capacity to exercise is an important issue, because the ability to perform physical activity to reduce co-morbidities and maintain health, as humans grow older, could help manage some of the multifaceted aspects associated with aging. Thus far, tart cherry research in the exercise domain has accumulated evidence for their application in recovery (Bell, Walshe, Davison, Stevenson, & Howatson, 2015; Howatson et al., 2010), however, only recently has focus turned to their effects on physical performance (Keane et al., 2018; Morgan, Barton, & Bowtell, 2019). Because of the possible interplay between tart cherries, exercise and health, the following section will outline the existing literature surrounding the use of tart cherries in exercise domains.

2.2.5.1 Tart cherries in Recovery

Exercise results in oxidative stress, inflammation and decreased muscle force production, the magnitude of which is dependent on the intensity and duration.

The high content of phytochemicals in tart cherries, via their antioxidant and anti-inflammatory effects, have been proposed to lessen muscle damage, reduce levels of pain, and ultimately improve recovery (Vitale, Hueglin, & Broad, 2017). For example, a series of investigations have demonstrated MC elicit recovery-promoting effects following endurance running (Dimitriou et al., 2015; Howatson et al., 2010; Kuehl, Perrier, Elliot, & Chesnutt, 2010b). Howatson et al. (2010) demonstrated faster recovery of isometric strength, measured as maximum voluntary isometric contraction, over a 48-hour period in marathon runners supplemented with tart cherries compared to the placebo group. The same authors also report significant reductions in inflammatory markers IL-6 and CRP and increased total antioxidant status in the cherry group. Similar findings regarding attenuation of muscle damage markers have been reported by others supplementing with MC (Dimitriou et al., 2015; Levers et al., 2015). Other benefits to tart cherry supplementation include reduced exercise-induced pain (Kuehl, Perrier, Elliot, & Chesnutt, 2010a) and potential attenuation of upper respiratory tract infections following prolonged exercise (Dimitriou et al., 2015), actions presumably also relating to their anti-inflammatory and antioxidant properties. Acceleration of recovery following tart cherry supplementation has also been reported in response to high-intensity and damaging eccentric exercise (Bell et al., 2015; Connolly, McHugh, Padilla-Zakour, Carlson, & Sayers, 2006; Levers et al., 2015), demonstrating a wide range of application for MC in exercise recovery.

This section although a caveat is important because it demonstrates existing research that tart cherries could directly or indirectly benefit exercise performance. Direct effects could involve the reduction of muscle fatigue and function. Whereas the indirect effects could include enhancement of training due to reduction of physiological stressors that negatively impact on training (illness or pain) and the improved recovery from previous exercise bouts.

2.2.5.2 Tart cherries in exercise capacity and performance

As previously mentioned the capacity to exercise is progressively reduced with age, likely because of concomitant impairments in vascular function (Houghton et al., 2016). Moreover, reduced physical activity as a result of decreased tolerance/capacity might perpetuate vascular dysfunction and predispose those individuals to cardiovascular and neurodegenerative diseases (Pandey et al., 2016). Thus, polyphenol-rich foods, such as tart cherries, are of great interest

because they might improve exercise capacity via several mechanisms. Firstly, through their previously described vasodilatory actions tart cherry supplementation might improve blood flow and tissue perfusion to the skeletal muscles (Morgan et al., 2019). Secondly, through their antioxidant capacity, because redox balance is imperative to muscle force production (Powers, Radak, & Ji, 2016), thus mitigating excess oxidative stress with MC supplementation might maintain muscle function during exercise. Lastly, some tart cherry anthocyanins have been shown to stimulate pathways associated with mitochondrial biogenesis (Matsukawa et al., 2017) and improved mitochondrial efficiency (Skemiene, Liobikas, & Borutaite, 2015). However, only in the last decade has research determining the efficacy of fruit-derived (including MC) polyphenols to enhance exercise tolerance and performance become an area of interest (Gao & Chilibeck, 2020; Myburgh, 2014; Somerville, Bringans, & Braakhuis, 2017).

Indeed, there is now accumulating evidence that polyphenols can improve exercise performance (Somerville et al., 2017). For example, Gao and colleagues (2020) conducted a meta-analysis on 10 studies that suggested that relatively short-term (1.5 hours – 7 days) tart cherry supplementation, in the concentrate or powdered form, improved endurance exercise performance (SMD: 0.36; 95% CI: 0.07 to 0.64; $p = 0.01$; $I^2 = 0\%$). This meta-analysis highlights several limitations with the existing literature in MC and exercise performance, for example of the studies included only 2 found significant improvements in half marathon time (Levers et al., 2016) and 15km cycling time (Morgan et al., 2019) but the others failed to show effects on overall performance, likely due to the small sample sizes of existing studies. Moreover, included studies adopt short-term dosing strategies in trained individuals which leaves the question of the longer-term effects of MC, and the potential for sustained consumption to benefit exercise capacity in an unconditioned cohort, entirely open. This important question to address for several reasons. Firstly, although an acute intake of tart cherries may influence vascular function, such as vasodilation (Keane et al., 2016b) and increased peripheral blood flow (Keane et al., 2016c) it has been speculated longer intake durations may be required to result in changes in cellular signalling including, but not limited to, those described above (Cook & Willems, 2019). Secondly, untrained individuals are likely to have deteriorated vascular and metabolic health

compared to their trained counterparts and therefore MC which could have a potential role in reducing the prevalence of age-related diseases by (i) improving these risk factors and (ii) increasing the exercise capacity and the ability to perform physical activity.

2.3 Summary of evidence

This overview summarises the recent additions to the literature regarding MC and other phytochemical-rich supplements on vascular function, cognitive and physical performance in the context of human health. Firstly, a number of studies have established that tart cherries and foods containing similar phytochemicals can increase circulating bioactive compounds (with much research directed towards anthocyanins and their downstream metabolites). Moreover, consumption of foods rich in these compounds have been shown to improve a number of risk factors associated with CVD, type II diabetes, and neurodegenerative disease. Particularly, here evidence for the vascular effects of tart cherries is the main focus. As highlighted, there is some evidence that MC may improve vascular function *in vivo* (**Table 3**). This has several implications in human health as; firstly, and foremost vascular dysfunction is implicated in the pathophysiology several age-related diseases, such as CVD. Secondly through improved vascular function this may in turn positively influence cognition and exercise capacity, which are important aspects of healthy aging, but there is a clear lack of studies examining the longer-term effects of MC on these indices. Despite some promising evidence of the influence of MC on vascular and metabolic function there is a paucity of original research in humans relating to their longer-term influence on cardiovascular and health in general. Moreover, existing clinical studies are small in size, of short duration, paradoxical and inconsistent. This is likely a result of the different populations and dosing strategies used as exampled in Table 3. While the exact physiological dose of MC have yet to be established based on previous work from our research institution 60 ml of MC concentrate has beneficial effects on vascular function and exercise performance/recovery (Brown, Stevenson, & Howatson, 2019; Keane et al., 2018; Keane et al., 2016b; Keane et al., 2016c). While other analogues and doses have recently been adopted 30ml given bi-daily appears to have the most consistent evidence (Bell et al., 2014b; Chai et al., 2018). In light of the limitations highlighted in this review, the overarching aim of this thesis is to

provide novel insight into potential role of dietary anthocyanins in cardiovascular health, with the specific aims outlined in Chapter 1.

Chapter 3:

Dietary intake of anthocyanins and risk of cardiovascular disease: a systematic review and meta-analysis of prospective cohort studies

Publication arising from this chapter: Kimble, R., Keane, K.M., Lodge, J.K. and Howatson, G., 2018. Dietary intake of anthocyanins and risk of cardiovascular disease: A systematic review and meta-analysis of prospective cohort studies. *Critical Reviews in Food Science and Nutrition*, **59**, 3032-3042.

3.1 Introduction

The average lifespan of humans is increasing, and with this there is an increase in mortality and morbidity from non-communicable diseases. Amongst mortality rates CVD remains the most predominant cause worldwide (Townsend et al., 2016). However, strong epidemiological evidence suggest that dietary factors, such as increased intake of F&V can reduce risk of CVD and premature death (Forouzanfar et al., 2016), which might in part be attributable to plant polyphenols. For example, higher intake of major classes of dietary polyphenols, e.g. anthocyanins, have been linked to reduced risk of type II diabetes (Guo, Yang, Tan, Jiang, & Li, 2016; Rienks et al., 2018), CVD and all-cause mortality (Grosso et al., 2017; Wang, Ouyang, Liu, & Zhao, 2014b). Anthocyanins (from the Greek *anthos*, a flower, and *kyanos*, dark blue) are important secondary plant metabolites that occur primarily as glycosides of their aglycone anthocyanidins. They are water-soluble pigments often responsible for the orange, red, and blue colours in fruits, vegetables, flowers, and other tissues in plants (Delgado-Vargas et al., 2000). Nonetheless, these compounds are not evenly distributed between fruits and vegetables, cherries in particular can account for almost a third of overall dietary intake of anthocyanins (Adriouch et al., 2018a; Tresserra-Rimbau et al., 2014a), but they are also abundant in other colourful berry fruits. As discussed in Chapter 2 there is accumulating evidence substantiates that anthocyanin-rich foods such as berries and cherries can favourably improve major risk factors of CVD (Fairlie-Jones, Davison, Fromentin, & Hill, 2017; Huang, Chen, Liao, Zhu, & Xue, 2016; Luís, Domingues, & Pereira, 2018).

Mechanistically, anthocyanins and their metabolites have been demonstrated to elicit cardiovascular-protective properties such as antioxidant, anti-inflammatory, anti-atherogenic and vasodilatory actions and there is now a growing body of evidence also supports that intake of dietary anthocyanins can improve functional vascular health *in vivo* (section 2.2.2). The potential underlying mechanisms for the aforementioned might involve the augmentation of endothelial-derived nitric oxide NO bioavailability. Anthocyanins can directly increase NO through upregulation of the endothelial nitric oxide synthase and L-arginine pathways, but also indirectly by potentiating the nitrate-nitrite-NO pathway, and minimising degradation of NO via their antioxidant actions (Edwards et al., 2015; Rocha, Nunes, Pereira, Barbosa, & Laranjinha, 2014). Nitric oxide is an integral molecule

in regulating endothelial homeostasis and anthocyanin-rich foods, including tart cherries, have previously been demonstrated to improve endothelial function (Aboo-Bakkar et al., 2018; Rodriguez-Mateos et al., 2013), which has been replicated in studies utilising purified anthocyanin compounds (Rodriguez-Mateos et al., 2019a; Zhu et al., 2011). However, despite promising evidence that these compounds might improve major risk factors of CVD, the relationship specifically between dietary intake of anthocyanins and actual risk of CVD has yet to be systematically evaluated. Therefore, this chapter addresses the specific aim; to synthesize and evaluate the relationship between dietary intake of anthocyanins and the risk of CVD and related mortality from prospective cohort studies.

3.2 Methods

3.2.1 Search strategy

This review followed the preferred reporting items for systematic reviews and meta-analysis (PRISMA) guidelines (Stroup et al., 2000). The protocol of this systematic review was pre-registered with PROSPERO, the International Prospective Register of Systematic Reviews [registration number: CRD42018089121]. A systematic literature search was performed using SCOPUS, MEDLINE (ProQuest), CINAHL (EBSCO) and Cochrane Library from inception until January 2018 (Appendix A). The search strategy was conducted using Boolean operations and formed from existing reviews of anthocyanin-rich foods (Fairlie-Jones et al., 2017), collated in three key concepts; (i) anthocyanins, (ii) cardiovascular disease and (iii) prospective study design. Furthermore, the reference list of retrieved systematic reviews and included studies were hand searched to find potential articles that could be included in the current meta-analysis. A follow-up search was conducted in December 2019 to identify any additional studies that had been published.

3.2.2 Study selection

Studies were included for analysis if they met the following inclusion criteria: 1) were a prospective study in adults (≥ 18 years) including; prospective cohort, nested case-control and case-cohort studies; 2) included population distribution of ≥ 2 intakes of anthocyanins or anthocyanin-rich foods as exposure; 3) reported fatal or non-fatal CVD events as outcome of interest [i.e. coronary heart disease, ischemic heart disease, coronary artery disease, angina, myocardial infarction, heart failure, cerebrovascular disease (ischemic stroke and haemorrhagic

stroke), peripheral artery disease, CVD mortality]; 4) reported relative risk (RR) or hazard ratio (HR) and their corresponding 95% confidence intervals (or sufficient data to compute them). Titles and abstracts were independently reviewed using Covidence systematic review software (Veritas Health Innovation, Melbourne, Australia) by two researchers (RK and GH). Only full texts that were published in English or had an existing translation were retrieved and examined.

3.2.3 Data extraction and quality assessment

Data was independently extracted into piloted forms by two investigators (RK and KMK) in accordance with the Meta-analysis of Observational Studies in Epidemiology (MOOSE) guidelines (Stroup et al., 2000). Any discrepancies were resolved by reviewing the original article. The following data was extracted from each study: the first author's last name(s), publication date, location, the cohort name, participant's age (at baseline) and sex, the follow up period, the method of dietary assessment and anthocyanin exposure range, type of outcome, number of cases, HR or RR and their corresponding 95% CI and any covariate adjustments. Quality of each study was evaluated using the nine-star Newcastle-Ottawa scale (Stang, 2010), which assesses three major domains; selection of the study group (0–4 stars), comparability (0–2 stars) and outcome in the cohorts (0–3 stars). A maximum of 9-stars could be awarded, where 0-3, 4-6 and 7-9 stars were regarded as low, moderate and high quality, respectively.

3.2.4 Statistical Analysis

A random-effects meta-analysis was conducted because of unexplained heterogeneity between studies, where the common measure was RR. All included studies used COX's models, therefore HR were directly considered as RRs. The multivariate-adjusted RR and standard errors (SEs; derived from their corresponding 95% CIs) were logarithm transformed. If individual studies reported RR for multiple outcomes of CVD, or were stratified by sex, the risk estimates were pooled by fixed-effects model and the summary estimate was used in the main meta-analysis (Aune et al., 2017).

Sensitivity analysis was performed by omitting one study at a time to evaluate the potential bias and robustness of the overall risk estimate. Heterogeneity between studies was determined by χ^2 by Cochran's Q test (significance level at $P_h < 0.10$) (DerSimonian & Laird, 1986) and the I^2 statistic. For the I^2 statistic, I^2 values ≤25%, ≤50%, ≤75% and >75% indicated no, little, moderate and significant

heterogeneity, respectively. Subgroup analysis was conducted to determine possible sources of heterogeneity. Subsequently, the effect of exposure, type of outcome, sex, location, sample size and follow-up length of the included studies evaluated. Potential publication bias was evaluated by Egger's test ($P < 0.10$) and visual inspection of funnel plots (Begg & Mazumdar, 1994). All statistical analysis was conducted using STATA v.16.0 (StataCorp, College Station, Texas, USA), $P < 0.05$ was considered significant unless otherwise stated.

3.3 Results

3.3.1 Literature search

The original literature search and study selection results are presented in **Figure 6**. There were 1689 studies initially identified by the search strategy. After exclusion of duplicates 1277 titles and abstracts were reviewed. Seventy-six full texts were retrieved and after irrelevant articles were excluded for; wrong exposure (29), wrong study design (22), wrong outcomes (5), not complete (1), not English (2); 17 articles remained. Two additional study was identified by hand searching reference lists. After re-running the search strategy an additional 12 full texts were screened and four found to fit the inclusion criteria.

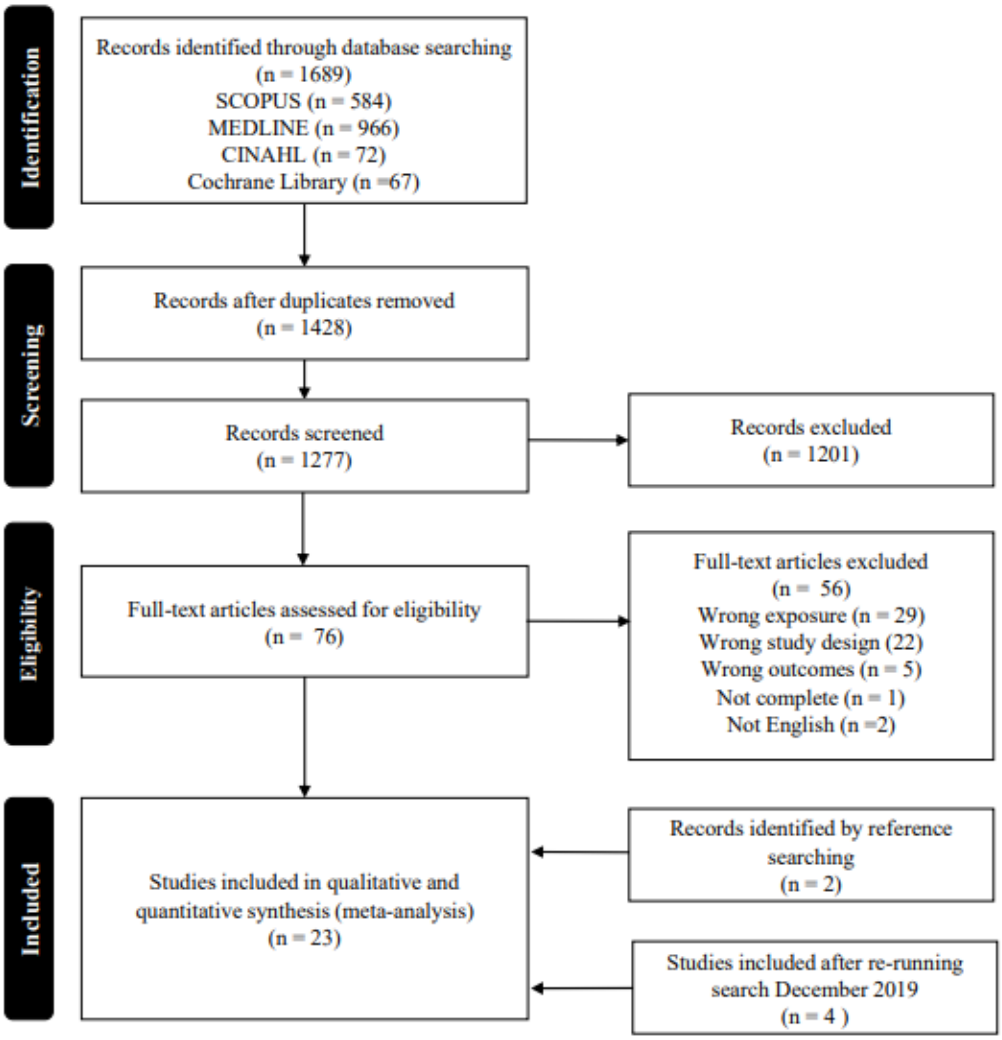

Figure 6. PRISMA flow chart for literature search and study selection.

3.3.2 Study characteristics

The characteristics of the included studies are presented in **Table 4**. Twenty-three studies involving 741,053 participants (aged between 25-81 years) with more than 37,054 incidences of non-fatal or fatal CVD were included in the current meta-analysis. All studies were published between 1996-2019, with seven studies conducted in USA, fourteen in Europe and two in Australia. Eight studies reported anthocyanin intake, eight studies reported anthocyanidin intake and seven studies reported berry intake. The follow-up length of the included studies ranged from 4.3 to 41 years. All studies provided covariate-adjusted risk models (e.g. age, sex, BMI, smoking). Quality scores were ≥ 6 stars (**Table 4**), two studies were moderate quality (6 stars) (Hirvonen et al., 2000; Ivey, Lewis, Prince, & Hodgson, 2013) and all other studies considered high quality (≥ 7 stars).

Table 4. Characteristics of prospective cohort studies on dietary anthocyanin intake and risk of cardiovascular disease

Reference	Country	Study cohort and no. of participants	Sex	Age range or mean (years)	Follow-up duration (years)	Exposure type, highest intake and assessment Method	Outcome and no. of cases	Covariate adjustments	Study Quality
Adriouch et al. (2018b)	France	NutriNet-Santé study 84158	Males/Females	44.1	4.9	**Anthocyanins** 55.0 (69.7) mg/d Web-based 24-h dietary records	Total CVD 602 CHD 309 Stroke 293	Age, BMI, physical activity, smoking status, numbers of dietary records, alcohol intake, energy intake, family history of CVD, educational level, and season of completion of 24-h dietary records	7
Bondonno et al. (2019a)	Denmark	Danish Diet, Cancer, and Health cohort study 52492	Males/Females	52-60	23	**Anthocyanins** 70.7 (53.2-396.9) mg/d FFQ	CVD mortality 4065	Age, sex, BMI, smoking status, physical activity, alcohol intake, hypertension, hypercholesterolemia, social economic status (income), diabetes, and prevalent disease, intakes of fish, red meat, processed meat, dietary fiber, polyunsaturated fatty acids, monounsaturated fatty acids, and saturated fatty acids	8

Bondonno et al. (2019b)	Australia	The Blue Mountains Eye Study 2349	Males/Females	64.7	14	**Anthocyanidins** 200.4 (155.5 - 747.8) mg/d FFQ	CVD mortality 548 CHD mortality 432	Age, sex, BMI, smoking status, physical activity, alcohol intake, hypertension, hypercholesterolemia and social economic status.	8
Cassidy et al. (2012)	USA	Nurses' Health Study 69622	Females	30-55	14	**Anthocyanins** >20.21 mg/d FFQ	Ischemic stroke 943 Hemorrhagic stroke 253 Stroke 1803	Age, BMI, physical activity, alcohol consumption, energy intake, use of multivitamin supplements, use of aspirin, menopausal status, smoking and history of Type 2 diabetes, coronary heart disease, hypercholesterolemia, or hypertension.	7
Cassidy et al. (2013)	USA	Nurses' Health Study II 93600	Females	25-42	18	**Anthocyanins** 25.1 mg/d FFQ	MI 405	Age, BMI, physical activity, alcohol consumption, energy intake, cereal fibre intake, fat intake, caffeine intake, use of aspirin, menopausal status, postmenopausal hormone use, oral contraceptive use, smoking, and family history of MI.	7

Cassidy et al. (2016)	USA	Health Professionals Follow-Up Study 43880	Males	32-81	24	**Anthocyanins** 26.3 mg/d FFQ	MI 863 Stroke 346 Ischemic stroke 200 Hemorrhagic stroke 48	BMI, physical activity, alcohol consumption, smoking, marital status, history of hypertension, history of hypercholesterolemia, quintiles of energy intake, cereal fibre, fat intake and folate, and family history of MI.	7
Dalgaard et al. (2019)	Denmark	Danish Diet, Cancer, and Health study 53552	Males/females	50-65	21	**Anthocyanins** 70 (53–397) mg/d FFQ	Total CVD 8773	Age, sex, body-mass index, smoking status, physical activity, alcohol intake, and social economic status (income), intakes of fish, red meat, processed food, polyunsaturated fatty acids, monounsaturated fatty acids, and saturated fatty acids.	8
Goetz et al. (2016)	USA	Reasons for Geographic and Racial Differences in Stroke 16678	Males/Females	>45	6.06	**Anthocyanidins** 24.7 (≥18.6) mg/d FFQ	CHD 589	Age, energy intake, sex, physical activity, smoking demographic factors (race and region of residence), socioeconomic factors (household income and educational attainment), energy intake from sweetened foods and beverages, reported beer, liquor and fat intake.	7

Hirvonen et al., (2000)	Finland	Alpha-Tocopherol, Beta-Carotene Cancer Prevention 26593	Males	50-69	5-8	**Berries** >49 g/d FFQ	Cerebral infarction 736 Subarachnoid haemorrhage 83 Intracerebral haemorrhage 95	Age, BMI, height, supplementation group, systolic and diastolic blood pressures, serum total cholesterol, serum HDL cholesterol, smoking-years, number of cigarettes daily, history of diabetes or coronary heart disease, alcohol intake, and education.	6
Hjartaker et al., (2015)	Norway	Norwegian Migrant cohort 10718	Males	33-73	41	**Berries** >8 servings/m FFQ	CVD mortality 4595 Stroke 1034	BMI, physical activity, beer, spirits, coffee, socioeconomic status (professional, administration, agricultural, industrial and other) and total smoking.	7

Ivey et al., (2013)	Australia	The Calcium Intake Fracture Outcome Age Related Extension Study 1063	Females	>75	5	**Anthocyanidin** >41 mg/d FFQ	CVD mortality 64	Age, energy intake, BMI, previous atherosclerotic vascular disease, energy expended in physical activity, previous diabetes, anti-hypertensive medication use, history of smoking and intakes of saturated fat, fibre, protein, starch, vitamin C and alcohol at baseline. Sensitivity analysis was performed by repeating logistic regression analysis in participants without previous atherosclerotic vascular disease and diabetes at baseline.	6
Jacques et al., (2015)	USA	Framingham Heart Study 2880	Males/Females	28-62	20	**Anthocyanins** 17.8 mg/d FFQ	Total CVD 518 CHD 261	Age, sex, current smoking status, BMI, total energy intake and fruit/vegetable intake.	8
Knekt et al., (1996)	Finland	The mobile clinic of the Finnish Social Insurance Institution 2748	Males	35-69	26	**Berries** ≥19 (<3) g/d Dietary history interview	CHD 324	Age, BMI, smoking, serum cholesterol and hypertension.	7

Knekt et al., (1996)	Finland	The mobile clinic of the Finnish Social Insurance Institution 2385	Females	35-69	26	**Berries** ≥24 (<7) g/d Dietary history interview	CHD 149	Age, BMI, smoking, serum cholesterol and hypertension.	7
Lai et al., (2015)	UK	UK Women's Cohort Study 30458	Females	30-69	16.7	**Berries** 15.4-365 g/d FFQ	CHD 138 Stroke 148 Total CVD 286	Age, BMI, physical activity, smoking status, socio-economic status, alcohol intake, total vegetable intake, and mutual adjustments for fruits that are not in the exposure category.	7
Larsson et al. (2013)	Sweden	Swedish Mammography Cohort and the Cohort of Swedish Men 74961	Males/Females	45-83	10.2	**Berries** 0.5 servings/d FFQ	Cerebral infarction 3159 Intracerebral haemorrhage 435 Subarachnoid hemorrhage 347 Stroke 4089	Age, sex, BMI, smoking status and pack-years of smoking, education, total physical activity, aspirin use, history of hypertension, diabetes, family history of myocardial infarction, and intakes of total energy, alcohol, coffee, fresh red meat, processed meat, fish, total vegetable consumption and mutually adjusted for total fruit consumption.	8

McCullough et al., (2012)	USA	Cancer Prevention Study II Nutrition Cohort 98469	Males/Females	69.5	7	**Anthocyanidins** 22.2 (≥16.7) mg/d FFQ	CVD mortality 2771	Age, smoking, beer and liquor intake, history of hypertension, history of cholesterol, family history of myocardial infarction, BMI, physical activity, energy intake, aspirin use, hormone replacement therapy (in women only), and sex (in combined model only).	8
Mink et al., (2007)	USA	Iowa Women's Health Study 34489	Females	55-69	16	**Anthocyanidins** 0.2 (0.01-1040) mg/d FFQ	CVD mortality 2361 CHD mortality 1329 Stroke 469	Age, energy intake, marital status, education, blood pressure, diabetes, BMI, waist-to-hip ratio, physical activity, smoking, and oestrogen use.	7
Mizrahi et al., (2009)	Finland	Finnish Mobile Clinic Health Examination Survey 3932	Males/Females	40-74	24	**Berries** 19-308 g/d Dietary history interview	Intracerebral haemorrhage 58 Ischaemic strokes 335 Stroke 625	Age, sex, BMI, smoking, physical activity, serum cholesterol level, blood pressure and energy intake.	8
Mursu et al., (2008)	Finland	Kuopio Ischaemic Heart Disease Risk Factor Study 1950	Males	42-60	15.2	**Anthocyanidins** mean 6.2 mg/d 4-day food diary	Ischaemic stroke 102 CVD Mortality 153	Age, examination years, BMI, systolic blood pressure, hypertension medication, serum HDL- and LDL-cholesterol, serum TAG, maximal oxygen uptake, smoking,	7

								CVD in family, diabetes, alcohol intake, energy-adjusted intake of folate and vitamin E, total fat (percentage of energy) and saturated fat intake (percentage of energy).	
Ponzo et al., (2015)	Italy	Local Health Units of the province of Asti 1658	Males/Females	45-65	12	**Anthocyanidins** mean 32.9 mg/d FFQ	CVD Mortality 84 Total CVD 125	Age, sex, BMI, education, living in a rural area, METs (hour/week), fibre, and saturated fatty acid intakes, alcohol intake, smoking, values of systolic and diastolic blood pressure, total and HDL cholesterol, fasting glucose, CRP, statin and aspirin use.	7
Sesso et al., (2007)	USA	Women's Health Study 38176	Females	54	10.1	**Strawberries** ≥2 servings/w FFQ	MI 289 Stroke 339 CVD mortality 204 Total CVD 1004	Age, randomized aspirin treatment, randomized vitamin E treatment, randomized beta-carotene treatment, and total energy intake, body mass index, exercise, alcohol intake, smoking, post-menopausal hormone use, and parental history of myocardial infarction 60 years, plus clinical factors: hypertension, hypercholesterolemia, and diabetes, plus dietary	7

								components related to strawberry intake: fruit and vegetables, fibre, folate, vitamin C, potassium, saturated fat, and total flavonoid intake.	
Tressera-Rimbau et al., (2014b)	Spain	Prevención con Dieta Mediterránea 7172	Males/Females	66	4.3	**Anthocyanins** 74.6 mg/d FFQ	Total CVD 273	Age, sex, smoking, BMI, alcohol, energy, physical activity, family history of CVD, aspirin use, antihypertensive drugs, cardiovascular drugs, and diabetes status, plus intake of proteins, saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids, and cholesterol.	7
Zamora-Ros et al., (2013)	Spain	European Prospective Investigation into Cancer and Nutrition 40622	Males/Females	29-70	13.6	**Anthocyanidins** >33.5 mg/d Computerized diet history questionnaire	CVD mortality 416	Age, sex, BMI, education level, physical activity, tobacco smoking, alcohol lifetime, total energy, vitamin C and fibre intakes.	7

Abbreviations: BMI, body mass index; CAD, coronary artery disease; CHD, coronary heart disease; CVD, cardiovascular disease; HDL, high-density lipoproteins; LDL, low-density lipoproteins; food frequency questionnaire, FFQ; METs, metabolic equivalents; MI, myocardial infarction.

3.3.3 Intake of anthocyanins and cardiovascular disease risk

The random-effects pooled results for fully adjusted risk estimates by type of CVD (**Figure 7**) showed an inverse association for intake of dietary anthocyanins and reduced risk of CVD mortality (RR = 0.91, 95% CI: 0.87, 0.95; $I^2 = 0.0$, $P_h = 0.772$) and CHD mortality (RR = 0.88, 95% CI: 0.78, 0.99) with no between-study heterogeneity ($I^2 = 0.0$, $P_h = 0.928$). However, there was no relationship between the intake of these compounds and reduced risk of MI, CHD, stroke or total CVD. Subgroup analyses can be found in **Table 5**. Dietary intake of anthocyanidins was associated with reduced risk of CVD mortality (*n* = 7), CHD mortality (*n* = 2), total CVD (*n* = 1) and CHD (*n* = 1). Anthocyanin intake was associated with reduced risk of CVD mortality (*n* = 1) only, whereas dietary intake of berries was not associated with reduced risk of any CVDs. Studies in female cohorts showed an inverse relationship between intake of these compounds and CVD mortality (*n* = 4), CHD mortality (*n* = 1) and CHD (*n* = 2) and those with combined sexes associated with reduced risk of CVD mortality (*n* = 5) and total CVD (*n* = 5). Total CVD was also reduced (RR = 0.84; 95% CI: 0.72, 0.98) in those studies located in Europe (*n* = 6). Studies conducted in Europe were associated with reduced risk of CVD mortality (RR = 0.93; 95% CI: 0.88, 0.99; *n* = 4) but was lower in those in the USA (RR = 0.89; 95% CI: 0.83, 0.96; *n* = 3). Risk of MI remained non-significant when stratified by exposure, sex, location, follow up and sample size. Stroke was further stratified by type (**Figure 8**), but no association was found between the intake of these compounds and reduced risk of cerebral infarction, ischaemic or haemmorrhagic strokes.

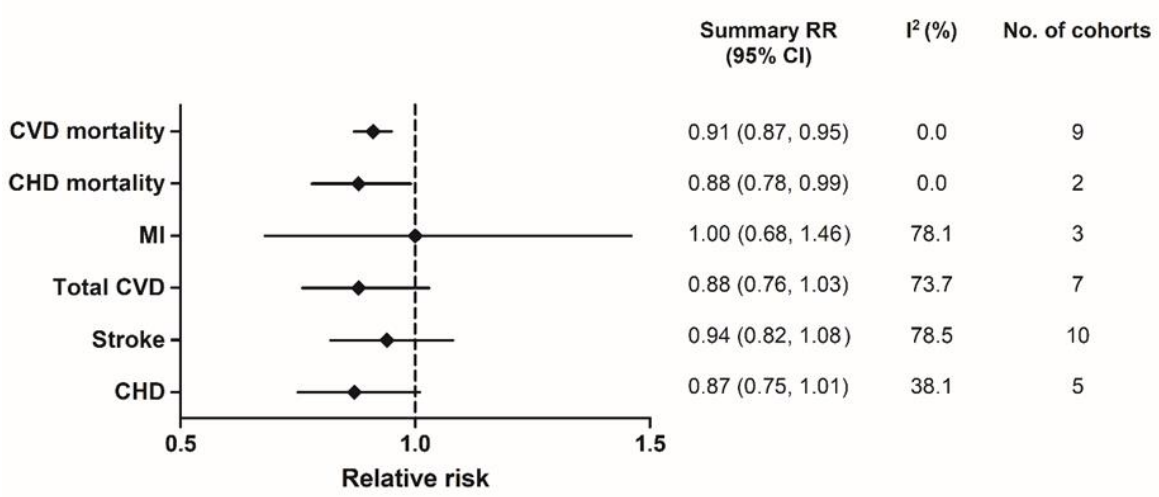

Figure 7. Random-effects analysis of fully-adjusted risk estimates of cardiovascular diseases (CVD) by highest versus lowest dietary anthocyanin intake. Summary relative risk for CVD mortality, coronary heart disease (CHD) mortality, myocardial infarction (MI), total CVD, stroke and CHD, respectively.

Table 5. Subgroup analysis of different cardiovascular diseases

	Outcome								
	CVD mortality			CHD mortality			MI		
Subgroup	Summary RR (95%CI)	I^2 (%)	No. of cohorts	Summary RR (95%CI)	I^2 (%)	No. of cohorts	Summary RR (95%CI)	I^2 (%)	No. of cohorts
Exposure									
Anthocyanins	0.90 (0.82, 0.99)	-	1				0.84 (0.60, 1.18)	74.5	2
Anthocyanidins	0.92 (0.87, 0.97)	0.0	7	0.88 (0.78, 0.99)	0.0	2			
Berries	0.76 (0.38, 1.53)	-	1				1.84 (1.04, 3.25)	-	1
Sex									
Males	0.92 (0.79, 1.07)	0.0	2				0.97 (0.87, 1.08)	-	1
Females	0.89 (0.82, 0.96)	0.0	4	0.88 (0.78, 0.99)	-	1	1.09 (0.41, 2.88)	88.5	2
Males/Females	0.91 (0.86, 0.97)	3.3	5	0.90 (0.56, 1.45)	-	1			
Location									
Europe	0.93 (0.88, 0.99)	0.0	4						
USA	0.89 (0.83, 0.96)	0.0	3	0.88 (0.78, 0.99)	-	1	1.00 (0.68, 1.46)	78.1	3
Australia	0.77 (0.54, 1.08)	0.0	2	0.90 (0.56, 1.45)	-	1			
Follow up									
≥10 years	0.91 (0.87, 0.96)	0.0	8	0.88 (0.78, 0.99)	0.0	2	1.00 (0.68, 1.46)	78.1	3
<10 years	0.67 (0.33, 1.35)	-	1						
Samples size									
≥10,000	0.78 (0.55, 1.10)	0.0	2	0.88 (0.78, 0.99)	0.0	2	1.00 (0.68, 1.46)	78.1	3
<10,000	0.91 (0.87, 0.96)	0.0	6						

Table 4 continued

	Total CVD			Stroke			CHD		
	Summary RR (95%CI)	I^2 (%)	No. of cohorts	Summary RR (95%CI)	I^2 (%)	No. of cohorts	Summary RR (95%CI)	I^2 (%)	No. of cohorts
Exposure									
Anthocyanins	0.88 (0.75, 1.03)	78.5	4	0.84 (0.62, 1.14)	91.0	3	0.88 (0.64, 1.18)	68.9	2
Anthocyanidins	0.56 (0.36, 0.88)	-	1	1.01 (0.83, 1.23)	-	1	0.73 (0.55, 0.96)	-	1
Berries	1.12 (0.80, 1.56)	33.5	2	1.00 (0.88, 1.14)	49.9%	6	0.95 (0.75, 1.21)	0.0	2
Sex									
Males				0.98 (0.87, 1.11)	0.0	2	1.21 (0.89, 1.64)	-	1
Females	1.12 (0.80, 1.56)	33.5	2	1.00 (0.89, 1.13)	0.0	4	0.64 (0.43, 0.95)	0.0	2
Males/Females	0.83 (0.70, 0.98)	79.5	5	0.86 (0.58, 1.29)	94.5	3	0.83 (0.65, 1.04)	65.5	3
Location									
Europe	0.84 (0.72, 0.98)	74.5	6	0.89 (0.71, 1.11)	86.8	6	0.92 (0.80, 1.06)	19.8	4
USA	1.27 (0.94, 1.72)	-	1	1.00 (0.91, 1.10)	0.0	4	0.73 (0.55, 0.96)	-	1
Follow up									
≥10 years	0.97 (0.86, 1.10)	59.6	5	1.03 (0.96, 1.10)	0.0	8	0.97 (0.87, 1.09)	0.0	3
<10 years	0.68 (0.53, 0.82)	0.0	2	0.71 (0.52, 0.98)	84.2	2	0.72 (0.58, 0.89)	0.0	2
Samples size									
≥10,000	0.93 (0.72, 1.21)	79.6	4	0.95 (0.82, 1.10)	80.9	9	0.72 (0.59, 0.89)	38.1	3
<10,000	0.75 (0.52, 1.09)	68.7	3	0.92 (0.73, 1.15)	-	1	0.98 (0.87, 1.10)	0.0	2

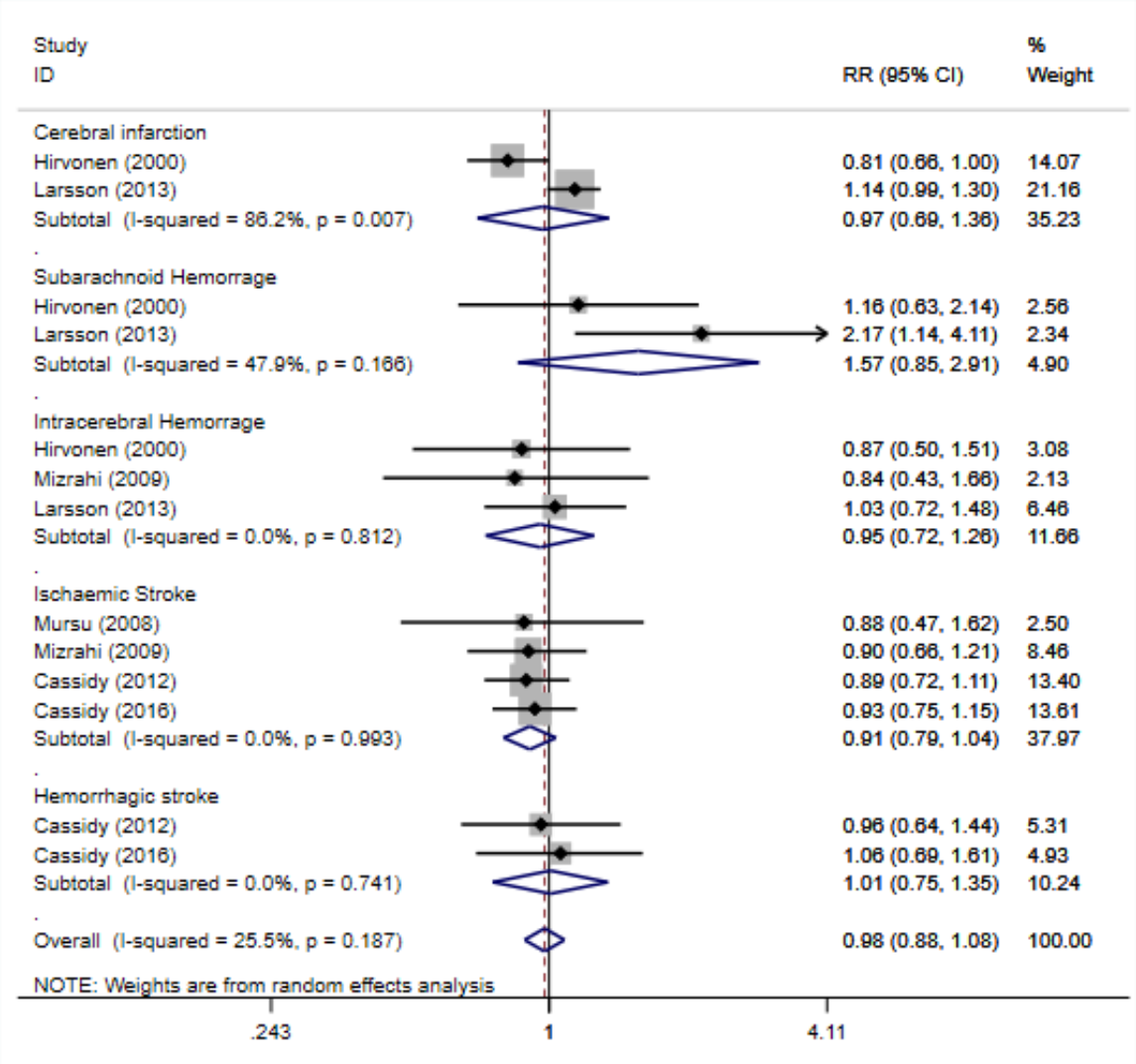

Figure 8. Random-effects analysis of fully-adjusted risk estimates of stroke by highest versus lowest dietary anthocyanin intake. Grey shaded area represents proportional weighting and the diamond represent summary relative risk for stroke subgroups.

3.3.4 Publication bias and sensitivity analysis

Egger's test suggested the absence of significant publication bias for CHD, MI, stroke and total CVD ($P \geq 0.178$). This was confirmed by visual inspection of the funnel plots (Appendix B) which showed no substantial asymmetry. There was weak evidence of potential publication bias for CVD mortality ($P = 0.057$). The sensitivity analysis determined no materially different risk estimates indicating stable results for CVD mortality or MI (Appendix B). However, there was some uncertainty between risk estimates of CHD, stroke and total CVD.

3.4 Discussion

The current meta-analysis of twenty-three prospective cohort studies is the largest, most comprehensive and contemporary to evaluate the association between dietary anthocyanin intake and risk of CVD. The findings indicate that dietary intake of anthocyanins is inversely associated with both CHD and CVD mortality (**Figure 7**), which is consistent with the findings of others (Grosso et al., 2017; Wang et al., 2014b). In the current meta-analysis, we found that those with the highest anthocyanin intake were associated with a 12% and 9% reduced risk of CHD and CVD mortality, respectively. This is of great interest because CVD remains the most common cause of death worldwide, accounting for 45% of all deaths in Europe (Townsend et al., 2016) and is estimated to cost healthcare services £15.7 billion in the UK alone (Luengo-Fernandez, Leal, Gray, Petersen, & Rayner, 2006). Our findings are of a similar magnitude to a recent meta-analysis that found that berry intake was associated with a significant reduction (RR = 0.92, 95% CI: 0.88, 0.97; $I^2 = 8.0$, $P_h = 0.34$) in all-cause mortality (Aune et al., 2017). Moreover, Guo and colleagues (2016) previously reported that risk of type II diabetes was reduced (5%) by increasing anthocyanin intake (7.5 mg/day; RR = 0.95; 95% CI: 0.93, 0.98) and berry intake (17 g/day; RR = 0.95; 95% CI: 0.91, 0.99), suggesting these compounds have a number of health benefits.

Conversely, we found no relationship between the intake of these compounds and the risk of stroke, MI or total CVD. This is surprising given that there is commonality in the aetiology of CVDs and a recent meta-analysis of randomised controlled trial showed and berry supplementation improved risk factors of CVD; specifically lipid profiles, systolic and diastolic blood pressure, which the authors speculate might be, at least partly, attributable to the anthocyanin content (Luís et al., 2018). A number of studies in the current meta-analysis reported berry intake as the exposure. Berries are a major source of anthocyanins, but these compounds are not uniformly distributed between these and other sources (Clifford, 2000; Horbowicz, Kosson, Grzesiuk, & Dębski, 2008). Importantly, because the included studies reported sources other than berries to be major sources of overall anthocyanin intake (Adriouch et al., 2018a; Ponzo et al., 2015; Tresserra-Rimbau et al., 2014b), perhaps the specific foods need more careful consideration. For example, total CVD was reduced in those cohorts from Europe

(**Table 5**) and foods such as red wine and cherries have been reported to be significant contributions of anthocyanindins in these cohorts (Ponzo et al., 2015).

Higher anthocyanin intake was not associated with reduced risk of strokes, even when further stratified by types of stroke (because of possible differences in underlying mechanisms; **Figure 8**). The reasons for the different risk estimates between anthocyanin intake and CVD mortality versus stroke remains unknown, but might be because 1) anthocyanins do not circulate the brain in large enough doses to elicit neurovascular protection, or 2) the cerebrovasculature is more susceptible to atherosclerosis because of the smaller vessel diameters (Mursu et al., 2008). Our findings are consistent with a recent meta-analysis that reported highest berry intake was not associated (RR = 0.98, 95% CI: 0.86, 1.12) with reduced risk of stroke (Aune et al., 2017). Previous meta-analytic evidence has suggested that increased polyphenol intake is associated with reduced risk of stroke (Tang, Li, Zhang, & Hou, 2016a). However, most of the prior epidemiologic evidence on flavonoids and CVD risk, specifically stroke, has focused on flavanones, flavones, flavonols, and flavan-3-ols (Tang et al., 2016a) that might have different bioactivities compared to anthocyanins. Interestingly, a number of these compounds are also found in tart cherries (**Figure 1**) and they have previously been shown to have synergistic and additive actions in combination with MC anthocyanins (Keane et al., 2016a; Kirakosyan et al., 2010) and protective properties against stroke related phenotypes (Seymour et al., 2013) against the findings of this review.

A strength of this meta-analysis was the inclusion of multivariate-adjusted risk models that adjusted for important confounding variables. However, a limitation of the included studies is that intake of anthocyanins is positively correlated to fruit and vegetable intake and the beneficial nutrients associated with these, such as other flavonoids, vitamins, carotenoids and fibre (Zamora-Ros et al., 2013). Thus, because of multicollinearity, and because only some studies adjusted for some of these as covariates (**Table 4**), it is not possible to isolate whether the observed reduction in risk estimates is solely in response to intake of dietary anthocyanins and not other bioactive compounds. Nonetheless, recent *in vitro* and human studies of anthocyanins and their relevant metabolites suggests that these compounds are responsible for some degree of cardiovascular protection (Fairlie-Jones et al., 2017; Reis et al., 2016; Rodriguez-Mateos et al., 2019a).

There are also a number of weaknesses in the available data that warrant discussion. Firstly, the majority of included studies in the current meta-analysis used FFQs to assess dietary intake of anthocyanin-rich foods and these may not always be comprehensive or reliable indicators of anthocyanin intake due to measurement error and misclassification. Secondly, there is inherent difficulty in determining the intake of these compounds because the databases have missing foods and limited information on retention of these compounds following cooking (Zamora-Ros et al., 2011), and there were major variations between the highest categories (**Table 4**). Moreover, studies were pooled which reported anthocyanidin, anthocyanin and berry intake indiscriminately (although they were later sub-grouped). This is thought to be appropriate because studies likely report anthocyanidin or anthocyanin based on database used (e.g. Phenol-explorer report anthocyanin content whereas USDA database reports just the aglycones (Haytowitz, Wu, & Bhagwat, 2018; Rothwell et al., 2013)) moreover C3G (a major anthocyanin) is a biomarker of berry intake in a dose-dependent manner (Sandoval-Ramírez et al., 2019). Nevertheless, the different associations between CVDs might be confounded by suboptimal quantification of dietary intake of anthocyanins (and because it was not possible to distinguish different types of these compounds), but could also be because of the limited number of studies that examined these variables (Jackson & Turner, 2017) or that the meta-analysis was less robust (as per the sensitivity analysis; Appendix B).

In summary, this systematic review and meta-analysis addressed the first specific aim of this thesis by synthesising and evaluating the relationship between dietary intake of anthocyanins and the risk of CVD and related mortality from prospective studies. Importantly this chapter has established empirical evidence that a higher dietary intake of anthocyanins is associated with reduced risk of both CHD and CVD mortality in cohorts of mostly middle-aged and older adults. Results from meta-analyses have to be interpreted with some caution given the limitations summarised above, however, the meta-analytical technique is currently the best method to systematically review previous work (Haidich, 2010) and hence adds to the body of knowledge through data synthesis. This data taken together with the findings of recent randomised controlled trials (Chai et al., 2018; Desai et al., 2019; Keane et al., 2016b) provides further support, that anthocyanin-rich foods (such as tart cherries) may have a putative role in cardiovascular health.

Moreover, there is existing evidence that these compounds might also reduce risk factors of other non-communicable (Guo et al., 2016) and neurodegenerative diseases (Devore, Kang, Breteler, & Grodstein, 2012) which from a public health perspective might help lessen the socioeconomic burdens associated with aging. This chapter provides additional rationale for longer-term randomised controlled trials determining the effect of anthocyanin intake on cardiovascular risk factors, to try and establish a causal link. Resultantly, this will be investigated in the future chapters of this thesis. Future work should try and establish the optimal dose-response and type of anthocyanins responsible for any reduced risk of CVD mortality.

Chapter 4:

The test-retest repeatability of a battery of non-invasive measures of vascular function *in vivo*

Publication arising from this chapter: Kimble, R., Keane, K.M., Lodge, J.K. and Howatson, G. (2019). Methodological Considerations for a Vascular Function Test Battery. *International Journal of Sports Medicine*, **40**, 601-608.

4.1 Introduction

Cardiovascular diseases (CVD) remains the leading cause of morbidity and mortality in men and women in the United Kingdom and other developed societies (Townsend et al., 2016). Thus, effective interventions and prevention strategies are needed to reduce CVD risk in these populations. Hypertension is amongst the top five most robust modifiable risk factors for CVD (Yusuf et al., 2004) and BP has been widely targeted by nutritional (including MC) interventions (e.g. Section 2.2.2.1). However, in recent years the prognostic and clinical utility of measurements of endothelial function and arterial stiffness, particularly in the context of vascular aging, have become increasingly more apparent (Anderson & Phillips, 2015). Moreover, because endothelial dysfunction and stiffening of the arteries precede hypertension, these measures collectively represent valuable nutritional targets in the prevention and management of CVD (Houston, 2018). Currently, there are now a number of non-invasive methods, that when used in combination, can determine both morphological and functional changes in the micro- and macro-vascular system that occur with age and these are being widely adopted in nutritional research (Desai et al., 2019; Keane et al., 2016b; Rodriguez-Mateos et al., 2013).

Importantly, endothelial function has been demonstrated to be responsive to both acute and chronic nutritional interventions, e.g. polyphenols (Fairlie-Jones et al., 2017; Kay, Hooper, Kroon, Rimm, & Cassidy, 2012). However, the effects of such interventions on arterial stiffness is less clear. For example, in a recent meta-analysis anthocyanin supplementation has been reported to improve arterial stiffness acutely, measured by pulse wave velocity (PWV), but not following chronic supplementation (Fairlie-Jones et al., 2017). Per contra, epidemiological evidence suggests that higher intake of these compounds is associated with reduced PWV (Jennings et al., 2012). Moreover, other indices of arterial stiffness, such as AIx have produced inconsistent findings following MC and indeed other anthocyanin-rich food consumption (Desai et al., 2019; Fairlie-Jones et al., 2017; Keane et al., 2016b).

In addition, despite the purported inter-relationship between endothelial function and arterial stiffness (Nigam, Mitchell, Lambert, & Tardif, 2003), these measures are not always measured concurrently. In studies where the aforementioned are measured together, improvements in some, but not all, measures are not always

observed, suggesting some might be more sensitive to interventions (Collier et al., 2008; Hobbs et al., 2013). These discrepancies demonstrate a clear benefit of being able to measure both endothelial function and arterial stiffness in a single testing session, i.e. as a 'test battery'. However, to be clinically useful, multimodal vascular function assessment needs to be sensitive (as highlighted in section 2.2.2.1) and repeatable, yet there is a paucity of data with regards to the reliability of multiple indices of vascular function taken in a single session (Ibrahimi et al., 2018; Woodman et al., 2005).

Woodman et al. (2005) reported the repeatability of a combination of common techniques used to assess peripheral and central arterial stiffness taken one week apart; however, some methods exhibited increased variability when taken in combination rather than when taken independently. For instance, the coefficient of variations (CVs) for the stiffness index (SI) derived from digital volume pulse (DVP) are typically less than 10% in healthy individuals (Millasseau, Kelly, Ritter, & Chowienczyk, 2002; Millasseau, Kelly, Ritter, & Chowienczyk, 2003), but were higher (20.7%) when taken together with other measures (Woodman et al., 2005). This study highlights the importance of assessing the repeatability of multiple measurements taken in a single testing session. In this instance, the assessment of arterial stiffness measures are calibrated by peripheral BP and are influenced by heart rate that would likely change depending on the testing duration (Mahe et al., 2017). In addition, commonly used methods to assess endothelial function such as laser Doppler imaging with iontophoresis (LDI) and flow mediated dilation (FMD) are reliant on NO release (Green, Dawson, Groenewoud, Jones, & Thijssen, 2014; Turner, Belch, & Khan, 2008) and NO might alter arterial stiffness (Sugawara et al., 2007). Therefore, measurements of endothelial function and arterial stiffness taken in a single testing session, which is becoming increasingly common, might exhibit different levels of repeatability than those taken independently. In this context, the aim to of this chapter addresses the second specific aim of determining the reliability of a battery of commonly used non-invasive measures of vascular function *in vivo.*

4.2 Methods

4.2.1 Participants and study design

A convenience sample of twenty-one healthy, normotensive, non-smoking males aged 21-46 years (Mean ± SD; stature: 180 ± 6.5 cm; mass: 79.9 ± 15.1 kg; BMI:

24.7 ± 4.0 kg/m^2) took part in this study. The study was ratified by the Northumbria University Research Ethics Committee and participants gave written informed consent prior to taking part. Participants were required to visit the laboratory on two separate occasions. For all testing visits they were required to arrive fasted (≥ 10h), avoid alcohol, strenuous exercise, any medication or nutritional supplements, 24 h before and caffeine 12h prior to each testing day. Vascular function was measured by a battery of tests, as described below, which were taken twice in the same session (intra-session; repeatability [*n* = 20], *n* =1 missing due to time commitments) and again a week later (inter-session; reproducibility [*n* = 21]) to determine the short-term reliability of these measures. The order of measurement was; automated BP, LDI, DVP, PWV, AIx measured by pulse wave analysis (PWA) and FMD, which took approximately 1.5 h to measure. To reduce the potential for variation, participants were required to wear loose fitting garments; whilst testing was performed in the same sequence on each participant, by the same researcher on each occasion. As LDI and FMD are both reliant on the release of NO, these measures were done at the beginning and end of the sequence, in contralateral arms. All vascular measures were taken at the same time of day (8 am ± 1 h) in ambient temperature (22 ± 2°C) with the participants in the supine position following a minimum of 5-minute acclimation period at the beginning of each test battery.

4.2.2 Vascular function assessment

4.2.3 Blood pressure

Blood pressure (BP) and heart rate (Hr) were measured using a validated (Reinders, Reggiori, & Shennan, 2006), non-invasive, automated vital signs monitor (Carescape V100; Dinamap) closely adhering to the guidelines specified by the European Society of Hypertension (O'Brien et al., 2003). Supine brachial BP measurements were taken with the arm supported at the level of the heart in triplicate. Three BP measurements were taken, each separated by 1 min , and the mean of the last 2 readings of SBP, DBP and Hr used for analysis (Pickering et al., 2005).

4.2.3 Laser Doppler imaging with iontophoresis (LDI)

All subjects had an acclimation period of at least 15 min before the measurements were taken. Two Perspex chambers (ION6, Moor Instruments Limited, UK) with an internal platinum wire electrode were attached to the skin using adhesive discs

(MIC-1AD; Moor Instruments Limited, UK) on the ventral aspect of the left forearm and connected to the iontophoresis controller (MIC2, Moor Instruments Limited, UK). A 2.5 ml volume of 1% acetylcholine chloride (ACh) dissolved in 0.5% NaCl solution was placed in the anodal chamber and the same volume of 1% sodium nitroprusside (SNP) in 0.5% NaCl solution was placed in the cathodal chamber (all reagents acquired from Sigma-Aldrich, UK). Circular glass coverslips (MIC-ION6-CAP; Moor Instruments Limited, UK) were placed over each chamber to prevent loss of solutions. Current delivery was controlled by a laser Doppler imager Windows software v.6.1 (Moor Instruments Limited, UK). Measurement of skin perfusion was carried out using a moor LDI2-IR laser Doppler imager (Moor Instruments Limited, UK). The scanner head was positioned 30 cm above the chambers. The laser beam was directed by a moving mirror in a raster fashion over both chambers. A total of twenty repeat scans were taken; the first set with no current to act as a control, then four scans at 5 µA, four at 10 µA, four at 15 µA and two at 20 µA, the final five scans were measured with no current. The back scattered light (flux) is measured in arbitrary perfusion units (PU) and area under the median flux PU vs. time curve over the twenty scans was calculated (using the trapezoidal rule) as a measure of microvascular response to ACh (endothelium-dependent vasodilation) and SNP (endothelium-independent vasodilation), respectively (**Figure 9**).

4.2.4 Digital volume pulse

A PulseTrace PCA 2 with a photoplethysmograph transducer transmitting infrared light at a wavelength of 940 nm (MicroMedical, Kent, UK) was placed on the index finger of the right hand and used to calculate the DVP stiffness index (DVP-SI) and DVP reflection index (DVP-RI). The DVP records the systolic and diastolic waveforms of the pulse by measuring infrared-light transmission through the finger. The DVP-SI (in m/s) is defined as the height of the subject divided by the time between the peaks of the first and the second wave, and it is correlated with the stiffness of large arteries (Millasseau, Ritter, Takazawa, & Chowienczyk, 2006; Woodman et al., 2005). The DVP-RI is the relative height of the second peak compared with the first and is associated vascular tone of small arteries (Millasseau et al., 2003).

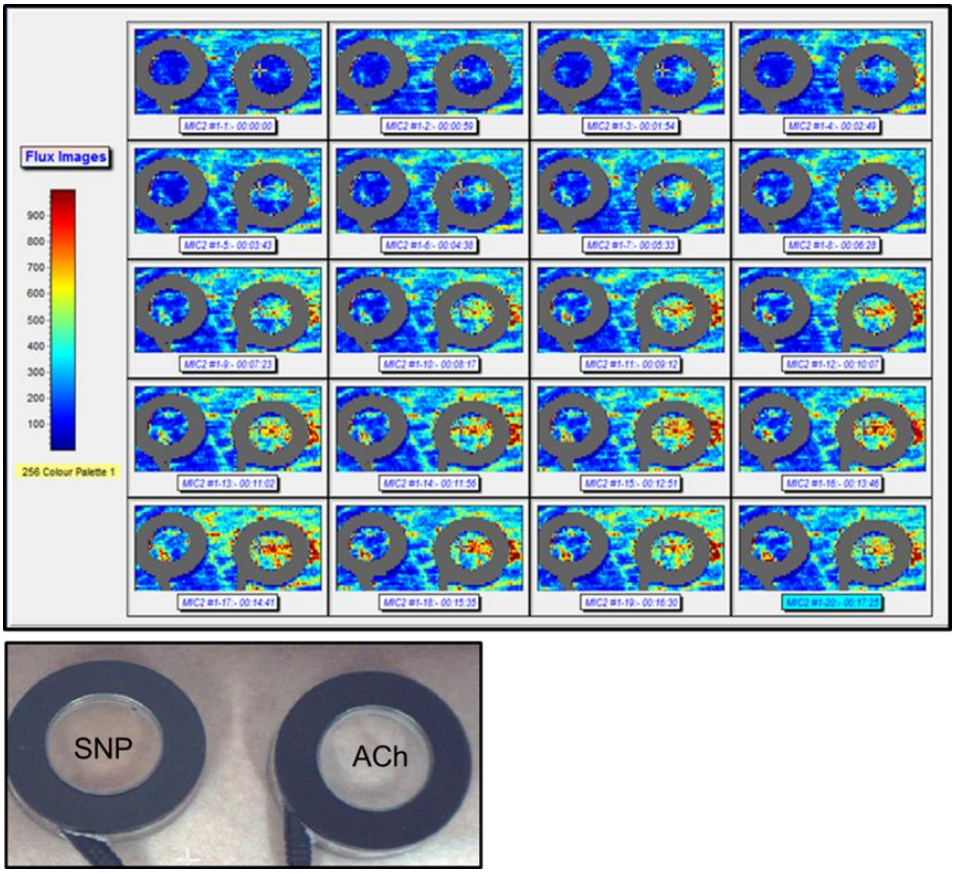

Figure 9. Analysis of LDI was determined by backscattered light intensity (flux) of specified regions of interest for SNP (Left circle) and ACh (right circle) over 20 scans. Flux is colour-coded with lowest perfusion in dark blue (0 perfusion units; PU) and highest in dark red (1000 PU).

4.2.5 Pulse wave velocity

The PWV was determined between carotid and femoral sites. A pencil-like pressure tonometer (SphygmoCor CPV system, ScanMed Medical, UK) was held at the base of the neck over the carotid artery and at the inguinal crease over the femoral artery on the right side of the body. The distance between carotid and femoral sites was measured and electrocardiogram gating permitted the time lapse between pulse waves at the carotid and femoral sites to be calculated. The PWV (in m/s) was calculated as the ratio of the distance between the two sites and the pulse transit time (PTT). Recordings were taken when a consistent signal was obtained with a high amplitude excursion. The SphygmoCor software (version 9.0, ScanMed Medical, UK) provides indices of quality control, if the measurement did not meet these control criteria, it was discarded and replaced

by a new measurement. A minimum of two acceptable readings were obtained for PWV and the average used for analysis.

4.2.6 Pulse wave analysis

The PWA was recorded at the radial artery using the same pencil-like pressure tonometer and software (SphygmoCor CPV system, version 9.0, ScanMed Medical, UK). Peripheral pulse waveforms were recorded for a minimum 11 s and the aortic artery waveform determined using a generalised transfer function (Pauca, O'rourke, & Kon, 2001). The AIx was calculated by the software as the: $\frac{augmentaion\ pressure}{pulse\ pressure} \times 100$; where augmentation pressure is the difference between the "shoulder" of the wave and "peak" systolic pressure. Since AIx is influenced by heart rate (Wilkinson et al., 2000), AIx normalised for a standard heart rate of 75 bpm (AIx@75) is also reported. The AIx@75 is only calculated when a participants heart rate is between 40 and 110 bpm, outside this range it is not computed by the software (Stoner, Young, & Fryer, 2012). A minimum of two acceptable readings were obtained for PWA (Quality Index ≥ 80%; pulse height ≥ 80 units; pulse height variation ≤ 5%; and diastolic variation ≤ 5%) and the average used for analysis.

4.2.7 Flow mediated dilation

Flow mediated dilation of the brachial artery was determined according to previously established guidelines (Corretti et al., 2002; Thijssen et al., 2010) using an ultrasound (HDI-5000 SONO CT ultrasound machine; Philips Medical System) and semi-automated computer software (Brachial Analyzer; Medical Imaging Applications; **Figure 10**). Briefly, a blood pressure cuff placed around the forearm (approximately 55 mm below the antecubital fossa), using a 7.5 MHz linear-array transducer, baseline images of the brachial artery were recorded for 60 s while the cuff remained deflated. The cuff was then inflated to 50 mmHg above systolic BP and following 5 min of occlusion, the pressure was rapidly released to allow reactive hyperaemia. Images were recorded for the last minute of occlusion and continuously for 3 min after release. Peak diameter was defined as the maximum diameter obtained post-occlusion. Average baseline diameter (60 s pre-occlusion) and the peak diameter were used to calculate the absolute change (peak diameter – baseline diameter) and percentage FMD: $\left(\frac{absolute\ change}{baseline\ diamater}\right) \times 100.$

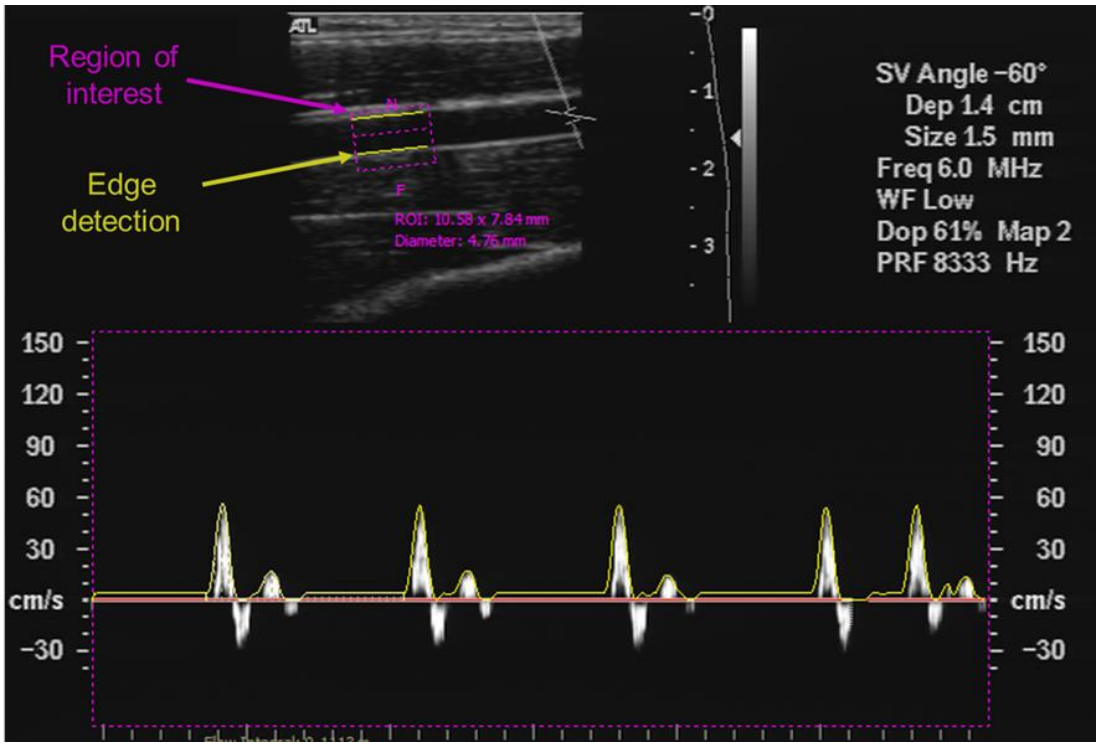

Figure 10. Analysis of brachial artery, regions of interest were defined (purple) and edge detection of the brachial artery diameter (top) and rate of flow (bottom) are shown in yellow.

4.2.8 Statistical analysis

All measures are expressed as mean ± standard deviation (SD) unless otherwise stated. Variables were tested for normality (Shapiro-Wilks test) and Log transformed where appropriate. For intra-session repeatability and inter-session reproducibility; relative consistency of all vascular measures was assessed using intra-class correlation coefficient ($ICC_{3,1}$(Hopkins, 2000)), where an ICC >90, 0.75-90, 0.50–0.75 and <0.50 indicate excellent, good, moderate and poor reliability, respectively (Koo & Li, 2016). Typical error (TE) was calculated as the between-subject SD of the measurement pairs divided by √2 to represent absolute index of repeatability that encapsulates both the random and systematic error associated with each measurement (Batterham & George, 2000). For non-negative values (Bedeian & Mossholder, 2000), the within-subject variability was also assessed using coefficients of variation (CV) for each pair of measurements, determined by using the following equation: $(\frac{SD}{mean}) \times 100$. A CV ≤ 10% was considered good, 10–25% moderate and ≥ 25% poor reproducibility (Tew et al., 2011). Paired samples *t*-tests or Wilcoxon's rank test were analysed for

systematic error (Atkinson & Nevill, 1998). All data were analysed using IBM SPSS statistics (v 24.0 for Windows; SPSS, Chicago, IL).

4.3 Results

4.3.1 Intra-session repeatability

Results for repeatability of all measures are presented in **Table 6**. Intra-session SBP, DBP and Hr showed a moderate-good level of repeatability (ICCs ≥ 0.73; CV ≤ 8.9%). Repeatability of LDI perfusion response to SNP taken within-session was poor (ICC: 0.34; CV = 28.4%). The AUC for ACh displayed a good level of consistency (ICC: 0.84) however CV was high. There was also evidence of systematic error for intra-session LDI-ACh. Repeatability of DVP-SI, DVP-RI, PWV, AIx and AIx@75 were moderate-excellent within-sessions (ICC ≥ 0.64; CV ≤ 9.5%). However, a consistent pattern of pulse waveforms could not be established in one participant, therefore all AIx data presented is for 19 participants. Additionally, three participants heart rates fell below 40 bpm and AIx normalised to 75 bpm was not calculated. Pulse wave velocity was highly repeatable within-session (ICC ≥ 0.89). For FMD two participants data was excluded from analysis, one due to substantial movement of the arm during deflation and the other because clear images of the artery could not be obtained (n = 18). Baseline diameter had excellent repeatability (ICC: 0.90; CV = 3.1%) and both FMD absolute and percentage FMD had a moderate level of repeatability (ICC ≥ 0.53).

4.3.2 Inter-session reproducibility

Inter-session reproducibility data is presented in **Table 6**. The SBP, DBP and Hr displayed a similar level of reproducibility between-sessions (ICC ≥ 0.73; CV ≤ 5.8%). Both LDI measures (ACh and SNP) displayed poor reproducibility inter-session (ICC ≤ 0.40; CV ≥ 32.7%). The DVP-SI was highly reproducible, whereas DVP-RI had poor consistency between measures (ICC: 0.17) despite only a moderate level of within-subject variability (CV = 15.1%). The PWV and AIx was highly reproducible (ICC ≥ 0.83), however paired samples t-test suggested systematic bias between inter-session AIx measures (P = 0.030). No systematic error was found for AIx@75, which remained highly reproducible between-sessions (ICC = 0.93). Absolute FMD and percentage FMD displayed a moderate-good level of reproducibility (ICC ≥ 0.71) and the reproducibility of the baseline diameter was again excellent (ICC = 0.90; CV = 2.7%).

Table 6. Intra-session repeatability and inter-session reproducibility of vascular function assessment

	Measure 1 (mean ± SD)	Intra-session					Inter-session				
		Measure 2 (Mean ± SD)	TE (raw units)	CV (%)	ICC	P value	Measure 3 (Mean ± SD)	TE (raw units)	CV (%)	ICC	P value
SBP (mmHg)	117 ± 6	116 ± 7	3.3	2.2	0.733	0.741	117 ± 6	3.0	1.9	0.753	0.385
DBP (mmHg)	65 ± 7	66 ± 7	2.9	3.5	0.812	0.113	64 ± 5	3.0	3.4	0.734	0.256
Hr (BPM)	56 ± 11	52 ± 11	4.8	8.9	0.815	0.006	58 ± 10	4.2	5.8	0.846	0.318
LDI-ACh (AUC; PU)	2125 ± 1494	1664 ± 1147	542	29.4	0.837	0.007	2459 ± 1543	1265	36.7	0.306	0.566
LDI-SNP (AUC; PU)	2262 ± 1271	1985 ± 1026	946	28.4	0.339	0.433	2488 ± 1150	943	32.7	0.395	0.274
DVP-SI (m/s)	5.5 ± 0.6	5.6 ± 0.5	0.2	2.7	0.876	1.000	5.5 ± 0.5	0.3	3.3	0.763	0.927
DVP-RI (%)	59.6 ± 13.3	61.0 ± 14.9	8.6	9.5	0.635	0.839	63.3 ± 13.1	11.9	15.1	0.173	0.337
PWV (m/s)	6.0 ± 0.9	6.0 ± 0.7	0.3	3.1	0.894	0.319	5.8 ± 1.1	0.4	6.2	0.825	0.149
AIx† (%)	-0.4 ± 11.3	-1.2 ± 12.0	3.2	–	0.926	0.156	-2.2 ± 11.3	2.5	–	0.955	0.030
AIx @75† (%)	-13.0 ± 11.9	-12.9 ± 12.5	3.4	–	0.920	0.483	-13.7 ± 11.6	3.1	–	0.932	0.541
FMD (%)	8.2 ± 2.2	8.8 ± 2.9	1.8	16.6	0.528	0.391	8.6 ± 2.8	1.3	14.2	0.714	0.408

†CV is not reported for AIx or AIx@75 as these produce both negative and positive values.
Systolic and diastolic blood pressure (SBP and DBP); heart rate (Hr) laser Doppler imaging with iontophoresis (LDI); acetylcholine chloride (ACh); sodium nitroprusside (SNP); perfusion units (PU); area under the curve (AUC); digital volume pulse (DVP); stiffness index (SI); reflection index (RI); pulse wave velocity (PWV); augmentation index (AIx); flow mediated dilation (FMD); absolute (Abs); typical error (TE); coefficient of varation (CV); intraclass correlaction (ICC)
For intra-session SBP, DBP, HR, LDI-ACh and LDI-SNP (n = 20), DVP- SI, DVP-RI and AIx (n = 19); PWV, percentage FMD (n = 18); and AIx@75 (n = 16)
For inter-session SBP, DBP, HR, LDI-ACh, LDI-SNP, DVP-SI and DVP-RI (n = 21); PWV and AIx (n = 20); percentage FMD (n = 19); and AIx@75 (n = 17)
Reasons for excluding data from analyses include: technical problems with equipment (DVP), no consistent pattern of waveforms (PWV and AIx), substantial arm movement (FMD) and heart rate <40 bpm (AIx@75)

4.4 Discussion

The aim of the current study was to determine the repeatability and reproducibility of a battery of vascular function tests. The main finding of this study was that taken together, there is a considerable range of variability regarding the repeatability and reproducibility (poor-excellent; **Table 6**) of non-invasive measures of endothelial function and arterial stiffness, which holds important information for the future experimental chapter in this thesis. Before comparing the reliability of a vascular function test battery to measures taken independently, there is a number of important factors relating to the technical and biological variability that need to be considered. Firstly, there are extensive differences between the degree of skill needed to perform the measures reported in the current study (Anderson & Phillips, 2015), and therefore to reduce the amount of variability several trained researchers might be needed to carry out each measurement, but this is an unlikely scenario in a single site research study. Secondly, the testing duration to do multiple methods can be much longer than a single measurement, which increases participant burden and the potential for additional stress that might influence vascular function variables (Poitras & Pyke, 2013). Lastly, the close interplay between the BP, endothelial function and arterial stiffness could also contribute to sources of variability. With this in mind, the automated BP and Hr was found to be measured with an adequate level of repeatability and reproducibility (**Table 6**). The between-day reproducibility appears to be consistent with previous reports of resting SBP and DBP (Stanforth et al., 2000). However, in this scenario non-invasive measurements of arterial stiffness were more reliable (within- and between-sessions) than methods used to assess endothelial function in the test battery.

Endothelial dysfunction is a hallmark of the early development of atherosclerosis and CVD (Bonetti et al., 2003; Grover-Páez & Zavalza-Gómez, 2009). Moreover, because of the systemic nature of endothelial function, dysregulation of microvascular endothelial function is thought to be a surrogate marker of coronary endothelial function (Hansell, Henareh, Agewall, & Norman, 2004). As such, the micro-vascular endothelial function, as measured by LDI and DVP-RI, and macro-vascular endothelial function measured by FMD, are important therapeutic targets (Anderson, 2006). With regards to micro-vascular endothelial function, laser Doppler methods are increasingly popular, because they are relatively easy

to perform and do not require extensive operator experience (Roustit & Cracowski, 2013). That said, there are a number of extraneous factors that introduce variability into these measures, especially when used in conjunction with iontophoresis (Loader et al., 2017). Moreover, despite the putative clinical utility of these methods, there is currently no standardisation of methods and/or data analysis (Roustit & Cracowski, 2013; Turner et al., 2008). In the current study an iontophoresis protocol that had previously been reported to be reliable was adopted (Jadhav, Sattar, Petrie, Cobbe, & Ferrell, 2007) and is presented as AUC (**Table 6**) as this is commonly used in randomised controlled trials (Jin, Alimbetov, George, Gordon, & Lovegrove, 2011; Keane et al., 2016b; Macready et al., 2014). In contrast to our findings, Jadhav *et al.* (2007) reported good reproducibility of the AUC for both ACh and SNP taken 8 weeks apart in females with cardiac syndromes, which might be attributable to the longer rest period before the measure, correction for skin resistance and the different populations between studies. Nonetheless, the findings in the current study are in line with others, in that, laser Doppler methods in conjunction with iontophoresis have been reported to produce high day-to-day CVs (Kubli, Waeber, Dalle-Ave, & Feihl, 2000; Morris & Shore, 1996), low ICCs (Tibiriçá, Matheus, Nunes, Sperandei, & Gomes, 2011) and SNP perfusion response can be less reproducible compared to ACh (Jadhav et al., 2007; Kubli et al., 2000; Tibiriçá et al., 2011). Moreover, it is particularly noteworthy that a second measurement taken within the same testing session produced a lower endothelial-dependent (ACh) perfusion response; despite identical placement of the Perspex chambers, limiting variations and spatial differences in capillary density. Although the reason for this remains unknown, it might relate to the lower heart rate (Maio et al., 2013) and warrants careful consideration in research designs.

The DVP method is quick-to-perform, operator-independent and the DVP-RI is strongly related to the vascular tone of small arteries (Millasseau et al., 2003) but there are limited published data concerning the reproducibility of DVP-RI. Notwithstanding, in agreement with others, the current study found that RI had higher intra-individual variability and poorer reliability within and between-sessions than SI (Millasseau et al., 2003). The current study also found better intra-session reliability for DVP-RI than inter-session, which has been reported elsewhere (Millasseau et al., 2003; Scholze et al., 2007). The within-subject

variability (15.1%) is also similar to those reported by Millasseau and colleagues (2003) who found inter-session CVs of 13.8% in a very small cohort of 8 healthy males. However, in the current study the low ICC demonstrated poor reproducibility between-sessions. On the other hand, macro-vascular endothelial function was assessed using FMD which showed moderate repeatability and reproducibility, despite requiring more operator skill than LDI and DVP-RI. Moreover, FMD produced a comparable level of technical and biological variation as previous studies (median CV of 17.5% (van Mil et al., 2016) and TE ranges from 0.4-4.8 in healthy individuals (Greyling et al., 2016)).

Arterial stiffness, which is an independent predictor of CVD (Mattace-Raso et al., 2006), was measured in the current test battery by several different methods due to an absence of a true 'gold standard' (Woodman et al., 2005). Firstly, we measured DVP-SI, which has been associated with stiffness of the large arteries (Millasseau et al., 2002), vascular aging (Millasseau et al., 2003) and risk of CVD (Gunarathne, Patel, Hughes, & Lip, 2008; Vakalis et al., 2015). The current study demonstrated that the intra- and inter-session DVP-SI can be measured consistently with very little variability (ICC ≥ 0.76; CV ≤ 3.3%), which is supported by the literature (Millasseau et al., 2002; Millasseau et al., 2003). In the current study, arterial stiffness was also measured by PWV and PWA, which were both highly reproducible (ICC ≥ 0.83) within- and between-sessions, and compare favourably with previous research (Tripkovic, Hart, Frost, & Lodge, 2014; Woodman et al., 2005). Notably, there was also evidence of systematic bias between-days for AIx, although all other statistical tests supported excellent repeatability and reproducibility. Conversely, AIx normalised for heart rate (75 bpm) was not different between-session, suggesting Hr might have contributed to the observed systematic bias, however it should be acknowledged that fewer individuals contributed to AIx@75 analysis (n = 17). This is because some participants Hr fell below 40 in the supine position, which might pose a problem and minimises the utility of this measure in athletic or bradycardic populations.

This study has various strengths such as the use of well-established, commonly used methodologies, but it is conceivable that other methods used to assess endothelial function might have been more reproducible. There are several other limitations that warrant discussion, firstly, the population in the current study were healthy, non-smoking young males and different to those in Chapter 5, but

nonetheless was importantly not confounded by the influence of menstrual cycle or medications and hence represent a relatively stable population to examine vascular function. Secondly, as recommended, a minimum of 5 minutes acclimation period was utilised (O'brien et al., 2003; Pickering et al., 2005); however it should be acknowledged that a longer acclimation period might have provided a more stabilised BP reading (Sala, Santin, Rescaldani, & Magrini, 2006) and perhaps more reproducible results. Nonetheless, this would have increased the testing period and participant burden, so has to be weighed up with pragmatically.

The study has addressed the specific thesis aim of determining the reliability of a battery of commonly used non-invasive measures of vascular function *in vivo.* The current research demonstrates considerable inconsistencies in the repeatability and reproducibility of non-invasive measures of structural and functional vascular health. This study found that BP, Hr, indices of arterial stiffness and macro-vascular endothelial function can be taken in a battery with adequate reliability in a test battery. However, measurements of microvascular endothelial function, LDI and to a lesser extent DVP-RI, demonstrates poor reproducibility between-sessions. Particularly LDI responses were highly variable, as has been reported elsewhere (Kubli et al., 2000; Morris & Shore, 1996; Tibiriçá et al., 2011). This chapter was a prelude to investigating the influence of anthocyanins on cardiovascular risk factors given the role of these human health discussed in section 2.2. This data has been used to inform the final experimental chapter of this thesis, as a result LDI measurements will be excluded from the randomised controlled trial. Moreover, this study has added to the literature as it highlights the importance of testing the reliability of multiple measures of vascular function taken in a single session and methodological considerations for future research designs.

Chapter 5:

The influence of tart cherry (*Prunus Cerasus*, cv Montmorency) concentrate on physiological and cognitive function: a randomised, placebo-controlled trial in free-living, middle-aged adults

5.1 Introduction

Recent advances in technology and medicine have resulted in increased life expectancy. Subsequently due to an aging population, it is now estimated that non-communicable diseases account for 71% of all deaths globally (WHO, 2019). In particular, CVD and type II diabetes combined are the primary cause of global mortality, increasing in prevalence with age (Danaei et al., 2014). Midlife risk factors have been proposed to underlie the development of these diseases, which evolve over years or decades before the emergence of clinical manifestations (Samieri et al., 2013). Thus, midlife has consistently been highlighted as a pivotal period for lifestyle interventions to improve health and reduce disease trajectory to promote healthy aging (King, Mainous III, & Geesey, 2007; Samieri et al., 2014). For example, middle-aged individuals who decrease their BP to normal ranges (<120/<80 mm Hg) have a significantly lower risk of remaining lifetime CVD compared to those with hypertension (Allen et al., 2012). As reviewed in detail in Section 2.2 of this thesis MC are currently being investigated for their positive impact on cardiovascular risk factors, including potential antihypertensive properties. Moreover, Chapter 3 demonstrated that higher intake of anthocyanins, a major class of tart cherry polyphenols, was inversely associated with the risk of CHD and CVD death − and cherries can account for a considerable dietary intake of these compounds (Adriouch et al., 2018a; Igwe, Charlton, & Probst, 2019; Tresserra-Rimbau et al., 2014a). Nonetheless, despites some promising epidemiological and *in vitro* studies suggesting a putative role for MC in cardiovascular and overall health (Keane et al., 2016a; van de Klashorst et al., 2020), clinical trials so far have provided paradoxical and equivocal findings for any benefits associated with their intake. Thus, well designed clinical trials are needed to clarify any causal link.

In addition to reducing morbidity and mortality in aging populations, another important aspect of healthy aging is maintaining good physical and cognitive function (Rowe & Kahn, 1997). Importantly, as highlighted in section 2.2.4 and 2.2.5, by improving vascular function and other cardiovascular risk factors, MC could also improve cognition and exercise capacity, as both are heavily reliant on vascular function, delivery and redistribution of metabolic substrates to the brain and muscle, respectively (Ainslie et al., 2008). In addition, tart cherry anthocyanins have been shown to interact with cell signalling pathways relating

mitochondrial biosynthesis function (Matsukawa et al., 2017), cell survival and regeneration (Williams et al., 2008) conferring potential nootropic and ergogenic activities. However, research surrounding the influence of tart cherries on physical and cognitive function in humans has been less consistent. For example, previous work from this laboratory demonstrated that acute supplementation with 60 ml of MC concentrate enhanced cerebral oxygenation in middle-aged adults without any influence on measures of cognitive function (Keane et al., 2016c). However, longer-term cherry supplementation has been shown to improve some areas of cognitive performance, specifically those associated with attention and memory, in older adults with or without cognitive impairment (Chai et al., 2019b; Kent et al., 2017a). Hence, the question remains whether longer-term exposure to dietary anthocyanins could be beneficial to cognitive function in other populations. In addition, while C3G, a major tart cherry anthocyanin, has been shown to increase exercise capacity in rats (Matsukawa et al., 2017), the effect of MC on physical performance in humans has yielded conflicting results (Keane et al., 2018; Morgan et al., 2019). In a recent meta-analysis short-term tart cherry supplementation was demonstrated to improve endurance exercise (Gao & Chilibeck, 2020), but the effects of longer-term MC supplementation on exercise capacity remain unknown. Subsequently it was hypothesised is that MC would improve vascular function and in turn cognitive function and exercise capacity. Therefore, the aim of the current study was three-fold; to determine the influence of longer-term (3 month) MC supplementation on 1) vascular function; 2) cognitive function; 3) exercise capacity; in free-living middle-aged adults and thus addresses the final specific aim of this thesis.

5.2 Methods

5.2.1 Participants

Power calculations were performed for the primary end point: change in SBP after 3 month consumption. The power was based on the inter-individual variability for SBP measurement (SD = 6) from Chapter 4. Assuming an 80% power, and a 0.05 significance level, the total number of subjects required to provide sufficient power to detect a 5 mmHg, a clinically meaningful amount (Makai, IntHout, Deinum, Jenniskens, & van der Wilt, 2017), in a 2-arm, parallel study was estimated to be 50. A total of 60 participants were needed to allow for a 20% drop-out.

Non-smoking males and females between the ages of 40 to 60 years were recruited from Newcastle and the surrounding areas by the use of posters, email distributions, social media and word of mouth. To be included in the study, participants must have reported to consume (on average) less than 5 servings of fruits and vegetables per day, did ≤4 hours of moderate-vigorous physical activity per week and additionally had ≥1 risk factor for type II diabetes. These risk factors included body mass index (BMI) >25 kg/m^2; waist circumference >102 cm for males and >88 cm for females; family history of type 1 or type 2 diabetes; were a member of a type 2 diabetes high risk population (Aboriginal, Hispanic, Asian, South Asian, or African decent) or were hypertensive; >140/90 mmHG (Ekoé et al., 2013; Lindström & Tuomilehto, 2003). All participants were otherwise in apparent good health as assessed by a health-screening questionnaire, not regularly taking medication (or stabilised ≥ 3 months, with no adverse symptoms) or antioxidant supplements and willing to report any changes in health status or medication during the study period.

Exclusion criteria was defined as a history of cardiometabolic disease, uncontrolled hypertension (SBP >159 mmHg or DBP >99 mmHg), gastrointestinal disease or malabsorption syndromes, reported changes in dietary or physical activity patterns within 3 months prior or intention to change during the study period, had unusual dietary habits (vegetarians, vegans or known eating disorders), alcohol intake of more than 21 units per week for males or 14 units per week for females, or a BMI ≥40 kg/m^2. Additionally, participants who were pregnant or planning to become pregnant during the study, lactating, or initiating or changing a hormone replacement therapy regimen within 3 months of the start of the study were also excluded. The study was conducted in accordance with the Declaration of Helsinki and ratified by the University's Research Ethics Committee prior to participants providing written, informed consent. This study was registered as a clinical trial with clinicaltrials.gov [NCT04021342].

5.2.2 Study design

This study employed a randomised, double-blind, placebo-controlled, parallel design. After screening and recruitment, participants were familiarised with the testing equipment and procedures. Following this they were randomly assigned to receive either Montmorency tart cherry concentrate (MC) or an isocaloric

placebo for 3 months using a computer-generated plan (randomization.com), stratified by sex. The study comprised of two experimental visits, following a minimum of a 7-day low anthocyanin run-in. Outcome variables were assessed at baseline (visit 2; pre-supplementation) and at 3 months (visit 3; post-supplementation) as shown in **Table 7**. All experimental visits took place between 8:00 and 10:00 am and were preceded by an overnight fast (≥ 10h). Participants were also asked to arrive hydrated and to avoid strenuous exercise, alcohol, nutritional supplements 24 hours and caffeine 12 hours prior.

Table 7. Overview of study design

Study Event	Screening (V1)	Baseline (V2)	3 month (V3)
Informed consent	X		
International Physical Activity Questionnaire	X		X
General health and lifestyle questionnaire	X		
Anthropometrics	X	X	X
Resting blood pressure	X	X	X
Diet and exercise diary (3 day)	X		X
Urine collection		X	X
Pittsburgh Sleep Quality Index		X	X
SF-36 Health Survey		X	X
Body Composition (DXA)		X	X
Vascular Function*		X	X
Near infra-red spectroscopy		X	X
Cognitive test battery§	[Practice]	X	X
Plasma Samples†		X	X
Astrand-Rhyming exercise capacity	[Practice]	X	X

* Arterial stiffness (pulse wave analysis/velocity) and endothelial function (flow mediated dilation)
§ Bond-Lader mood scales, digital vigilance, rapid visual information processing and 3-back task
† Lipid profiles, insulin, glucose, and high-sensitivity C reactive protein
DXA = dual-energy X-ray absorptiometry

5.2.3 Dietary intervention

A concentrated MC juice stored at 4ºC was used in this study. The concentrate was supplied by CherryActive (Active Nutrition Ltd., Sunbury, UK) and King Orchards (USA). Once randomised participants received the same type of juice throughout the trial. Participants were instructed to consume either 30 ml of MC concentrate diluted in 240 ml of water (as recommended by the supplier) or the same volume of placebo twice daily, once in the morning and again in the

evening. According to the supplier each 30 ml dose of MC is estimated to be equivalent to approximately 90 whole cherries (equating to ~180 cherries per day). The placebo was prepared by mixing unsweetened black cherry flavoured Kool-Aid (Kraft Foods, United States), dextrose (MyProtein Ltd., Northwich, UK), fructose (Sports Supplements Ltd., Essex, UK) with bottled water to best match the calorie content of the MC concentrate (Energy = 102 kcal, volume = 30 ml, carbohydrates = 25.5 g, protein = 0 g and fat = 0 g). Additional lemon juice, for tartness, and artificial food colouring was added (Chai et al., 2018; Losso et al., 2017) so the final product had identical visual properties (**Figure 11**). The placebo was packed in clear polyethylene terephthalate containers (provided by ActiveEdge Nutrition® Ltd). The assigned treatment and a 30 ml measuring cup were supplied to the participants by a researcher (independent to the project) to ensure the study remained double-blinded. Compliance was measured by daily tick sheets and return of any unconsumed juice. On the second experimental visit, following the supplementation period, participants were asked to guess which treatment they thought they had been taking for the duration of the study.

Throughout the study participants were encouraged to maintain their habitual diet and exercise routines, however they were asked to refrain from consuming cherries, cherry products, or any antioxidant supplements. Additionally, they were given verbal and written instructions to limit berry fruits, red grapes (including extracts/ juices) and red wine [which are the top contributors of dietary anthocyanins (Adriouch et al., 2018a; Tresserra-Rimbau et al., 2014a)] to ≤1 portion per day throughout the study period. Participants recorded their pre-evening meal before experimental visit one and were asked to replicate this before the second experimental visit. To monitor dietary intake, participants recorded a 3-day food and exercise diary (two consecutive weekdays and one weekend day), which was analysed retrospectively (Nutritics software, v5.09, Dublin, Ireland). Participants also completed the International Physical Activity Questionnaire (IPAQ) and a short form quality-of-life survey (SF-36 (Ware & Kosinski, 2001)) at the beginning and end of the study to determine physical activity levels and tolerance to the intervention, respectively.

Figure 11. Montmorency tart cherry concentrate (left) and isocaloric placebo (right). Labels were removed by an independent researcher before assigning treatments to the participants.

5.2.4 Total Anthocyanin Content

The monomeric anthocyanin pigment content of the cherry juice was determined using the pH-differential method (Giusti and Wrolstad, 2001). The cherry juices were diluted 1:20 in 0.025 M potassium chloride buffer at pH 1.0 and 0.4 M sodium acetate buffer at pH 4.5, respectively. The absorbance was measured spectrophotometrically at 510 and 700 nm with a Jenway 7135 UV/Visible spectrophotometer (Cole-Parmer Ltd., United Kingdom). The absorbance difference A was calculated as A=(A510-A700)pH1.0 - (A510-A700)pH4.5. The total anthocyanin concentration C (mg/L) was expressed as mg cyanidin-3-glucoside equivalents according to the following equation: C=A.MW.DF.1000/(ε.l), where MW = molar mass for cyanidin-3-glucoside (449.2 g/mol); DF the dilution factor, 1000 the conversion from g to mg; ε the molar extinction coefficient for C3G (26900 L/mol.cm); l the path-length (1 cm).

5.2.5 Total Phenolic Content

Total phenolic content (TPC) was measured using a modified Folin-Ciocalteu colorimetric method as previously described (Keane et al., 2016b). Briefly, the cherry juice and placebo was diluted in deionised water (1:10) and 50 µL of the diluted sample, 50 µL of Folin-Ciocalteu reagent diluted in water (1:25) and 100

µL of 6% (w/v) sodium carbonate were added into corresponding sample wells of a 96 well plate. Absorbance readings were taken at 725 nm, at 5 minute intervals, over a 30 minute period at 25°C using a BioTek Synergy HT Multi-Mode Microplate Reader (BioTek, Winooski, USA). The quantification was performed on the basis of a standard curve (R^2=0.99) in the range 0-500 mg/L (Waterhouse, 2009). The analysed samples were measured versus a blank and are expressed as mean mg/L of gallic acid equivalents (corrected for the dilution factor) ± SD for 6 replications. The CV for this method was <3%.

5.2.6 Anthropometry and body composition

Stature was measured to the nearest 0.1 cm using a stadiometer and body mass measured to the nearest 0.1 kg using the same digital scale (Seca Scales 703, Seca Ltd. Birmingham, UK). Waist circumference was measured in accordance with the International Society for the Advancement of Kinanthropometry guidelines. Body composition (fat mass, fat percentage, android/gynoid ratio and lean body mass) was measured by dual-energy X-ray absorptiometry (DXA; Hologic, Horizon, Manchester, UK). The scanner was calibrated before each scan in accordance with manufacturer's guidelines and participants were instructed to wear the same clothing for each visit. All measurements were performed at baseline and on the return visit, 3 months later.

5.2.7 Vascular function

Measurements of BP, Hr, arterial stiffness (PWV and PWA) and endothelial function (FMD) were taken as described in Chapter 4.2.

5.2.8 Cerebral blood flow, cognitive function, sleep and mood

5.2.8.1 Near infra-red spectroscopy (NIRS)

The NIRS is a non-invasive imaging technique in which two nominal wavelengths of light, which are differentially absorbed by oxygenated and deoxygenated haemoglobin, respectively, are introduced through the body tissues via a laser emitter. The wavelengths are then measured, following transit through the upper surface of the tissue of interest, by an optode placed at a pre-set distance from the light source. Recently NIRS has been applied to demonstrate changes in cerebral haemodynamics following acute (Keane et al., 2016c; Wightman et al., 2015) and chronic nutritional interventions (Jackson, Reay, Scholey, & Kennedy, 2012). Accordingly, NIRS has been deemed sensitive enough to assess changes in cerebral haemodynamics within the prefrontal cortex in response to neural

activity following supplementation with nutritional interventions (Jackson & Kennedy, 2013).

In the current study, relative change in CBF was assessed using continuous wave NIRS (NIRO-200NX, Hamamatsu Photonics K.K., Japan). Two near-infrared sensors were placed over the left and right frontal lobe region of the forehead corresponding to the International 10–20 system Fp1 and Fp2 EEG positions; these signals were averaged to determine cerebral oxygenation. The sensors were secured to the skin using double-sided adhesive tape and shielded from ambient light using an elastic head band. The sensors alternately emit two wavelengths of near-infrared light (~765 and 855 nm) with an emitter/optode separation distance of 4 cm. A 5-minute rest (which acted as the NIRS baseline for CBF calculations) was taken at each testing session and data were acquired continuously throughout a cognitive task battery. Output was time stamped at the start of each task segment to assure that data corresponded to the relevant period of task performance. Baseline adjusted data with respect to the 5 min of NIRS data collected immediately prior to completing the tasks (Jackson et al., 2016; Wightman et al., 2015), was then calculated for each task offline. NIRS data is reported as changes in cerebral oxy- (HbO_2), deoxy- (hHb) and total-(tHb) haemoglobin concentrations.

5.2.8.2 Cognitive function, sleep and mood assessment

Participants completed the Pittsburgh Sleep Quality Inventory, PSQI (Buysse, Reynolds III, Monk, Berman, & Kupfer, 1989), to assess sleep quality. Cognitive function and mood measures were assessed using a test battery and administered via the Computerised Mental Performance Assessment System (COMPASS, Northumbria University, Newcastle upon Tyne, UK), a purpose-designed software application for the flexible delivery of randomly generated parallel versions of standard and novel cognitive assessment tasks. The test battery included three tasks; digital vigilance (DV; 3 min), rapid visual information processing (RVIP; 5 min) and *N*-back task (~3 min). These tests were chosen because firstly they have previously been shown to activate the prefrontal cortex where cerebral haemodynamics were being assessed (Coull, Frith, Frackowiak, & Grasby, 1996; Jansma, Ramsey, Coppola, & Kahn, 2000). Secondly they have been frequently applied to evaluate the efficacy of polyphenol supplementation on cognitive function (Cook et al., 2020; Keane et al., 2016c; Wightman et al.,

2015) and lastly they asses the domains attention and memory which have previously been shown to be influenced by dietary anthocyanin and cherry supplementation (Chai et al., 2019b; Kent et al., 2017a; Kent, Charlton, Netzel, & Fanning, 2017b). The cognitive tests (described below) were repeated twice in order to induce some level of cognitive fatigue, which was assessed immediately after each battery by a visual analogue scale (VAS). Participants also completed Bond-Lader VAS (Bond & Lader, 1974) before and after the test-battery to assess subjective mood.

5.2.8.3 Bond-Lader VAS

The VAS required participants to indicate how they currently feel "at this moment in time" by clicking, using the mouse, at the appropriate point along a 100 mm scale on screen. Sixteen scales are presented with antonyms at either end, e.g. 'alert' vs. 'drowsy', 'lethargic' vs. 'energetic' and 'troubled' vs. 'tranquil', with these 16 scores (% along the line towards the right end) combining to create three overall measures of mood factors: 'alert', 'content' and 'calm'.

5.2.8.4 Digit Vigilance (DV)

The DV task is a measure of sustained attention and psychomotor speed. A single target digit was randomly selected and constantly displayed on the right hand side of the screen. A series of single digits appeared on the left hand side of the screen, one at a time, at the rate of 150 per minute. The participant was required to press the spacebar on the keyboard as quickly as possible every time the digit in the series matched the target digit. Task outcomes included accuracy (%) and reaction time for correct responses (ms). This task has been shown to identify age-related declines in attention and the test-retest correlation coefficient for reaction time is 0.81 (Wesnes et al., 2017).

5.2.8.5 Rapid Visual Information Processing (RVIP)

The RVIP task is a measure of sustained attention and working memory. The task requires the participant to monitor a continuous series of single digits for targets of three consecutive odd or three consecutive even digits. The digits are presented on the computer screen one at a time at the rate of 100 per minute in pseudo-random order, and the participant responds to the detection of a target string by pressing the spacebar on the keyboard as quickly as possible. Task outcomes included number of target strings correctly detected (%) and average reaction time for correct detections (ms). The test-retest correlation for these is

(>0.70) in older adults and has been reported as reliable in the detection and monitoring of cognitive deficits (Goncalves, Pinho, & Simoes, 2016).

5.2.8.6 N-Back

The 3-back task measures working memory and memory capacity. The task requires participants to indicate whether the letter presented on screen was also presented 3 letters previously in the letter sequence. Participants are required to respond by pressing buttons corresponding to 'yes' or 'no' on the keyboard, to each letter, as quickly as they can. Participants were presented with 45 stimuli (letters), however the task is dependent on speed (i.e. slower reaction times will result in a lengthier task). The task outcomes included accuracy of correct yes responses (%) and reaction time for correct yes responses (ms). The test-retest correlation coefficient for this task has been reported to be 0.73 for accuracy and 0.81 for reaction time, respectively (Hockey & Geffen, 2004).

5.2.10 Exercise capacity

Aerobic exercise capacity ($\dot{V}O_{2max}$) was estimated using a frequently applied sub-maximal cycle test; the Astrand-Rhyming single-stage, 6-min test (Noonan & Dean, 2000) on an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands). A submaximal protocol was chosen as this method is less strenuous and safer for individuals who are relatively inactive and/or at increased cardiovascular risk. The Astrand-Rhyming test has been shown to be a valid (r = 0.85) and reliable (r = 0.83) estimate of $\dot{V}O_{2max}$ (Cink & Thomas, 1981) and has been utilised in other lifestyle interventions assess the influence on exercise capacity (Fibbins et al., 2020; Vadstrup, Frolich, Perrild, Borg, & Roder, 2012). Following a 3 minute warm up, participants were instructed to maintain a pedalling rate of 50 revolutions per minute (rpm) for the six minutes, which was started with a constant workload 100 W for males and 75 W for females (ACSM, 2017). During cycling, Hr was continuously recorded (T31, Polar, Finland) and workload was adjusted where necessary to reach a target Hr of 125-170 bpm. The average HR, which differed by no more than 5 bpm during the last two minutes of the test was used to estimate $\dot{V}O_{2max,}$ adjusted for age (Astrand, 1960).

5.2.11 Biological samples

5.2.11.1 Collection

All samples were collected following an overnight fast on the morning of each experimental visit. Spot urine samples (Langer, Kennel, & Lodge, 2018), avoiding

the first morning void, were collected into 30 ml sterile tubes (Sterillin, Thermo Scientific, UK). Aliquots of urine were immediately stored at −80 °C for later analysis. Venous blood samples (~12 ml) were collected in lithium-heparin vacutainers (Becton, Dickinson and Company, USA). Due to blood sampling error samples were only available for 40 participants. These were centrifuged at 3000×g (4°C) for 10 min and the plasma aliquotted and stored at −80 °C to be analysed later.

5.2.9.2 Preparation and Analysis

Plasma samples were analysed by the Department of Blood Sciences in the Royal Victoria Infirmary Hospital, Newcastle. Cholesterol, HDL cholesterol, non-HDL Cholesterol, triglycerides and total/HDL cholesterol ratio, as well as high sensitivity CRP (hs-CRP), glucose and insulin levels were analysed for the available samples (Cherry *n* = 19; Placebo *n* = 21). The LDL cholesterol was calculated using the Friedewald equation (Friedewald, Levy, & Fredrickson, 1972). The homeostatic model assessment of insulin resistance (HOMA-IR) was calculated according to Matthews et al. (1985) using the following formula:

$$\mathrm{HOMA-IR} = \frac{Fasting\ insulin \times Fasting\ glucose}{405}$$

Urine samples were defrosted on ice and normalised to the same reflective index (Edmands, Ferrari, & Scalbert, 2014). Then 100 µl of normalised urine was mixed with 100 µl of chilled methanol (-20°C), vortex mixed and kept on ice for 30 minutes. Samples were then centrifuged for 2 minutes at 10,000 rpm. The top 100 µl of the sample was aliquotted, filtered, and transferred to an LCMS vial for analysis.

Analysis of the urinary metabolome was intended to take place, but because of the issues raised by COVID-19, this has been indefinitely postponed. Consequently, this thesis does not contain these data, but the intention is to analyse non-targeted metabolomic profiles of the spot urine samples.

5.2.12 Data and Statistical Analysis

All data were analysed using IBM SPSS statistics (v 26.0 for Windows; SPSS, Chicago, IL), measures are reported as means ± standard deviation (SD) in tables and standard error (SE) in figures unless otherwise stated. Normality of

distribution for outcome measures was tested using the Shapiro Wilks test and assumptions were tested prior to analysis. Baseline characteristics where analysed by Wilcoxon signed-rank test where data were continuous, and Chi squared where data were categorical. Dietary, physical activity and SF-36 data were analysed using a two-way (treatment × time) analysis of variance (ANOVA). Treatment guess data was analysed by Chi squared. The effect of the intervention on vascular function variables was evaluated using a one-way analysis of covariance (ANCOVA) adjusted for baseline (Vickers & Altman, 2001) and also sex and use of medication.

The PSQI data was analysed using a Friedman non-parametric test. Despite familiarisation with the cognitive function tasks some participants did not perform the tasks correctly. Therefore, cognitive function and mood data was cleaned by generating box plots for each outcome variable to identify potential outliers. Values that were more than one and a half and three deviations from the interquartile range were identified as outliers, and extreme outliers, respectively (Tukey, 1977). Outliers were removed, meaning a number of data points were missing for each participant. Therefore, to accommodate missing data points cognitive function variables were analysed using a linear mixed model with; treatment (cherry juice/placebo) and repetition (1,2) as fixed factors and baseline (pre-treatment) values, sex and use of medication as covariates.

For NIRS data, task length was fixed for the DV (180 s) and RVIP (300 s), but NIRS data from the N-Back test was truncated so that the same amount of data was analysed for all participants during each task period. The data was adjusted for resting baseline data (5 mins prior) and the 2 channels were averaged across hemispheres. If the participant's data had been omitted from the cognitive function task (both accuracy and time) they were excluded for that task. The analysis of resting baseline adjusted data was performed using a linear mixed model with as described above.

For blood samples where values fell below the limits of detection the sensitivity threshold of the assay were used to maintain participant numbers. The hs-CRP, HOMA-IR and insulin values were non-normally distributed so were log transformed before analysis. The variables were analysed using a linear mixed model with; treatment (cherry juice/placebo) as a fixed factor and baseline (pre-treatment) values, sex and use of medication as covariates. Sidak adjusted *post-*

hoc comparisons were then carried out between cherry juice and placebo as appropriate.

5.3 Results

A total of 56 individuals were enrolled in the study and randomised to the intervention. There was no difference between the group characteristics at baseline (**Table 8**). Three participants from each group did not complete the study as shown in **Figure 12.**

Table 8. Baseline Characteristics

Characteristic	**All** (*n* = 56)	**Cherry** (*n* = 28)	**Placebo** (*n* = 28)	P-Value
Age (y)	48 ± 6	49 ± 6	47 ± 6	0.160
Sex (m/f)	37/19	19/9	18/10	0.778
Stature (cm)	173.1 ± 8.8	173.7 ± 8.9	172.4 ± 9.0	0.494
Body Mass (kg)	81.8 ± 12.9	81.7 ± 14.0	82.0 ± 11.9	0.793
BMI (kg/m2)	27.3 ± 3.7	27.0 ± 3.8	27.4 ± 3.7	0.569
Ethnicity (*n*; %)				0.368
White	54 (96.4)	27 (96.4)	27 (96.4)	
Education (*n*; %)				0.798
Less than high school	-	-	-	
High school or equivalent	24 (43)	11 (39)	13 (46)	
Bachelor's degree	19 (34)	9 (32)	10 (36)	
Postgraduate degree	13 (23)	8 (29)	5 (18)	
Left handed (*n*; %)	5 (9)	3 (11)	2 (7)	0.639
Medication (*n*; %)	17 (30)	10 (36)	7 (25)	0.771

values are mean ± standard deviation

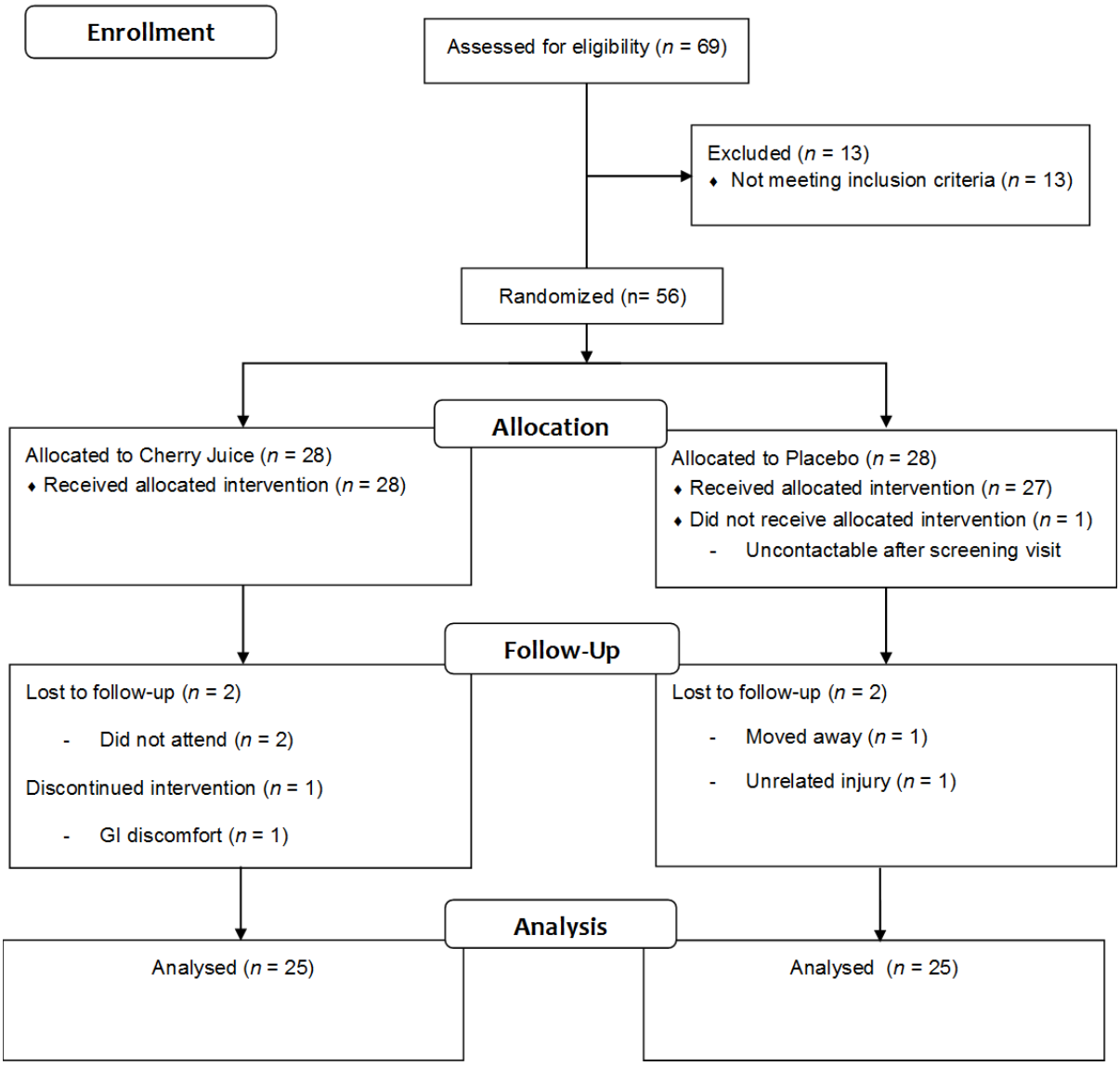

Figure 12. Consort flow diagram of participants

5.3.1 Polyphenol content, safety and tolerance of the interventions

The total anthocyanin and polyphenol content of the intervention beverages are shown in **Table 9**, below. The mean (± SD) self-reported compliance (as assessed by tick sheets) was 94 ± 9% and 98 ± 4% in the cherry and placebo group, respectively. Five participants (20%) correctly guessed they were in the placebo group, but Chi squared (Table 1; Appendix D) suggested successful blinding (P = 0.386). One participant in the cherry group discontinued the juice and withdrew from the study due to gastrointestinal discomfort and bloating. The

treatments were otherwise well tolerated as suggested by the SF-36 (Appendix E) which showed no treatment, time or interaction differences between groups.

Table 8. Juice analysis

Beverage	TACN (mg/L)	TPC (mg/L)
Placebo	-	358.3 ± 37.6
Cherry Active	258.0	3089.9 ± 155.7
King Orchards	482.6	3400.0 ± 154.9

Abbreviations: TACN; total anthocyanin content (cyanidin 3 glucoside equivalents); TPC; total polyphenol content (gallic acid equivalents). Placebo was not analysed because it contained artificial colourant (E129) which causes interference with the assay (Giusti & Wrolstad, 2001).

5.3.2 Physical activity, diet and body composition

There was no treatment, time or treatment × time interaction effects observed for physical activity, sitting time or exercise capacity (**Table 10**). Analysis of 3-day diet records showed that there were no significant differences between the two groups for mean intake of total energy, fat or saturated fat intake. Protein intake showed a treatment × time interaction ($F= 7.8$, $P = 0.011$). Planned *Post hoc* tests revealed protein intake at baseline was on average 12g higher in the placebo group compared to the cherry group ($F = 6.7$, $P = 0.016$). There was also an increase in carbohydrate (36g) intake at 3 months in both groups, main effect of time ($F= 17.3$, $P < 0.001$). Body mass ($F = 8.5$, $P = 0.007$), BMI ($F = 12.6$, $P = 0.002$) and fat mass ($F = 4.8$, $P = 0.040$) increased relative to baseline in the placebo group, main effect of time. Fat mass ($F= 7.7$, $P = 0.011$) and fat percentage ($F= 4.38$, $P = 0.047$) also increased after 3 months in the cherry group There was no treatment effect observed at baseline or 3 months for body mass. There was no treatment, time or treatment × time interaction effects between lean mass or android/gynoid ratio (**Table 10**).

Table 9. Physical activity, diet and body composition

	Cherry	Placebo	ANOVA		
			Treatment	Time	Interaction
METs (min/week)					
Baseline	2462 ± 2038	1953 ± 1527	0.267	0.959	0.961
3 months	2464 ± 2336	1978 ± 1217			
Sitting time (h)					
Baseline	6.6 ± 3.1	6.7 ± 3.0	0.856	0.852	0.486
3 months	6.7 ± 2.8	6.3 ± 3.0			
$\dot{V}O_{2max}$ ($ml \cdot kg^{-1} \cdot min^{-1}$)					
Baseline	35.9 ± 11.1	37.3 ± 7.5	0.319	0.187	0.386
3 months	33.7 ± 9.6	37.1 ± 6.2			
Energy (Kcal)					
Baseline	1921 ± 340	1896 ± 439	0.997	0.212	0.673
3 months	1977 ± 439	2001 ± 423			
Carbohydrates (g)					
Baseline	208.8 ± 38.6	196.1 ± 47.3	0.671	<0.001	0.350
3 months	237.3 ± 70.0[b]	239.6 ± 66.7[b]			
Fat (g)					
Baseline	76.5 ± 18.5	78.4 ± 24.2	0.681	0.231	0.999
3 months	72.1 ± 18.1	74.1 ± 21.2			
Saturated fat (g)					
Baseline	28.2 ± 8.6	27.5 ± 8.0	0.849	0.224	0.836
3 months	25.8 ± 8.3	25.7 ± 10.6			
Protein (g)					
Baseline	80.2 ± 21.9	92.3 ± 16.9*	0.193	0.051	0.006
3 months	80.3 ± 15.7	78.5 ± 19.3[b]			
Body mass (kg)					
Baseline	82.8 ± 13.9	82.4 ± 12.3	0.968	0.007	0.048
3 months	83.4 ± 14.2	84.1 ± 13.2[b]			
BMI (kg/m^2)					
Baseline	27.3 ± 3.8	27.5 ± 3.8	0.757	0.001	0.057
3 months	27.5 ± 3.8	28.1 ± 4.0[b]			
Body fat (%)					
Baseline	37.1 ± 7.9	36.1 ± 6.8	0.642	0.031	0.862
3 months	37.7 ± 6.8[b]	36.7 ± 6.8			
Fat mass (kg)					
Baseline	30.2 ± 9.1	29.6 ± 7.1	0.856	0.007	0.691
3 months	31.1 ± 9.5[b]	30.7 ± 7.5[b]			
Lean mass (kg)					
Baseline	48.1 ± 9.4	49.8 ± 8.5	0.360	0.248	0.199
3 months	48.1 ± 8.5	50.4 ± 9.2			
Android/gynoid ratio					
Baseline	1.09 ± 0.17	1.13 ± 0.18	0.394	0.403	0.529
3 months	1.08 ± 0.17	1.13 ± 0.18			

Mean ± SD

Body mass index (BMI)

[b] Significantly different from baseline (P <0.05): * significantly different between groups (P <0.05)

5.3.3 Vascular function

After adjusting for baseline (pre-treatment) values, sex and medication there were no group differences between cherry juice and placebo for SBP (**Figure 13**), DBP, Hr, arterial stiffness (PWV, AIx and AIx@75) or endothelial function (FMD) after three months (**Table 11**).

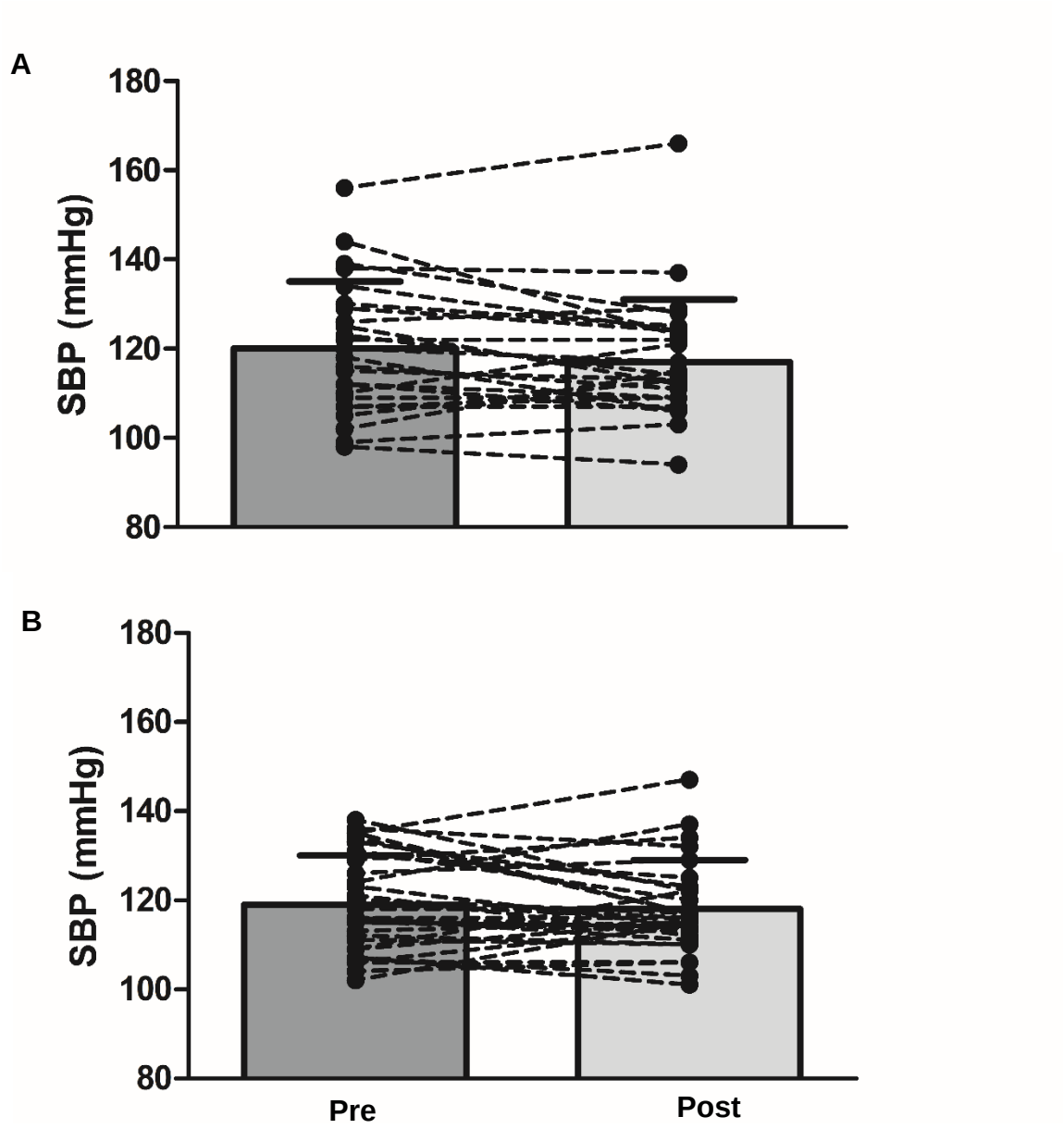

Figure 13. SBP before and after 3-month supplementation with cherry juice (A) and placebo (B).

Table 10. Effect of cherry juice compared to a placebo on vascular function

	Cherry Juice	Placebo	**ANCOVA** adjusted for baseline			adjusted for baseline, sex and medication		
			Difference (95%CI)	F	P-Value	Difference (95%CI)	F	P-Value
SBP (mmHg)								
Baseline	120 ± 15	119 ± 11	-0.5 (-4.9, 3.8)	0.056	0.814	-0.6 (-5.1, 3.9)	0.079	0.780
3 months	117 ± 14	118 ± 11						
DBP (mmHg)								
Baseline	73 ± 10	73 ± 8	-0.4 (-3.0, 2.3)	0.086	0.770	-0.5 (-3.1, 2.2)	0.127	0.723
3 months	73 ± 9	73 ± 8						
Hr (BPM)								
Baseline	59 ± 11	59 ± 10	-0.3 (-3.7, 3.1)	0.033	0.858	-0.2 (-3.6, 3.2)	0.014	0.908
3 months	59 ± 12	59 ± 10						
PWV (m/s)								
Baseline	6.7 ± 1.0	6.4 ± 0.8	0.3 (-0.3, 0.8)	1.051	0.312	0.2 (-0.2, 0.7)	0.967	0.332
3 months	6.8 ± 1.3	6.2 ± 0.8						
AIx (%)								
Baseline	22.1 ± 8.9	17.8 ± 11.4	0.3 (-3.6, 4.2)	0.021	0.886	0.3 (-3.6, 4.2)	0.022	0.884
3 months	20.4 ± 9.6	17.0 ± 10.0						
AIx@75 (%)								
Baseline	13.4 ± 8.1	9.6 ± 12.9	0.02 (-3.6, 3.6)	<0.001	0.991	-0.06 (-3.6, 3.5)	0.001	0.937
3 months	12.3 ± 9.2	9.3 ± 11.0						
FMD (%)								
Baseline	8.3 ± 3.5	9.3 ± 3.5	1.1 (-1.1, 3.3)	0.972	0.330	1.2 (-1.0, 3.3)	1.256	0.269
3 months	9.7 ± 3.5	9.0 ± 4.0						

Mean ± SD; Abbreviations; augmentation index (AIx); AIx normalised for a heart rate of 75 bpm (AIx@75); diastolic blood pressure (DBP); flow-mediated dilation (FMD); heart rate (Hr); pulse wave velocity (PWV).

5.3.4 Cerebral blood flow

There was no treatment or treatment × repetition interaction effects for HbO_2, hHb or tHb concentrations during any of the tasks (**Figure 14**).

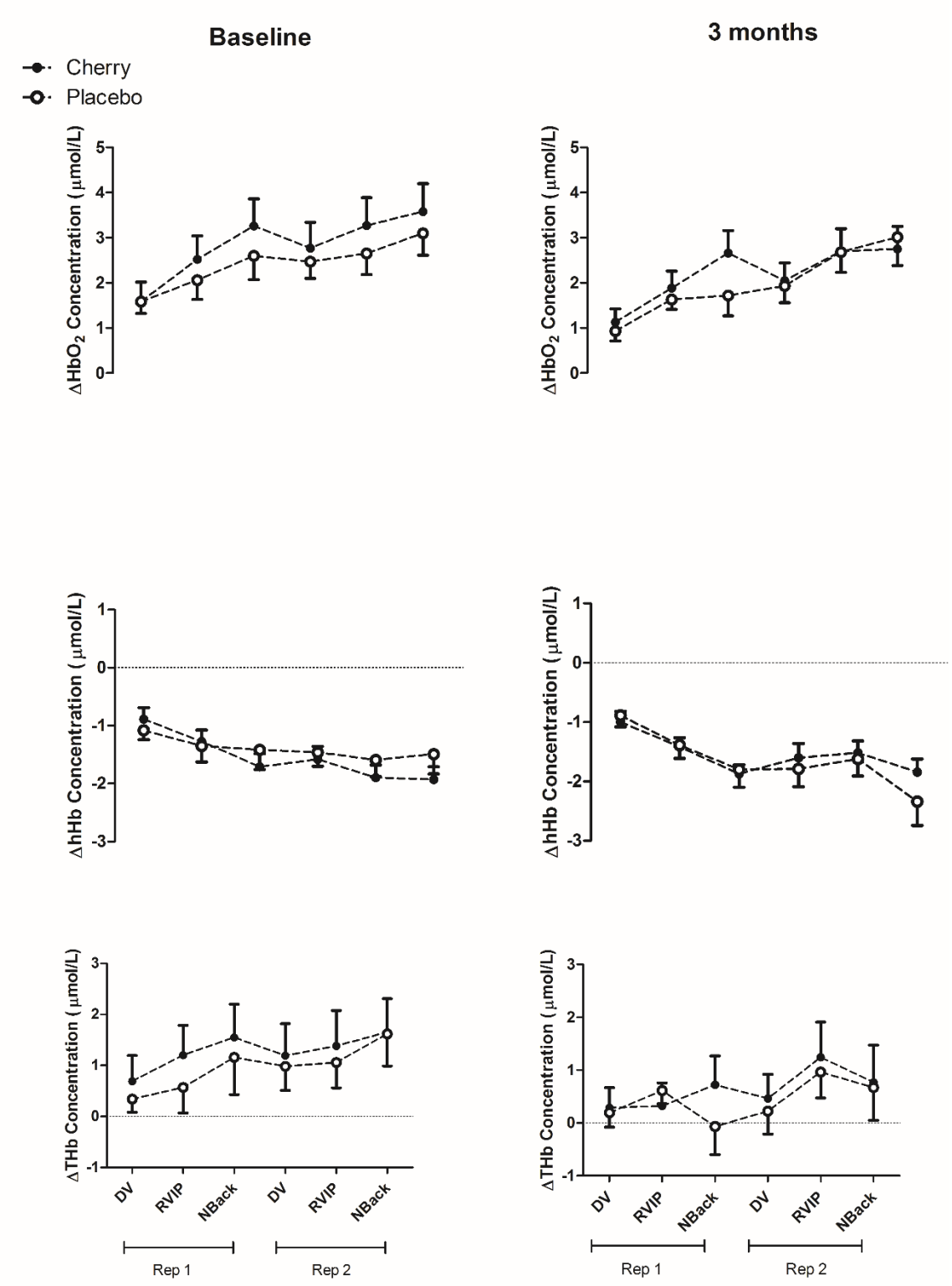

Figure 14. The baseline-adjusted oxygenated (HbO_2; top panel), deoxygenated (hHb; middle panel) and total- haemoglobin (THb; bottom panel) concentrations for each task repetition pre-supplementation (left) and post-supplementation (right). Rep = repetition

5.3.5 Cognitive function, sleep and mood

There was no difference between treatments for the sleep measures as assessed by the PSQI (**Table 12**). There was also no treatment or treatment × repetition interaction effects between cherry juice or the placebo between any of the cognitive function variables (**Table 13Table 13**). There was a treatment × repetition interaction for alertness, *post-hoc* revealed a difference between treatments after the second repetition (P = 0.041). The post-supplementation mental fatigue VAS was significantly lower in the cherry group (F = 4.38, P = 0.042), main effect of treatment.

Table 11. The composite Pittsburgh Sleep Quality Inventory (PSQI) scores

	Cherry	**Placebo**	**Friedman**	
			X^2	P-Value
Subjective Sleep Quality				
Baseline	1.0 (1.0)	1.0 (1.3)	1.43	0.699
3 months	1.0 (1.0)	1.0 (0.3)		
Sleep Latency				
Baseline	1.0 (1.0)	1.0 (1.0)	1.75	0.626
3 months	1.0 (1.3)	1.0 (1.3)		
Sleep Duration				
Baseline	1.0 (1.0)	1.0 (0.0)	7.25	0.064
3 months	1.0 (1.0)	1.0 (1.0)		
Habitual Sleep Efficiency (%)				
Baseline	91 (8)	87 (9)	5.50	0.138
3 months	84 (10)	88 (21)		
Sleep Disturbances				
Baseline	1.0 (0.0)	1.0 (1.0)	2.70	0.440
3 months	1.0 (0.0)	1.0 (0.0)		
Use of Sleeping Medication				
Baseline	0.0 (0.0)	0.0 (0.0)	3.00	0.392
3 months	0.0 (0.0)	0.0 (0.0)		
Daytime Dysfunction				
Baseline	1.0 (1.0)	1.0 (2.0)	3.01	0.378
3 months	1.0 (1.0)	1.0 (1.0)		
Global PSQI score				
Baseline	4.0 (2.3)	5.0 (2.0)	3.58	0.310
3 months	4.0 (2.3)	4.0 (3.0)		

Data is presented as median (interquartile range)

Table 12. Cognitive function tasks (data are presented as Mean ± SD)

			Baseline		3 months		Mixed Model	
Measure	**Treatment**	*n*	**Rep 1**	**Rep 2**	**Rep 1**	**Rep 2**	**Effect**	P-Value
DV accuracy (%)	Cherry	45	94.1 ± 5.9	88.2 ± 13.1	95.7 ± 3.5	88.6 ± 11.6	T	0.091
	Placebo		93.0 ± 6.0	84.7 ± 14.7	89.0 ± 9.8	85.9 ± 10.3	T*R	0.407
DV RT (ms)	Cherry	46	477.8 ± 37.8	492.5 ± 39.5	477.4 ± 33.9	495.3 ± 38.8	T	0.357
	Placebo		473.5 ± 31.4	500.1 ± 37.2	484.7 ± 37.5	511.4 ± 34.2	T*R	0.149
RVIP accuracy (%)	Cherry	42	57.2 ± 17.3	63.0 ± 18.3	63.0 ± 20.9	71.5 ± 15.3	T	0.116
	Placebo		50.4 ± 18.7	53.8 ± 17.2	56.3 ± 21.8	56.7 ± 20.5	T*R	0.240
RVIP RT (ms)	Cherry	41	555.6 ± 62.1	568.1 ± 50.6	555.5 ± 53.5	549.6 ± 35.0	T	0.448
	Placebo		557.9 ± 63.9	540.1 ± 47.3	536.4 ± 51.7	545.1 ± 37.1	T*R	0.339
3-Back accuracy (%)	Cherry	40	80.2 ± 5.4	85.3 ± 8.4	79.7 ± 9.1	85.4 ± 9.0	T	0.398
	Placebo		76.9 ± 9.3	81.8 ± 8.8	77.5 ± 9.4	83.3 ± 7.9	T*R	0.823
3-Back RT (ms)	Cherry	40	1110 ± 231	980 ± 234	1111 ± 248	942 ± 195	T	0.562
	Placebo		946 ± 239	901 ± 205	1054 ± 286	920 ± 247	T*R	0.694

Abbreviations; Digital vigilance (DV); rapid visual image processing (RVIP); reaction time (RT); repetition (Rep). Effects are treatment (T) and treatment by repetition interaction (T*R).

Table 13. Mood and visual analogue scale measures (data are presented as Mean ± SD)

			Baseline		3 months		Mixed Model	
Measure	**Treatment**	***n***	**Rep 1**	**Rep 2**	**Rep 1**	**Rep 2**	**Effect**	**P-Value**
Alert	Cherry	48	71.8 ± 9.5	52.6 ± 14.8	69.3 ± 11.3	59.7 ± 18.5*	T	0.134
	Placebo		71.8 ± 11.1	47.8 ± 14.2	65.6 ± 15.3	43.2 ± 14.8	T*R	**0.025**
Content	Cherry	48	77.5 ± 9.2	68.1 ± 12.6	80.9 ± 7.9	74.4 ± 12.2	T	0.095
	Placebo		76.7 ± 10.6	60.7 ± 18.5	75.8 ± 13.8	66.1 ± 17.8	T*R	0.142
Calm	Cherry	49	74.2 ± 9.6	57.3 ± 13.5	76.0 ± 13.1	64.7 ± 15.3	T	0.573
	Placebo		65.1 ± 18.0	54.4 ± 13.1	74.8 ± 13.7	61.8 ± 18.4	T*R	0.748
Mental fatigue	Cherry	46	53.5 ± 18.1	72.3 ± 10.5	48.8 ± 21.9	56.0 ± 22.9	T	**0.042**
	Placebo		59.0 ± 15.1	72.4 ± 21.7	56.6 ± 13.3	64.3 ± 24.6	T*R	0.130

Abbreviations; repetition (rep). Effects are treatment (T) and treatment by repetition interaction (T*R). * Significant difference between treatments ($P < 0.05$).

5.3.6 Blood markers

Table 14. Plasma markers

	Cherry (*n* = 19)	Placebo (*n* = 21)	Mixed model		
			Difference (95% CI)	F	P-Value
Insulin¶ (pmol/L)					
Baseline	20.3 ± 15.4	19.6 ± 10.2	-0.02 (-0.2, 0.2)	0.050	0.824
3 months	17.8 ± 10.0	19.0 ± 13.0			
Glucose (mmol/L)					
Baseline	5.4 ± 0.5	5.5 ± 0.5	-0.03 (-0.3, 0.2)	0.049	0.826
3 months	5.4 ± 0.5	5.4 ± 0.3			
HOMA-IR¶					
Baseline	0.7 ± 0.6	0.7 ± 0.4	0.03 (-0.1, 0.2)	0.089	0.767
3 months	0.7 ± 0.4	0.7 ± 0.5			
hs-CRP¶ (mg/L)					
Baseline	1.6 ± 2.3	1.2 ± 1.2	0.1 (-0.2, 0.3)	0.488	0.489
3 months	1.4 ± 1.7	1.2 ± 1.0			
Triglycerides (mmol/L)					
Baseline	1.2 ± 0.6	1.2 ± 0.7	-0.08 (-0.5, 0.4)	0.115	0.736
3 months	1.2 ± 0.7	1.3 ± 0.7			
Cholesterol (mmol/L)					
Baseline	5.3 ± 1.2	5.0 ± 1.0	0.1 (-0.6, 0.8)	0.084	0.773
3 months	5.2 ± 1.1	5.1 ± 0.9			
LDL cholesterol (mmol/L)					
Baseline	3.2 ± 1.0	3.0 ± 1.0	0.08 (-0.6, 0.7)	0.060	0.808
3 months	3.1 ± 1.0	3.1 ± 1.0			
HDL cholesterol (mmol/L)					
Baseline	1.6 ± 0.3	1.5 ± 0.4	0.1 (-0.2, 0.3)	0.191	0.665
3 months	1.5 ± 0.4	1.5 ± 0.4			
Non-HDL cholesterol (mmol/L)					
Baseline	3.7 ± 1.2	3.5 ± 1.2	0.04 (-0.7, 0.8)	0.010	0.919
3 months	3.7 ± 1.1	3.6 ± 1.1			
Total/HDL cholesterol ratio					
Baseline	3.5 ± 1.0	3.7 ± 1.3	-0.2 (-1.7, 0.7)	0.224	0.693
3 months	3.6 ± 1.2	3.8 ± 1.4			

Mean ± SD. Abbreviations: high-density lipoproteins (HDL); homeostatic model assessment of insulin resistance (HOMA-IR); high-sensitivity C-reactive protein (hs-CRP) ¶ log transformed before analysis, raw values are presented

After adjusting for baseline (pre-treatment) values, sex and medication there were no group differences between cherry juice and placebo for lipid profiles, insulin, glucose, HOMA-IR or hs-CRP (**Table 15**).

5.4 Discussion

The aim of the current study was to determine whether long term (3 month) supplementation with Montmorency tart cherry juice (MC) could positively impact risk factors of CVD in middle-aged adults. The primary outcome of interest was a change in vascular function, specifically SBP based on previous studies. However, on the basis of the vascular function variables measured in the current study, contrary to the hypothesis, there was no effect on BP, endothelial function or arterial stiffness (**Table 11**).

Our research group has previously shown that an acute bolus of 60 ml MC concentrate, presumably because of its anthocyanin content, can reduce SBP by approximately 6 mmHg in middle-aged individuals (Keane et al., 2016c). More recently, Chai and colleagues (2018) reported that twice daily supplementation with 30 ml MC concentrate for 12 weeks reduced SBP by 4.1 mmHg in older adults. These data are of interest because hypertension is one of the leading risk factors for CVD and is responsible for 10.7 million deaths globally (Forouzanfar et al., 2016). Moreover, observational studies involving persons without CVD have shown there is a continuous relationship with risk throughout the normal range of usual SBP down to at least 115 mmHg, particularly in those aged 40-69 years (Lewington, 2003). Similarly, lower SBP in middle-aged and older adults has been associated with reduced risk of CVD and all-cause mortality (Antikainen, Jousilahti, & Tuomilehto, 1998; Bundy et al., 2017). Thus, determining if a natural anthocyanin-rich food such as concentrated MC juice, which could be incorporated to the diet to reduce SBP is of great importance.

In the current study we did not observe any difference in BP following 3-month MC consumption when compared to an isocaloric placebo. The reason for these discrepancies could be several-fold, for example MC concentrate has been shown to increase circulating phenolic acids, which modulate VSMC. In addition, peak plasma phenolic acids (PCA and VA) have been shown to coincide with the greatest post-prandial reductions in SBP (Keane et al., 2016a; Keane et al., 2016b). Moreover, MC studies have shown that, at least acutely, this postprandial

vasomodulation of SBP returns to basal levels within 3-4 hours (Desai et al., 2019; Keane et al., 2016b). If the vaso-relaxatory properties of MC are solely related PCA and VA (Keane et al., 2016a; Keane et al., 2016b), there is no information regarding to the long-term accumulation of these simple phenolic acids from tart cherries. However, results from other anthocyanin-rich foods (e.g. chokeberry) suggest that plasma concentrations of these are increased following acute intake (2 hours), but not chronic (12 weeks) intake (Istas et al., 2019a). Moreover, blueberry intake has shown no change in plasma PCA (Feliciano, Istas, Heiss, & Rodriguez-Mateos, 2016) and a decline in urinary anthocyanins (Kalt, McDonald, Vinqvist-Tymchuk, Liu, & Fillmore, 2017) after longer-term supplementation. These data suggest a reduction in exposure to anthocyanins metabolites following long-term ingestion, perhaps due to systemic saturation over time. In the current study vascular function was measured after an overnight fast, therefore peak vasodilation might have been missed because of the rapid metabolism and/or excretion of PCA and VA (Keane et al., 2016a); notwithstanding, this investigation was ultimately interested in the cumulative influence of MC consumption.

Both tart (Chai et al., 2018) and sweet cherry juice (Kent et al., 2017a) have been reported to reduce SBP in older adults following longer-term (12 week) supplementation. The reason for sustained changes in vascular function following chronic anthocyanin and other polyphenol supplementation are currently not known but are thought to be mediated via host-microbiome co-metabolism of these compounds and complex gene expression alterations (Istas et al., 2019a; Krga et al., 2016; Rodriguez-Mateos et al., 2019a). Therefore, the genetic changes and reductions in microbial diversity that occur with age (Barrera-Reyes, de Lara, González-Soto, & Tejero, 2020; Filosa, Di Meo, & Crispi, 2018), could partially explain the observed differences during periods across the lifespan or why some individuals benefit more than others. Indeed, in a recent study with a similar population to the current study, MC supplementation was not shown to have any influence on microbial diversity or richness (Lear et al., 2019), but these individuals were likely to have higher numbers and diversity of many protective species, compared to older adults (de la Cuesta-Zuluaga et al., 2019; Mariat et al., 2009). Although, another important distinction is the aforementioned studies (Chai et al., 2018; Kent et al., 2017a) used participants with elevated baseline

SBP (>130 mmHg). In a recent review of the factors that influence the efficacy of anthocyanins on BP regulation, the authors highlight that baseline BP was an important factor with changes only evident in those with elevated initial BP (Vendrame & Klimis-Zacas, 2019). This is consistent with antihypertensive drugs in which the magnitude of change in BP is directly proportional to baseline values (Hu et al., 2017; Motaweih et al., 2015). Despite recruiting middle-aged individuals with additional risk-factors for CVD, the participants in the current study were either pre-symptomatic or had controlled hypertension, thus had BP readings within the normal range, therefore any changes might have been too small to detect. In fact, their vascular function on a whole was similar to the healthy younger cohort in Chapter 4, and well within normal expected ranges, thus the most likely reason for a lack of response. The finding that longer-term tart cherry supplementation does not influence SBP in normotensive individuals, even those with increased CVD risk (Johnson et al., 2020; Martin et al., 2018), is in line with others (Desai et al., 2018; Lynn et al., 2014).

Endothelial dysfunction is the earliest indicator of subclinical atherosclerosis and precedes hypertension, thus is a major risk factor for CVD, but also modifiable by diet (Houston, 2018). There is a growing interest in the influence of fruit polyphenols on non-invasive measures of functional vascular health. For instance, in a recent meta-analysis, chronic supplementation (≥1 week) with anthocyanin-rich foods was shown to improve endothelial function (SMD: 0.84%, 95% CI: 0.55, 1.12), measured by FMD (Fairlie-Jones et al., 2017). It was therefore anticipated that an anthocyanin-rich food, such as MC concentrate, could improve endothelial function in middle-aged adults. Cherry juice did appear to improve FMD above the TE expected, but given the high variability (Chapter 4) this study might have been underpowered to detect the small changes that are reported in healthy populations following anthocyanin-rich foods (Istas et al., 2019a; Rodriguez-Mateos et al., 2019a). Nonetheless, the finding in the current study is consistent with Aboo-Bakkar et al. (2018) who found 4 week supplementation of MC powder (256 mg/day anthocyanins split into two doses, morning and evening) did not improve FMD response in overweight, hypertensive, yet healthy, middle-aged men following an overnight fast. Polyphenols, including anthocyanins, are purported to improve endothelial function through the ability to increase NO bioavailability (Edwards et al., 2015).

However, unlike some anthocyanin-rich foods which have been shown to increase systemic markers of NO under resting conditions (Johnson et al., 2015; Stote et al., 2017), tart cherries have not (Aboo-Bakkar et al., 2018; Keane et al., 2018; Keane et al., 2016b; Keane et al., 2016c). Albeit, MC powder has been reported to restore FMD and enhance recovery of plasma nitrite following occlusion induced ischemia reperfusion (Aboo-Bakkar et al., 2018), which could suggest that tart cherries might protect vascular cells in response to additional stress (i.e. pathological conditions).

In the present study arterial compliance was measured by PWV and PWA (AIx and AIx@75). Previous research has reported no effect of MC on these indices, at least acutely (Keane et al., 2016b). However, a recent addition to the literature suggested MC might influence AIx at 2 hours after a bolus MC concentrate in individuals with metabolic syndrome, which also coincided with peak reductions in SBP (Desai et al., 2019). In contrast, Lynn and colleagues (2014) reported no effect on brachial-knee PWV in healthy middle-aged adults, following 6 week supplementation with 30 ml MC concentrate in an open-labelled randomised controlled trial. More recently, Johnson and colleagues (2020) reported no effect of 12-week bi-daily MC consumption on AIx, AIx when corrected for a Hr of 75 beats/min or PWV in individuals with metabolic syndrome. Here we also found no noteworthy changes in PWV or PWA. These findings are in line with a recent meta-analysis which reported no change in PWV or AIx following longer-term (1 week – 4 month) supplementation with anthocyanin-rich foods (Fairlie-Jones et al., 2017). However, tart cherries have been shown to improve cholesterol (Chai et al., 2018), oxidised LDL (Johnson et al., 2020) and antioxidant status following a high fat meal (Polley et al., 2019). Moreover, epidemiological evidence suggests higher intake of anthocyanins is associated with lower arterial stiffness (Jennings et al., 2012) and a reduced risk of CHD and CVD mortality (Chapter 3). This suggests some anti-artherogenic properties and the ability to reduce stiffening of the arteries; however, it might be that the relatively short duration of existing studies is not sufficient to distinguish these changes. Furthermore, the acute influence on AIx is likely to represent a functional change in compliance (De Bruyne et al., 2019), whereas the time span needed to determine any preservation or even reversal of structural stiffening of blood vessels would likely require more longitudinal observations, such as those reported in Chapter 3.

Apart from vascular function we also measured the effects of MC concentrate on CBF, cognition, sleep and mood. It was found that MC improved alertness and mental fatigue (**Table 14**), consistent with the anti-fatigue effects in response to cognitive demand batteries reported with other polyphenols (Massee et al., 2015; Wightman et al., 2015). As this was not associated with any change in sleep or CBF measures it is unlikely that these were involved. However, tart cherry polyphenols have been shown to elicit anti-neuro-inflammatory properties and to suppress neuronal apoptosis and stimulate pro-survival signalling cascades (Kim et al., 2005; Shukitt-Hale et al., 2016; Thangthaeng et al., 2016) − mechanisms that might protect against depression pathophysiology (Ogle et al., 2013). In addition, anthocyanin-rich blackcurrant juice has been shown to inhibit Monoamine Oxidase (MAO)-B activity (Watson et al., 2015). As MAO is involved in the oxidation of neurotransmitters, including the regulation of serotonin, inhibiting MAO has been suggested as a viable method to reduce anxiety, depression and fatigue (Fiedorowicz & Swartz, 2004). Moreover, specific tart cherry polyphenols, e.g. quercetin, similarly to caffeine, has been shown to act as an adenosine receptor antagonist (Alexander, 2006). Thus, the ability of anthocyanins to influence neurotransmitters offers a potential avenue in which they may promote positive mood states following longer-term supplementation This data is of interest and warrants further attention, since the anti-fatiguing effect of polyphenols might improve physical and cognitive performance (Matsukawa et al., 2017; Wightman et al., 2015). Nonetheless, although these data seem promising, due to the subjective nature of the VAS used and that these effects were not mirrored in cognitive or physical performance in the current study, it should be interpreted cautiously.

With regards to CBF we did not find any differences following 3-month supplementation. CBF and velocity is an important factor in optimal brain functioning and subsequently cognition, because it regulates the supply of oxygen and glucose to the neurons, as well as the removal of CO_2 and metabolites (Ajmani et al., 2000). Previous work from our research team has shown that MC concentrate can modulate CBF variables, specifically HbO_2 and tHb during cognitive function tasks (Keane et al., 2016c). In the current study we did not identify any changes in these parameters in response to the MC intervention (**Figure 14**). There is no directly comparable study, and hence this

represents the first study to determine the longer-term effects of MC on NIRS in response to cognitive performance. However, Wightman and colleagues demonstrated that both resveratrol (Wightman et al., 2015) and *Sideritis scardica* (Wightman et al., 2018) supplementation induced acute but not chronic changes in CBF parameters measured by NIRS. In fact, in a recent review CBF changes following longer-term polyphenol supplementation was only apparent using MRI, highlighting the difficulty in the methodology surrounding measurements of CBF (Joris, Mensink, Adam, & Liu, 2018). For instance, Bowtell et al. (2017) reported regional changes in brain perfusion measured by MRI following 12-week supplementation with blueberry concentrate. In the current study, we used continuous wave NIRS and the limitations surrounding this are well documented, namely it only measure relative changes in cerebral activation and CBF as opposed to the measurement of absolute, quantifiable, amounts of haemoglobin present within the cortex (Jackson & Kennedy, 2013; Murkin & Arango, 2009). Moreover, NIRS was only used on the prefrontal cortex and it is therefore possible some changes occurring elsewhere in the cerebral cortex were not detected.

With regards to cognitive function, both tart and sweet cherries have failed to show an acute improvement in cognitive performance (Caldwell et al., 2016; Keane et al., 2016c). Contrastingly, in a chronic study by the same group (Kent et al., 2015) it was reported that the daily consumption of sweet cherry juice for 12 weeks improved cognitive performance across almost all tasks in older adults with mild-to-moderate dementia; this group showed improvements for category verbal fluency and tasks relating to verbal learning and memory and concluded the positive changes have clinical relevance for these cognitive improvements. More recently, Chai et al. (2019b) reported improvements in visual sustained attention and spatial working memory in healthy older adults after 12 week supplementation with MC concentrate. It would therefore appear that longer-term cherry interventions might have the potential to induce sustained modifications to cognition. Despite this hypothesis we did not observe any differences in cognitive performance compared to a placebo (**Table 13**). A probable reason for this disparity is that in both aforementioned studies, improvements in cognition were predicated by lower SBP post-supplementation with cherry juice. High BP, due to its role in hypoperfusion, is a risk factor for late-life cognitive decline and risk of dementia in middle-aged and older adults (Qiu, Winblad, & Fratiglioni, 2005).

Therefore, the previously observed changes in cognitive performance in older adults might be in direct response to improvements in cerebral perfusion, as supported by changes in peripheral blood flow, i.e. SBP (Chai et al., 2019b; Kent et al., 2017a). In the present study we did not observe any changes in SBP or CBF and therefore it is perhaps unsurprising there was no difference in cognitive performance. Alternatively, the differences between our findings and previous work might be because of the different tasks used, despite measuring similar domains (i.e. memory and sustained attention), some tasks may have been more sensitive in detecting changes. Also it may be related to the population, for instance, older individuals are could more amenable to changes in cognitive performance despite using a similar task as there is a rapid decline in the ability to organize incoming and outgoing information that happens with the progression of age (Kirchner, 1958). It should also be acknowledged that despite familiarisation, due to incorrect performance of the tasks, a number of participants were excluded from analysis in this study which reduced statistical power and might have confounded the results.

We also collected sleep measures in the current study. There was no difference found in sleep measures, as assessed by the PSQI (**Table 12**). This contradicts previous research which showed that tart cherries, due to their melatonin content, improved sleep quality (Howatson et al., 2012; Losso et al., 2017; Pigeon et al., 2010). However, this is likely because of the use of a questionnaire rather than any objective measures of sleep quality. For example, previous research from this institution reported improved sleep efficiency and total sleep time measured by actigraphy, following 7 day consumption of MC, but the same measures collected by subjective questionnaires did not differ (Howatson et al., 2012). Therefore, there might have been some subtle differences in sleep quality in response to the intervention that were not observed, but importantly there were no substantial changes in the sleep patterns of participants, which might affect outcome measures.

This study also identified no changes in markers of metabolic health or inflammation following the intervention (**Table 15**). This is somewhat surprising given that MC have previously been shown to reduce LDL cholesterol (Chai et al., 2018), insulin levels (Desai et al., 2019) and hs-CRP (Martin et al., 2018). However, these findings are not alone, given that tart cherries have failed to

influence cholesterol (Lynn et al., 2014), insulin concentrations/ resistance (Martin & Coles, 2019) and markers of systemic inflammation (Lear et al., 2019) in similar populations in this study. In the current study the levels of these markers before the intervention were all within normal ranges and thus, similar to BP, less likely to benefit from an intervention, relative to those with elevated baseline values, i.e. older individuals, those with metabolic or inflammatory conditions (Chai et al., 2018; Desai et al., 2019; Martin et al., 2018; Schumacher et al., 2013). Therefore, the discrepancies between studies might once again be due to the population in question. Importantly, early intervention in at-risk individuals (such as those in the current study) may be particularly opportunistic in reversing or reducing the disease risk trajectory preventing CVD and type II diabetes and thus remains a research priority. Certainly, there is longitudinal population studies (Jennings, Welch, Spector, Macgregor, & Cassidy, 2014; Wedick et al., 2012) that demonstrate higher intake of dietary anthocyanins is associated with reduced inflammation, insulin resistance and risk of type II diabetes (which are all also cardiovascular risk factors). Therefore, future studies should try to establish the optimum dosing, delivery and duration of anthocyanin intake in healthy cohorts, but also whether they are beneficial in clinical populations. However, there was a marked effect of the intervention on body composition in both groups. After 3 months both body mass and BMI was higher in the placebo group, whereas fat percentage was higher in the cherry group and fat mass had increased in both groups (**Table 10**). While changes in fat mass following longer-term cherry juice consumption have not been reported elsewhere (Johnson et al., 2020; Martin et al., 2018), changes in body composition are not in isolation. For example, Chai and colleagues (2018) reported a higher BMI (1.06 Kg/m^2; $P = 0.02$) in those consuming MC concentrate for 12 weeks. Moreover, Lynn and colleagues (2014) reported a trend for higher body mass in the placebo group ($P = 0.073$) following 6 week MC intervention in middle-aged adults. A limitation of the present study is that dietary records and IPAQ were only collected at the beginning and end of the study as opposed to throughout, although total energy intake, physical activity or exercise capacity did not appear to change, we cannot rule out that changes may be seasonal and un-related to the intervention (Ma et al., 2006). However, given the increase in calorie intake due to the concentrate future studies should modify dietary intake to offset the incorporation of this in the diet.

Finally, there were no change in exercise capacity from MC in middle-aged adults. A finding that contradicts a recent meta-analysis, which suggested MC can improve endurance exercise performance (Gao & Chilibeck, 2020). However, based on pre-existing literature, performance aspects appear to be most beneficial in all-out or pro-longed exercise (Keane et al., 2018; Levers et al., 2016; Morgan et al., 2019), whereas here a short sub-maximal test was applied Moreover, this represents the first longer-term study, where previous MC dosing strategies have been relatively short term (≤ 7 days). Therefore previous enhancements might be due to acute influences of the bioactive compounds, whereas it has been speculated longer intake durations may be required to result in beneficial changes in cellular signalling (Cook & Willems, 2019). To date, there have been few studies determining the effect of polyphenols on exercise capacity but our findings are in agreement that 7-day New Zealand blackcurrant (Cook et al., 2020) and 4-week resveratrol supplementation (Voduc, La Porte, Tessier, Mallick, & Cameron, 2014) had no effect on exercise capacity.

Due to the low number of adverse events, good compliance levels reported and no effect on quality of life indices, it is reasonable to suggest that cherry juice is a safe and tolerable intervention. This study has several strengths such that it was successfully blinded, relatively large sample size, longer-term supplementation period and well controlled compared to other studies of a similar nature (Ataie-Jafari et al., 2008; Chai et al., 2018; Desai et al., 2019; Johnson et al., 2015; Lynn et al., 2014). However, there are other limitations that warrant discussion. Firstly, compliance to the intervention was self-reported; as these were free living adults, there was no control over whether they adhered to the intervention, how they stored the concentrate or when they consumed the MC. Secondly, two different juices were used in the current study due to a change in supplier, however both were confirmed to contain anthocyanins and high levels of polyphenols, but in varying amounts (**Table 9**). Moreover, subgroup analysis of the two different juices was done on vascular function which did not change the findings, thus suggesting the use of two different juices did not influence the outcomes or conclusion of this study. Whilst the variation between the juices is a potential limitation of the current work, it equally represents the batch-to-batch variation that can naturally occur due to growing conditions (e.g. soil, use of fertiliser, time of year, weather), storage, and processing. Since analysis was

done at the end of the study after storage, it adds confidence that the bioactive compounds were in good availability over the study duration. Moreover, given that these compounds will likely be extensively metabolised (Section 2.1.3), determining the compounds in the urine as a means of compliance and bioefficacy might add more clarity to these limitations. Lastly, BP was measured in the laboratory which was shown to be reliable (Chapter 4) but 24-hour ambulatory BP could be more advantageous in establishing small changes in BP following MC supplementation (Desai et al., 2020) due to the large number of readings and avoidance of white-coat hypertension.

In conclusion, the current study investigated whether longer-term supplementation with MC concentrate was capable of improving vascular function and, in turn, cognitive performance or exercise capacity. The results of this study revealed that a 3-month intervention was unable to improve these indices in this cohort. This study addresses the final specific aim of the thesis to which was to determine the longer-term effects of MC on physiological and cognitive function in in free-living middle-aged adults. These data have not found a causal relationship for the reduced risk of CVD mortality in Chapter 3, but this should be taken in light of the limitations described throughout. The findings are in line with previous literature that suggests tart cherry polyphenols might only be effective in modulating vascular and metabolic function in select populations. Moreover, this study has for the first time investigated the effects of long-term supplementation on indices of CBF, cognitive function and exercise capacity in a middle-aged population. Although there was some evidence that MC could improve alertness and mental fatigue during cognitive demand task we did not find any effect of the intervention on CBF, cognitive or physical performance, which is an important addition to the body of knowledge. Ultimately, MC concentrate was shown to be safe and well tolerated and importantly did not have any deleterious effects on these outcomes, further investigation might be better suited in populations with greater impairments in vascular, metabolic or cognitive function at baseline. Nonetheless, given the current interest in the facilitation of healthy aging, investigation in to the intake of anthocyanin-rich foods in pre-symptomatic and at-risk individuals are important as a means to preserve cardiovascular and cognitive health, but more longitudinal observations are required.

Chapter 6:

General Discussion

6.1 Experimental chapter synopsis

The research presented in this thesis has systematically examined evidence for the cardiovascular health benefits of dietary anthocyanins, specifically through knowledge synthesis and knowledge generation. This is in response to a number of studies that have recently established that tart cherries and berries, purportedly due to their high anthocyanin content, can reduce a number of risk factors associated with CVD (Section 2.2). Nevertheless, the relationship specifically between dietary intake of anthocyanins and actual risk of CVD has yet to be systematically addressed. Therefore, in Chapter 3 the association between dietary intake of anthocyanins and the risk of CVD and related mortality was systematically evaluated and synthesized from existing prospective cohort studies. This is an important addition to the literature as to date this is the largest and most contemporary and comprehensive meta-analysis of dietary anthocyanins and CVD risk. This was a necessary step as although randomised control trials suggest that supplementation with these compounds may improve factors of CVD, whether this could translate to reduced risk (in light of the 'CVD detection gap') was unknown. The results of this Chapter suggest there is an inverse association between anthocyanin intake and reduced risk of both CHD and CVD mortality, which is an important addition to the body of knowledge. This perhaps represents an important steppingstone in informing policy makers into recommending incorporating foods rich anthocyanins as an adjunct to the diet, but dose-response data is still needed.

In Chapter 4 the test-retest reliability of a battery of non-invasive multimodal measures of vascular function *in vivo* was assessed. The use of these methods in clinical trials, including nutritional research is becoming more common. Despite the influence that these variables can have on each other, the variability or typical error of doing them together in a single testing session (i.e. as a test battery) had not been reported. Therefore, as a prelude to Chapter 5 the variability in vascular function measures which were investigated and considered when designing an intervention. For example, measures of microvascular endothelial function, measured by LDI and DVP-RI, where found to have low reproducibility when taken in a test battery. On the other hand, it was found that BP, arterial stiffness (PWV/A) and macrovascular endothelial function (FMD) could be measured with adequate reliability and hence were adopted in the final experimental study.

Moreover, the results were used to form the power calculation for the intervention. In addition, these data have been added to the literature to highlight the importance of testing the reliability of multiple measures taken in a single session and methodological considerations for future research designs.

Lastly, in an attempt to provide a causal relationship to support the findings of Chapter 3, Chapter 5 consists of the main experimental randomised controlled trial which investigated the influence of MC on cardiovascular risk factors, as well as cognitive function and exercise capacity in middle-aged adults. This study has several strengths such that it was successfully blinded, relatively large sample size, of longer duration and well controlled compared to other studies of a similar nature. However, despite this the results of Chapter 5 showed no significant effect of MC on modulation of vascular or metabolic function, cognitive performance or exercise capacity. There was some evidence that tart cherries could improve alertness and metal fatigue, but also might influence body composition.

These data extend previous findings which suggest that the influence of MC concentrate is directly related to the characteristics of the cohort. In the current thesis despite being at elevated risk of CVD the middle-aged adults had no identifiable issues with their vascular, metabolic and/or cognitive function at baseline and thus less responsive to the intervention. Importantly, midlife has been consistently highlighted as a critical turning point in prevention of CVD and neurodegenerative diseases, due to the progression of morphological and functional alterations to the vasculature (MacIntosh et al., 2020; Pandey et al., 2016). Although this study did not establish any difference in the risk factors or cognitive function measures in this study compared to a placebo it cannot be ruled out that the MC elicited some vasoprotective properties that were not observed, which may translate to the reduced risk established in Chapter 3. For example, Johnson and colleagues (2020) recently reported that MC juice had no influence on vascular function but did influence oxidized LDL and soluble vascular cell adhesion molecule-1 compared to a placebo, suggesting some anti-artherogenic properties that might have been apparent but missed. The final study of this thesis did however show that MC was a relatively safe and well tolerable intervention, but more longitudinal studies across different populations are still needed.

6.1.1 Limitations of the current findings

A number of limitations exist in the interpretation of the findings from this thesis, which have been discussed in each chapter. The following over-arching limitations are potential issues and criticisms of the work.

A limitation is the generalisation of dietary anthocyanins. In chapter 3 dietary intake of these compounds were shown to reduce risk of CHD and CVD mortality, but the pharmacodynamics of specific anthocyanins is not well understood. In the final intervention MC concentrate was used which has previously been shown to be a relatively rich source of cyanidin and to a lesser extent peonidin glucosides (Kirakosyan et al., 2009). However, other anthocyanin-rich foods such as blackcurrant and blueberries have shown promising health benefits and the main anthocyanins in these are delphinidin and malvidin, respectively (Cook & Willems, 2019; Stevenson & Scalzo, 2012), where the position of the hydroxyl group, and thus type of anthocyanin might be responsible for their biological effects (Rechner & Kroner, 2005).

Another important limitation of the current thesis is that emphasis has been placed on dietary anthocyanins in general. This was based on emerging evidence that these compounds might elicit some degree of cardioprotection (Keane et al., 2016b; Rodriguez-Mateos et al., 2019a). However, it should be acknowledged that these compounds were not studied in isolation. In fact, foods that are rich in anthocyanins (such as those in Chapter 3 and 5) are likely to be sources of additional health-promoting nutrients such as other phytochemicals, vitamins, minerals and fibre. The different pharmacokinetics, metabolites and thus cardiovascular health benefits of specific dietary anthocyanins and the synergistic activities of the other nutrients in these foods should be taken into consideration when interpreting the results of the present thesis. It was intended to analyse the urinary metabolome in order to shed some light on this limitation, unfortunately due to unprecedented circumstances this was not possible. But this would have for the first time shown potential metabolic pathways and excreted polyphenol metabolites following longer-term intake of MC.

6.2 Future research directions

There are several important factors that might impact the influence of dietary anthocyanins on cardiovascular health, which should be reflected in future research designs in order to strengthen the evidence base. Moreover, because

existing evidence for the health benefits of MC is so varied these factors need to be addressed to improve our understanding of the application of these to human health.

First of all, extrinsic factors (e.g. the dose, duration, timing, and food matrix) need to be considered. There is inherent difficulty in quantifying the dietary intake of anthocyanins, i.e. due to the method of quantification, different databases, limited information on retention of these compounds following cooking and seasonal variability. Moreover existing studies, see **Table 4** (Section 2.2) and **Table 4** (Section 3.3), are inconsistent in doses and the highest intake of these compounds, thus establishing an optimal dose was not possible. Therefore in the current thesis, MC was given as a concentrate, bi-daily in a dose that had previously been found to be physiologically relevant as described in Section 2.3. However, this is a rather reductionist approach. Future studies should try to elucidate the appropriate dosing strategy, duration and source of anthocyanin interventions, how and when they should be taken. Given that this area of research is expanding rapidly uniformity in the quantification and reporting of anthocyanin content of certain foods might afford the opportunity to address these areas and give specific recommendations based on the intake of these compounds. With regards to the matrix, while the majority of studies have utilised MC concentrate (Chai et al., 2018; Johnson et al., 2020; Keane et al., 2016b; Lynn et al., 2014; Martin et al., 2018), there is emerging evidence for the physiological effects of MC powder and extracts (Aboo-Bakkar et al., 2018; Desai et al., 2019; Levers et al., 2016; Morgan et al., 2019). Given the potential impact of the juice on body composition (seen in **Table 10**. Chapter 5) these might be more beneficial given the limited calorie content. Moreover, co-ingestion should be investigated given that there is some evidence that the bioavailability of anthocyanins can be reduced, delayed and/or inhibited by other commonly consumed foods such as oats (Walton, Hendriks, Broomfield, & McGhie, 2009), dairy (Xiao et al., 2017) and soy milk (Oksuz, Tacer-Caba, Nilufer-Erdil, & Boyacioglu, 2019). It therefore may be necessary to recommend with what and when to take the tart cherries but there is limited information available.

The second important point relates to intrinsic factors (e.g. age, health status, microbiome and genetic polymorphisms). As highlighted in the Section 2.2.2.1 the baseline characteristics of the population studied is likely to be a determinant

in the efficacy of anthocyanin supplementation. Anthocyanin-rich foods such as tart cherries have been shown to elicit antioxidant and anti-inflammatory properties (section 2.1), therefore it is plausible these interventions would likely benefit those with underlying pathologies due to the systemic pro-inflammatory and pro-oxidative state associated with these. Future studies should determine the influence of MC concentrate on clinical populations. However, it should be noted this is not to say that dietary anthocyanins might only beneficial in 'unhealthy' or symptomatic cohorts, but if continuing to research in healthy or at-risk population studies should examine the longitudinal effects as any physiological changes will likely be smaller and harder to detect. Moreover, it is likely that dependant on the population of interest there could be differences in dose-responses. For instance, in a recent dose-response study of purified anthocyanins serum IL-6 was lower after 12 week supplementation with higher doses of anthocyanins (80 and 320 mg) in individuals with dyslipidemia (Zhang et al., 2020). Whereas the same authors found that in healthy individuals the dose-response curve suggested 40 mg of anthocyanins reduced IL-6 more than higher doses in healthy young adults (Guo et al., 2020). It remains to be established whether clinical populations need higher doses of MC due to underlying inflammation associated with these. Finally, another important factor is the interplay between the microbiome and genetic variations and the consideration of how these might contribute to responders vs non-responders. Even in this relatively small study in Chapter 5 there were differing effects of the intervention on SBP (**Figure 13**). Large clinical trials investigating the effects of these interventions on microbial enterotypes (Mayta-Apaza et al., 2018) and nutrient-gene interactions (Krga et al., 2016) might shed some light on the intra- and inter-individual responses to anthocyanin interventions. Before we have a better understanding of the factors highlighted above, it is difficult to draw any conclusions on the benefits of these compounds in the diet since the existing evidence base and the results of this thesis are inconclusive and equivocal.

6.3 Concluding remarks

The research summarised above has addressed the over-arching aim of this thesis provide novel insight into potential role of dietary anthocyanins in cardiovascular health. Firstly, Chapter 3 established an association between higher intake of dietary anthocyanins and reduced risk of CVD mortality.

Secondly, Chapter 4 highlighted important methodological considerations when investigating cardiovascular risk factors which was used to inform a final intervention. Lastly, Chapter 5 reports the influence of anthocyanin-rich MC on vascular function, cognitive and physical performance. Collectively, these studies progress scientific evidence and importantly have contributed to knowledge around how dietary anthocyanins can impact health outcomes in humans and influence chronic diseases. Moreover, the future directions outlined above can provide further clarity and understanding to the findings presented within this thesis. Although this thesis addresses knowledge gaps in relation to the longer-term health effects of dietary anthocyanins and corroborates previous literature, several important areas relating to the dietary sources and intra- and inter-individual variability in health outcomes still require attention.

Chapter 7:

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Chapter 8:

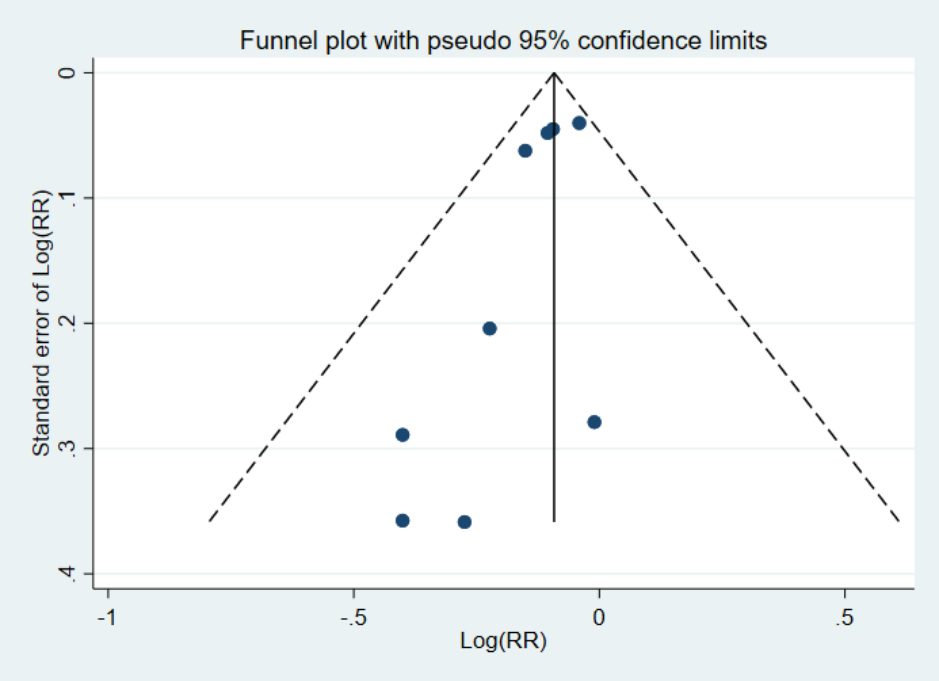
Appendices

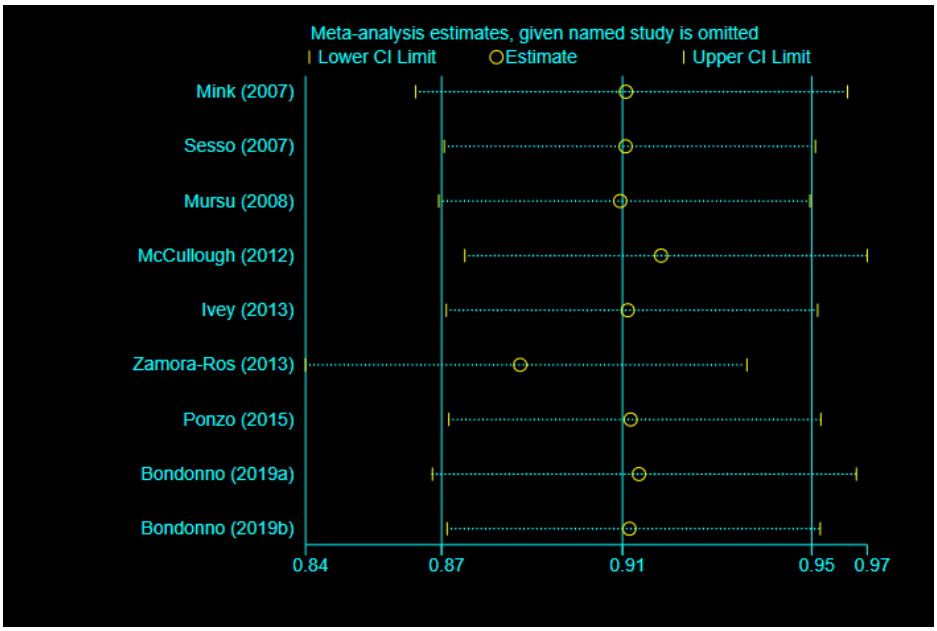
Appendix A: Systematic review search strategy

Table 1. Search Methods (originally run 25/01/2017)	
Item	
Search terms	((“flavonoid*” OR “anthocyani*” OR “berry” OR “berries” OR “chokeberry*” OR “aronia” OR “melanocarpa aubergine*” OR “brinjal*” OR “eggplant*” OR “solanum melongena*” OR “Guinea squash” OR “Solanum insanum” OR “black currant*” OR “Ribes nigrum” OR “blueberr*” OR “Vaccinium corymbosum” OR “Vaccinium cyanococcus” OR “blood orange*” OR “cherry” OR “cherries” OR “Cerasus vulgaris” OR “Prunus cerasus” OR “Prunus avium” OR “grape*” OR “rhubarb” OR “rheum rhabarbarum” OR “strawberr*” OR “fragaria vesca” OR “Fragaria ananassa” OR “blackberr*” OR “raspberr*” OR “rubus glaucus” OR “Rubus fruticosus” OR “plum” OR “plums” OR “red cabbage*” OR “purple cabbage*” OR “Brassica oleracea var capitata f rubra” OR “red wine” OR “cranberr*” OR “vaccinium macrocarpon*” OR “elderberr*” OR “sambucus Canadensis” OR “bilberr*” OR “vaccinium myrtillus” OR “whortleberr*” OR “pomegranate*” OR “Punica granatum”) AND ("heart disease*" OR "chd" OR "cardiovascular diseases" OR "cvd" OR "myocardial infarction" OR "ischemic heart disease" OR "stroke" OR "death" OR "mortalit*" OR "surviv*") AND ("prospective" OR "cohort" OR “nested case-control” OR “observational” OR “longitudinal”))
SCOPUS	
TITLE-ABS-KEY	*584*
MEDLINE (ProQuest)	
	966
Cochrane	
TITLE-ABS-KEY	67
CINAHL (EBSCO)	
	72
EndNote	*Total 1689*
	Duplicates Removed 1428

Appendix B: Funnel plot and sensitivity analysis

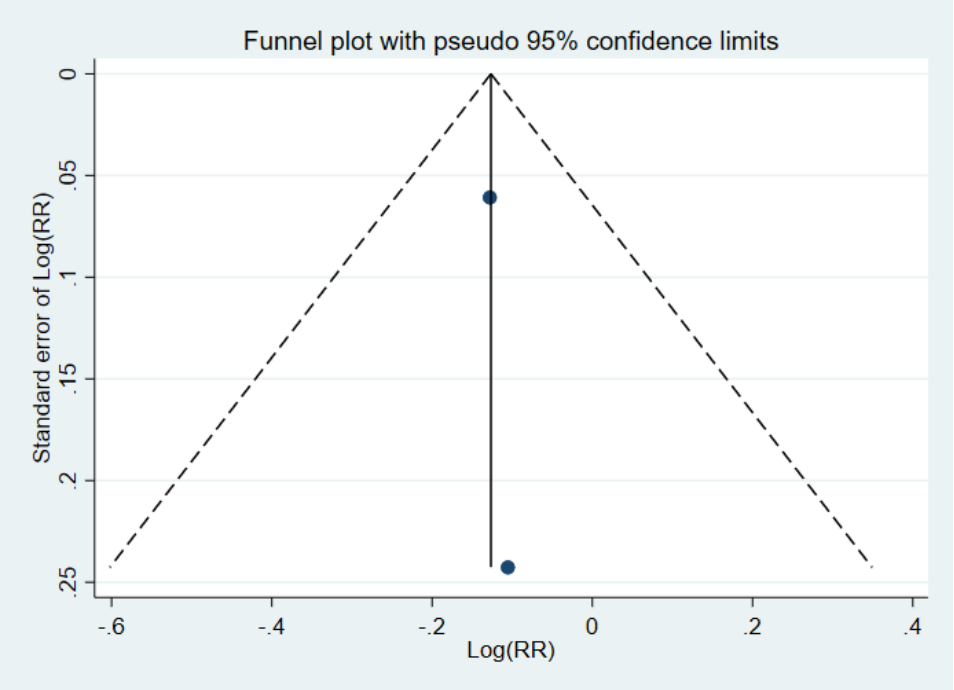
CVD mortality



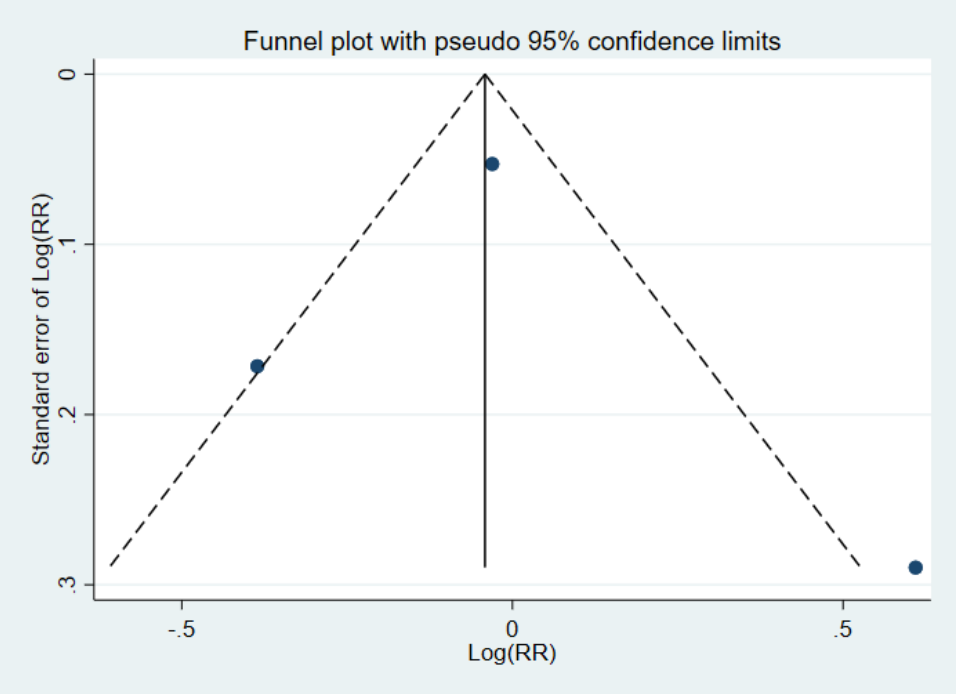


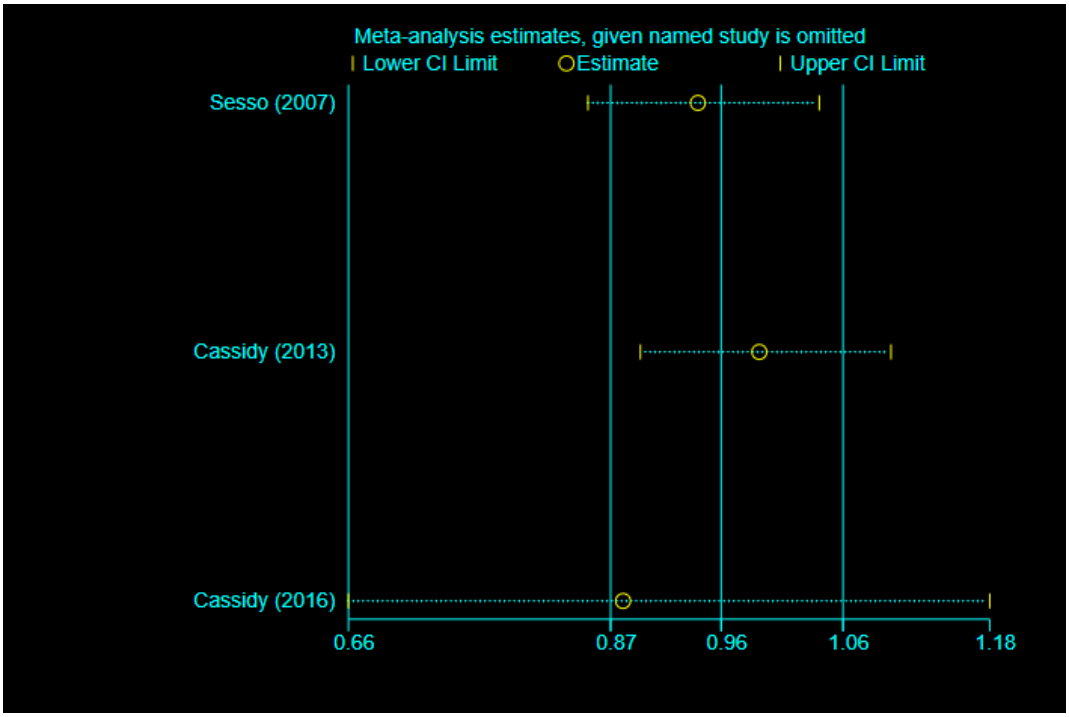
Study omitted	Estimate	[95% Conf.	Interval]
Bondonno (2019a)	.91579431	.86965281	.96438396
Bondonno (2019b)	.91366935	.87292892	.95631123
Ivey (2013)	.91329682	.87274605	.95573175
McCullough (2012)	.92074728	.87688029	.9668088
Mink (2007)	.91287488	.86588895	.96241039
Mursu (2008)	.91159666	.87106782	.9540112
Ponzo (2015)	.91392785	.8733049	.95644045
Sesso (2007)	.91280884	.87228024	.95522046
Zamora-Ros (2013)	.88924474	.84128881	.93993431
Combined	.91211388	.87169855	.95440303

CHD mortality



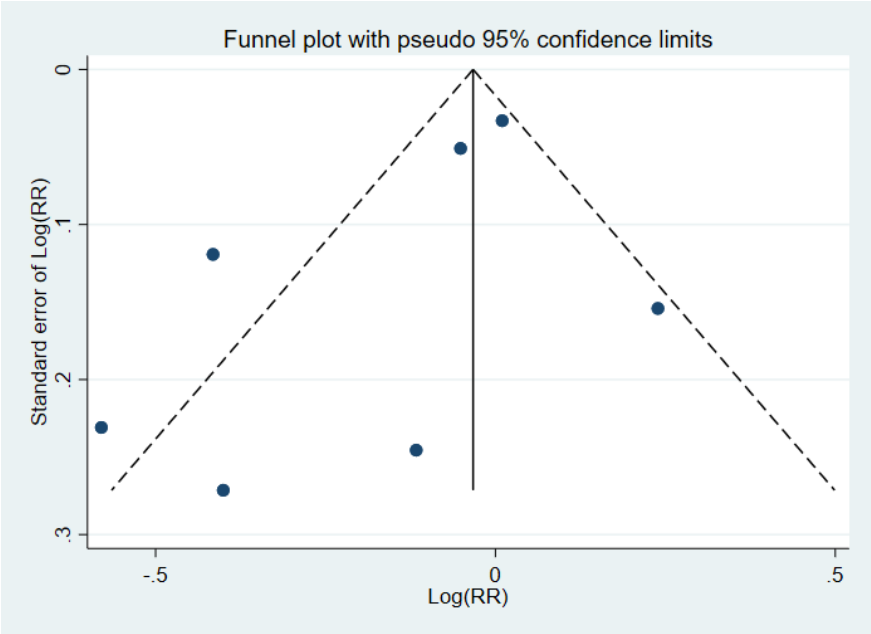
MI

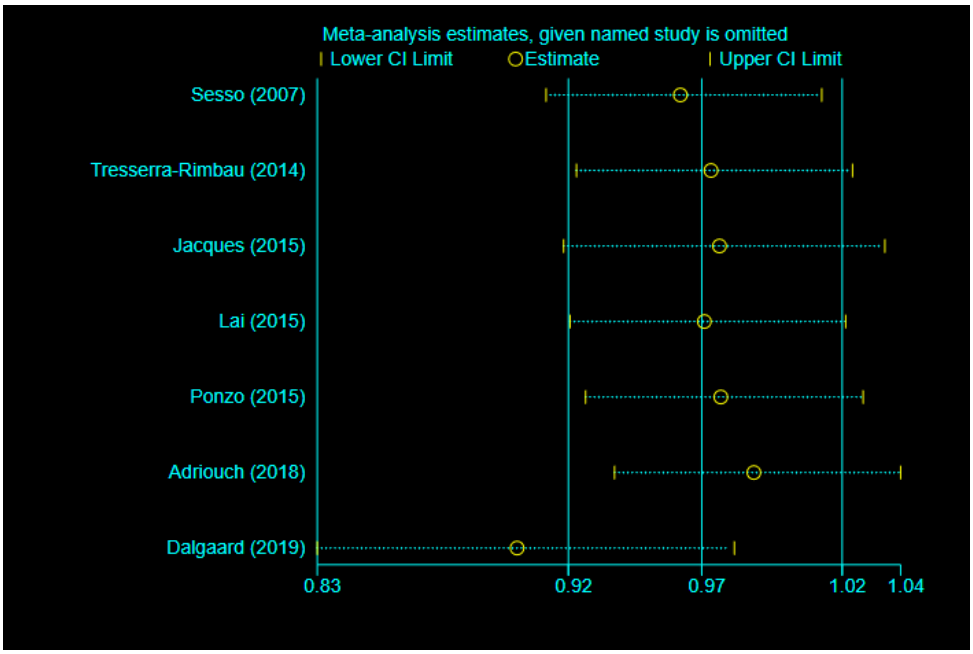




Study omitted	Estimate	[95% Conf.	Interval]
Cassidy (2013)	.99013674	.89431351	1.096227
Cassidy (2016)	.88034195	.65913522	1.175786
Sesso (2007)	.9406572	.85209078	1.0384291
Combined	.95939624	.87033911	1.0575661

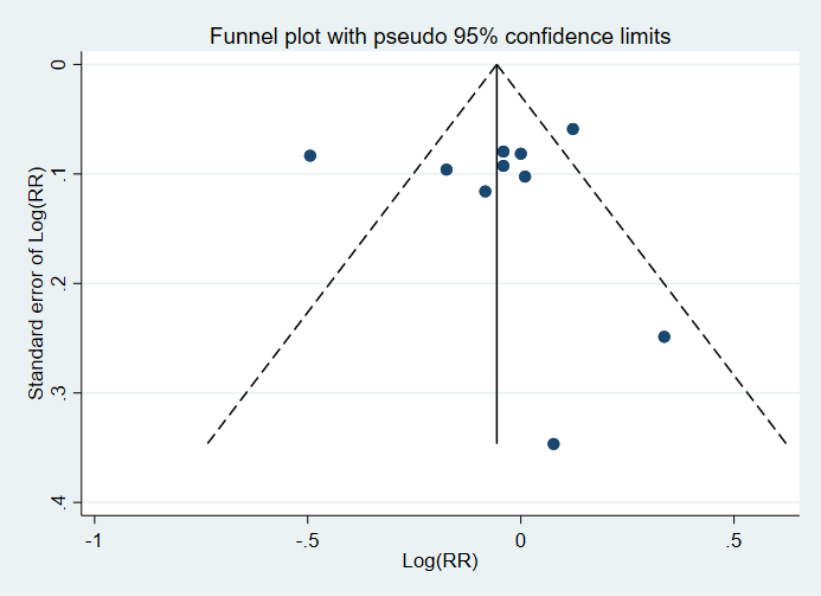
Total CVD

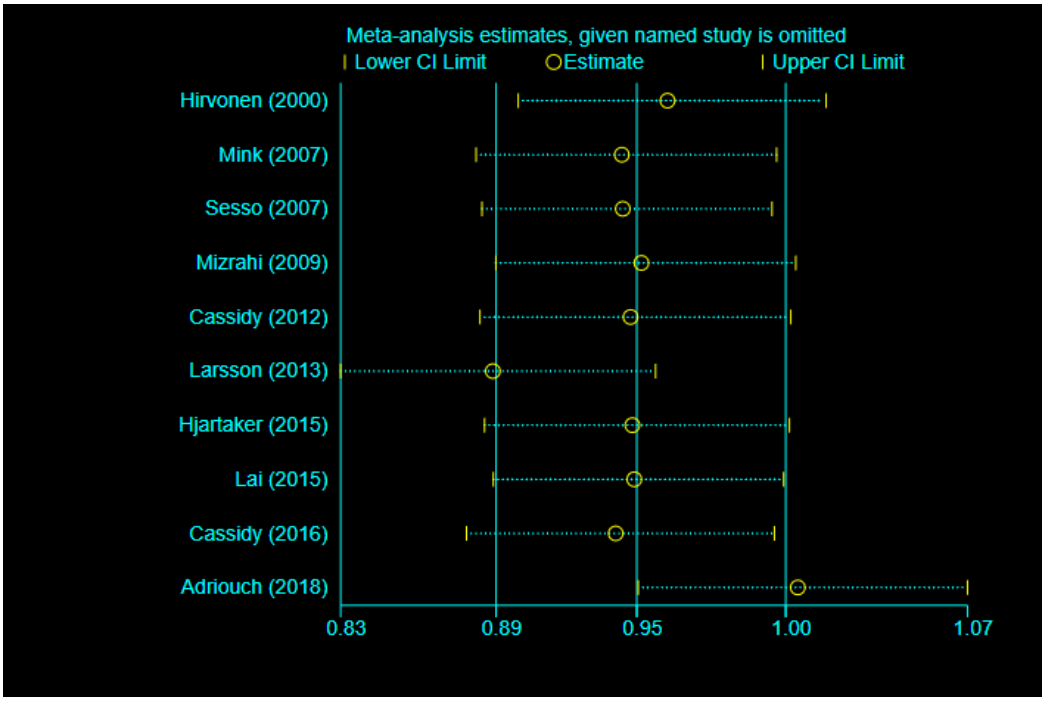




Study omitted	Estimate	[95% Conf.	Interval]
Adriouch (2018)	.98655128	.93607044	1.0397545
Dalgaard (2019)	.90075338	.82834011	.97949702
Jacques (2015)	.97409064	.91761994	1.0340365
Lai (2015)	.96860975	.91995203	1.0198411
Ponzo (2015)	.97458023	.92558676	1.026167
Sesso (2007)	.95991689	.91127598	1.0111541
Tresserra-Rimbau (2014)	.97101933	.92228997	1.0223234
Combined	.96768052	.91933884	1.0185642

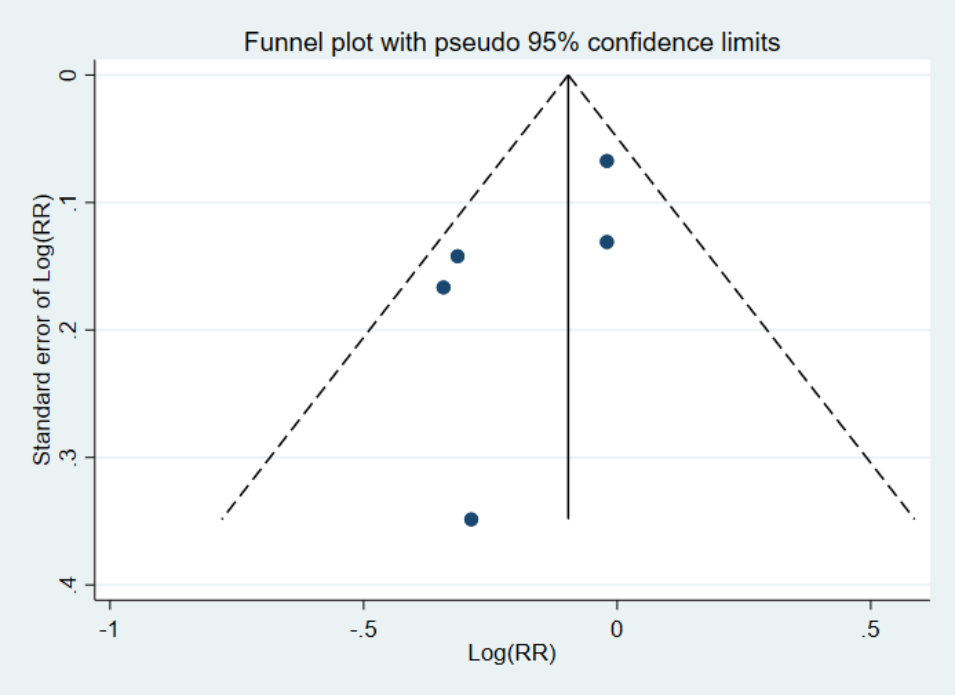
Stroke

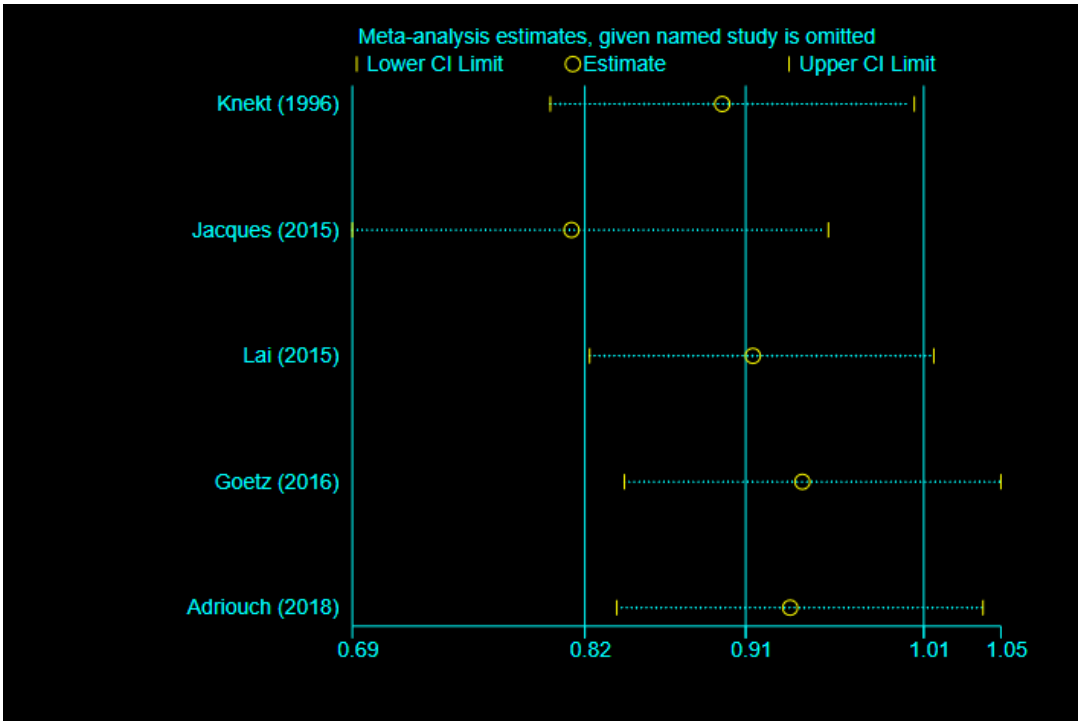




Study omitted	Estimate	[95% Conf.	Interval]
Adriouch (2018)	1.0057938	.94579893	1.0695943
Cassidy (2012)	.94293177	.88630426	1.0031773
Cassidy (2016)	.93733394	.8812477	.99698979
Hirvonen (2000)	.95681834	.9006896	1.0164449
Hjartaker (2015)	.94360352	.8880378	1.0026461
Lai (2015)	.9443301	.89132625	1.0004859
Larsson (2013)	.89116627	.83393335	.95232707
Mink (2007)	.93964517	.88485312	.99783009
Mizrahi (2009)	.94699609	.89229536	1.0050502
Sesso (2007)	.94000638	.88706988	.99610198
Combined	.94524057	.89237097	1.0012425

CHD





Study omitted	Estimate	[95% Conf.	Interval]
Knekt (1996)	.89526349	.80151957	.99997145
Jacques (2015)	.81306595	.69358194	.9531337
Lai (2015)	.91196311	.82293397	1.0106239
Goetz (2016)	.93895119	.84190416	1.0471849
Adriouch (2018)	.93227661	.83777159	1.0374422
Combined	.9080294	.82032044	1.0051162

Appendix C: Example informed consent form

INFORMED CONSENT FORM

Northumbria University NEWCASTLE

TITLE OF PROJECT: The effect of a fruit juice on physiological and cognitive function

Participant ID Number:

Researcher: Rachel Kimble

Researcher contact details: rachel.kimble@northumbria.ac.uk

Principal Investigator: Glyn Howatson

Please tick where appropriate

I have read and understood the Participant Information Sheet.	☐
I have had an opportunity to ask questions and discuss this study and I have received satisfactory answers.	☐
I understand I am free to withdraw from the study at any time, without having to give a reason for withdrawing, and without prejudice.	☐
I agree to take part in this study.	☐
I would like to receive feedback on the overall results of the study at the email address given below. I understand that I will not receive individual feedback on my own performance. Email address……	☐

Signature of participant..... Date.....……..

(NAME IN BLOCK LETTERS).....…….

Signature of researcher..... Date.....……..

(NAME IN BLOCK LETTERS)……..

INFORMED CONSENT FORM: REMOVAL AND STORAGE OF TISSUE

TITLE OF PROJECT: The effect of a juice on physiological and cognitive function

Participant ID Number:

Researcher: Rachel Kimble

Researcher contact details: rachel.kimble@northumbria.ac.uk

Principal Investigator: Glyn Howatson

I agree that the following tissue or other bodily material may be taken and used for the study:

Tissue/Bodily material	**Purpose**	**Removal Method**
Plasma	To analyse markers of inflammation, metabolic health and metabolites	Venepuncture
Buffy coat	To analyse for DNA genotypes	Venepuncture
Urine	To analyse for metabolomics profiles	Urination
Stool	To analyse for gut microbiome	Defecation

I understand that if the material is required for use in any other way than that explained to me, then my consent to this will be specifically sought. I understand that I will not receive specific feedback from any assessment conducted on my samples, but should any kind of abnormality be discovered then the investigator will contact me.

I understand that the University may store this tissue in a Licensed Tissue Bank only for the duration of the study, it will then be destroyed.

Method of disposal:

Clinical Waste [X]

Other [ ]
If other please specify.....

I consent to the University distributing this tissue to partners in this research study, outside of the University, for further testing (please tick the box if you agree). [ ]

Signature of participant..... Date.....……..

Signature of researcher..... Date.....……..

Appendix D: Treatment guess

Table 1. Chi-squared table for treatment guesses.

Treatment	**Guess**			Total
	Cherry	Placebo	Not sure	
Cherry	16	5	4	25
Placebo	12	5	8	25

Appendix E: Quality of life scores based on Short form-36

	Cherry	Placebo	ANOVA		
			Treatment	Time	Interaction
Physical functioning (%)					
Baseline	92 ± 2	89 ± 4	0.198	0.811	0.119
3 months	90 ± 2	96 ± 1			
RF physical (%)					
Baseline	96 ± 3	81 ± 7	0.724	0.825	0.150
3 months	90 ± 5	94 ± 5			
RF emotional (%)					
Baseline	87 ± 5	80 ± 6	0.802	0.147	0.652
3 months	93 ± 4	89 ± 5			
Energy/fatigue (%)					
Baseline	65 ± 3	56 ± 4	0.458	0.183	0.443
3 months	64 ± 4	60 ± 4			
Emotional well-being (%)					
Baseline	79 ± 3	71 ± 4	0.496	0.268	0.609
3 months	78 ± 3	74 ± 4			
Social functioning (%)					
Baseline	90 ± 4	85 ± 4	0.381	0.866	0.079
3 months	86 ± 6	93 ± 3			
Pain (%)					
Baseline	86 ± 3	84 ± 5	0.195	0.340	0.467
3 months	86 ± 6	88 ± 4			
General health (%)					
Baseline	68 ± 3	72 ± 3	0.722	0.391	0.652
3 months	68 ± 3	68 ± 3			
Health change (%)					
Baseline	54 ± 3	58 ± 3	0.871	0.149	0.452
3 months	53 ± 4	51 ± 3			

Mean ± SEM. Abbreviations; Role functioning (RF).