Polyphenol Pharmacokinetics and Cardiovascular, Cognitive and Exercise Pharmacodynamics following Montmorency Tart Cherry Intake in Humans

K Keane

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Polyphenol Pharmacokinetics and Cardiovascular, Cognitive and Exercise Pharmacodynamics following Montmorency Tart Cherry Intake in Humans

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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

Cardiovascular disease is the primary cause of global mortality (Naghavi, 2015). Given the global health issues associated with poor cardiovascular function, interventions that help reduce the severity and prevalence of these diseases would not only have economic implications, but would also improve health, wellbeing and quality of life. Epidemiological studies have suggested that polyphenol-rich foods can exert positive cardiovascular health benefits and as a result could reduce the severity of the primary pathology and increase the capacity to stay physically and mentally active (Joshipura et al., 1999; Bazzano et al., 2002; Hung et al., 2004).

One of most studied polyphenol-rich, functional foods in recent years, in both the clinical and exercise domains, has been tart cherries. Tart cherries and their derivatives are high in numerous polyphenols (Wang et al., 1999; Seeram et al., 2001; Seymour et al., 2014; Bell et al., 2014) that include the flavonoids isorhamnetin, kaempferol, quercetin, catechin, epicatechin, procyanidins, and anthocyanins (Kim et al., 2005; Kirakosyan et al., 2009). Indeed, there has been an enormous research effort over the past decade to delineate the physiological and biochemical effects that tart cherries (and its constituents) might afford, and how these effects could be exploited to improve health outcomes. There is now strong evidence that tart cherries attenuate inflammation (Wang et al., 1999), oxidative stress (Howatson et al., 2010; Bell et al., 2014) and accelerate exercise recovery (Howatson et al., 2010; Bowtell et al., 2011; Bell et al., 2014; 2016). Furthermore, cherry extracts have been shown, in cell and animal models, to exert a range of cardioprotective effects that include increasing nitric oxide production and antioxidant (AOX) status, reducing lipid oxidation and inhibiting inflammatory pathways (Wang et al., 1999; Seeram et al., 2001). However, data from human trials are not always consistent. Furthermore, it is yet to be explored whether MC concentrate can be used for performance enhancement. Thus, the overarching aim of this thesis was to elucidate the effects of Montmorency, a specific cultivar of tart cherry, (MC) supplementation on vascular function and exercise performance in humans.

The series of investigations that set out to address this aim have led to many novel and interesting findings. To begin, study 1 was the first to show that protocatechuic and vanillic acid were identified in the plasma post MC consumption. Furthermore, a combination of PCA and VA increased cell migration,

but had no effect on the proliferation of vascular smooth muscle cells. Secondly, and perhaps the most novel finding of this thesis was that MC supplementation showed promise as an effective adjuvant in the management of hypertension. This was a consistent finding throughout the thesis; in all instances MC supplementation was able to significantly reduce systolic blood pressure. Another important finding was that MC supplementation resulted in an acute modulation of cerebral blood flow parameters in the front cortex during task performance with no changes in cognition or mood. Finally, the final study of this thesis demonstrated that MC supplementation can improve aspects of exercise performance with no changes in $\dot{V}O_2$ kinetics, NO_2- concentrations or muscle oxygenation. All of these findings suggest that circulating phenolic metabolites derived from MC juice are at least partly responsible for these effects.

Collectively, findings of this thesis provide novel information to literature surrounding the application of MC in health maintenance and exercise performance. In addition to identifying and quantifying some of the primary downstream metabolites of the principal anthocyanins contained in the concentrate, this research is the first to provide efficacy for the use of MC supplementation to acutely modulate various aspects of vascular function including systolic blood pressure, total and oxy-Hb during a cognitively challenging task. The underlying mechanisms that govern these effects remain elusive, although data from study 1, 2 and 4 would suggest that it is not likely to be attributed to NO, at least systemically. A more likely idea by which MC supplementation improves factors associated with CVD is based on the uptake of polyphenols that possess cardio-protective properties. To conclude, this thesis highlights the ability of MC to improve aspects of cardiovascular function and exercise performance. Circulating phenolic metabolites derived from MC are at least partly responsible for these acute improvements. Further work is required to; fully elucidate the mechanisms by which MC exerts its protective effects, determine whether the effects reported would be amplified using chronic supplementation and demonstrate effects of MC supplementation within habitual dietary practices.

Publications

Peer reviewed publications arising from this course of investigation

- **Keane, K.M.,** Bell, P.G., Lodge, J., Constantinou, C., & Howatson, G. (2016). Polyphenol uptake following human consumption of Montmorency tart cherry (*L. Prunus Cerasus*) and influence of phenolic acids on vascular smooth muscle cells *in vitro.* European Journal of Nutrition, 55(4), 1695-705.

- **Keane, K.M.,** George, T.W., Constantinou, C.L., Brown, M.A., Clifford, T. & Howatson, G. (2016). Effects of Montmorency tart cherry (*Prunus Cerasus L*.) consumption on vascular function in men with early hypertension. The American Journal of Clinical Nutrition, 1-9

- **Keane, K.M.,** Haskell-Ramsay, C.F., Veasey, R.C. and Howatson, G. (2016) Montmorency Tart cherries (*Prunus Cerasus L*.) modulate vascular function acutely, in the absence of improvement in cognitive performance. British Journal of Nutrition, 116(11), 1935-1944.

Peer reviewed publications arising from studies conducted alongside this course of investigation

- Clifford, T., Constantinou, C., **Keane, K.M.**, West, D.J., Howatson, G. & Stevenson, E.J. (2016). The plasma bioavailability of nitrate and betanin from Beta vulgaris rubra in humans. European Journal of Nutrition, [Epub ahead of print].

- Clifford, T., Allerton, D.M., Brown, M.A., Harper, L., Horsburgh, S. **Keane, K.M.,** Stevenson, E.J. and Howatson, G. (2016). Minimal muscle damage after a marathon and no influence of beetroot juice on inflammation and recovery. Journal of Applied Physiology, Nutrition, and Metabolism, 1-34.

Conference communications and published abstracts during doctoral studies

- **Keane, K.M.,** George, T.W., Constantinou, C.L., Brown, M.A., Clifford, T. and Howatson, G. Effects of Montmorency tart cherry (*Prunus Cerasus L.)* consumption on vascular function in men with early hypertension. Centre for *In Vivo* Imaging Day, 2014, Newcastle University, United Kingdom.

- **Keane, K.M.,** George, T.W., Constantinou, C.L., Brown, M.A., Clifford, T. and Howatson, G. Effects of Montmorency tart cherry (*Prunus Cerasus L.)* consumption on vascular function in men with early hypertension. American College of Sports Medicine Annual Congress, 2016, Boston, USA.

- **Keane, K.M.,** Haskell-Ramsay, C.F., Veasey, R.C. and Howatson, G. (2016) Montmorency Tart cherries (*Prunus Cerasus L*.) modulate vascular function acutely, in the absence of improvement in cognitive performance. American College of Sports Medicine Annual Congress, 2017, Colorado, USA.

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List of Abbreviations

The following abbreviations have been defined in the text in the first instance.

ACh	Acetylcholine
AIx	Augmentation index
ANOVA	Analysis of variance
AOX	Antioxidant
ARE	Antioxidant response element
AUC	Area under the curve
BDNF	Brain-derived neurotrophic factor
BMI	Body mass index
BOLD	Blood-oxygenation-level-dependent
BP	Blood pressure
CBF(V)	Cerebral blood flow velocity
CHL	Chlorogenic acid
CIM	Cell invasion and migration
C_{max}	Maximum Plasma concentrations
COX	Cyclooxygenase
COX-1	Cyclooxygenase-1
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
COX-2	Cyclooxygenase-2
CRP	C-reactive protein
CV	Coefficient of variation
CVD	Cardiovascular disease
Cy3G	Cyanidin-3-glucoside
DAD	Diode array detector
DBP	Diastolic blood pressure
Deoxy-Hb	Deoxygenated haemoglobin
DF	Dilution factor
DPPH	2,2-diphenyl-1-picrylhydrazyl
DV	Digit vigilance
DVP	Digital volume pulse
EDTA	Ethylenediaminetetraacetic acid
EGCG	Epigallocatechin gallate
eNOS	Endothelial nitric oxide synthase
ES	Effect size
FLD	Fluorescence detector
fMRI	Functional magnetic resonance imaging
GET	Gas exchange threshold
GFAP	Glial fibrillary acidic protein
GPx	Glutathione peroxidase
HbA1c	Haemoglobin A1c
HCl	Hydrochloric acid
HPLC	High-performance liquid chromatography
HR	Heart rate
iAUC	Incremental area under the curve
IC50	The half maximal inhibitory concentration
IFN-γ	Interferon gamma
iNOS	Inducible nitric oxide synthase
LC-MS	Liquid chromatography–mass spectrometry
LDI	Laser Doppler Imaging

LDL-C	Low-density lipoprotein
LOD	Limit of detection
LPO	Lipid peroxidation
LSD	Least significant differences
MAP	Mean arterial pressure
MC	Montmorency tart cherry concentrate
MeOH	Methanol
mRNA	Messenger RNA
mTOR	Mechanistic target of rapamycin
MW	Molecular weight
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIRS	Near infrared spectroscopy
NOx	Nitric oxide
NOX1	NADPH Oxidase 1
Nrf2	Nuclear respiratory factor 2
Oxy-Hb	Oxygenated haemoglobin
PCA	Protocatechuic acid
PG	Propyl gallate
PGC-1a	Proliferator-activated receptor c coactivator
Pla	Placebo
PTFE	Polytetrafluoroethylene
PWA	Pulse wave analysis
PWV	Pulse wave velocity
RI	Reflection index
RT	Retention time
RVIP	Rapid visual information processing
SBP	Systolic blood pressure
SD	Standard deviation
SEM	Standard error of the mean
SI	Stiffness index
SIRT1	Sirtuins
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
TACN	Total anthocyanins
TCD	Transcranial Doppler
TEAC	Trolox equivalent AOX capacity
TFA	Trifluoroacetic acid
TIC	Total ion current
T_{max}	Time to achieve maximum Plasma concentrations
TOI	Tissue oxygenation index
Total-Hb	Total haemoglobin
TPC	Total phenolic content
UV	Ultraviolet
VA	Vanillic acid
VAS	Visual analogue scales
$\dot{V}O_{2max}$	Maximal volume of oxygen uptake
VSMC	Vascular smooth muscle cells

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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 44,894 words.

Name: Karen Keane

Signature:

Date:

1 Introduction

1.1 Introduction

Cardiovascular disease (CVD) is the primary cause of global mortality (Naghavi, 2015). In the United States, one in four deaths can be attributed to a cardiovascular related event, equating to roughly 610,000 people per annum (Centers for Disease Control and Prevention, 2015). In Europe, CVD is the major cause of death in adults and is responsible for nearly half (48%) of all annual deaths (Allender et al., 2008; Centers for Disease Control and Prevention, 2015). Risk factors for the development of atherosclerosis and CVD can be classified as modifiable, including obesity, dyslipidemia, hypertension, physical inactivity, and smoking, or non-modifiable, including age, sex and family history (Nelms et al., 2011).

A diet high in fruit and vegetables has been associated with a decreased prevalence of CVD (Hu et al., 2000; Ye et al., 2012; Oyebode et al., 2014) this is thought to be due to their high polyphenolic content. Despite extensive research efforts, the precise mechanism of polyphenols is not yet completely understood. Initially, the focal reason for this interest was based on their potential antioxidant (AOX) properties. Whilst this concept is no longer accepted, evidence suggests they may have several properties which make them potentially important compounds for the prevention of multiple diseases (cardiovascular, neurodegenerative, obesity and cancer) (Baur et al., 2006; de Pascual-Teresa et al., 2010; Feng et al., 2007; Hooper et al., 2008; Ramassamy, 2006).

One of most studied functional foods in recent years, in both the clinical and exercise domains, has been tart cherries. Tart cherries and their derivatives are high in numerous polyphenols (Wang et al., 1999; Seeram et al., 2001; Seymour et al., 2014; Bell et al., 2014) that include the flavonoids isorhamnetin, kaempferol, quercetin, catechin, epicatechin, procyanidins, and anthocyanins (Kim et al., 2005; Kirakosyan et al., 2009). Indeed, there has been an enormous research effort over the past decade to delineate the physiological and biochemical effects that tart cherries (and its constituents) might afford, and how these effects could be exploited to improve health outcomes. There is now strong evidence that tart cherries attenuate inflammation (Wang et al., 1999), oxidative stress (Howatson et al., 2010; Bell et al., 2014) and accelerate exercise recovery (Howatson et al., 2010; Bowtell et al., 2011; Bell et al., 2014; 2016). The significant decrease of markers of inflammation and oxidative stress afforded by cherry compounds and

derivatives may have implications for the management of clinical pathologies associated with high levels of inflammation and oxidative stress and suggests that their consumption may have the potential to reduce cardiovascular or chronic diseases in humans. In agreement, cherry extracts have been shown, in cell and animal models, to exert a range of cardio-protective effects that include increasing nitric oxide production and AOX status, reducing lipid oxidation and inhibiting inflammatory pathways (Wang et al., 1999; Seeram et al., 2001). However, data from human trials is uncertain. These protective effects are thought to be mediated by the high number of phenolic compounds in tart cherries (Ferretti et al., 2010). However, as with the aforementioned physiological effects of tart cherries, additive and synergistic effects with nitrate, cannot be ruled out. Furthermore, nitrate, via its conversion to NOx, has been shown to demonstrate potent anti-inflammatory effects independent of any interactions with phenolic compounds (Jädert et al., 2012; Justice et al., 2015).

In addition, polyphenol-rich food and supplements, in the form of grape seed extract (Wang et al. 2008), resveratrol (Giliberto et al. 2010) and red wine (Chen et al. 2009) have specifically demonstrated the ability to offer protection against the brain-aging processes leading to Alzheimer's disease in rodent models. On the other hand, evidence of polyphenol benefits on the young has been focused on various insults to the brain i.e. hypoxic ischemic brain injuries (Loren et al. 2005; West et al. 2007; Liu and Yu 2008). Few have demonstrated hard data in support of benefits to neuronal growth and brain development in healthy models (Scott et al. 2011). Considering the potential for brain bioactive nature of these polyphenols, it is not surprising that interest in research addressing the benefits for both young and old is growing, however research in tart cherry supplementation has been paradoxical with inconsistent results reported (Caldwell et al., 2015; Kent et al., 2015).

From an exercise perspective, while tart cherries, via a juice beverage, has emerged as an effective and popular recovery aid from exercise induced muscle damage (Howatson et al., 2010; Bell et al., 2014; 2015; 2016), it is yet to be explored whether they can be used for performance enhancement. However, the proposed relationship between oxidative stress, inflammation and exercise makes the expectation tenable that a polyphenol-rich tart cherry rich supplement has the potential to improve performance. Consequently the overarching aim of this

thesis was to investigate the application of Montmorency, a specific cultivar of tart cherry, supplementation in both health and exercise paradigms. Specifically, the course of research is broken in to five aims that are systematically addressed in four experimental chapters

1) Establish the polyphenol content of a commercially available Montmorency tart cherry juice, and the plasma bioavailability of principal downstream metabolites.
2) Examine the effects of phenolic acids on vascular smooth muscle cells *in vitro*.
3) Examine whether MC supplementation can improve indices of vascular function in males with early hypertension.
4) Examine whether MC supplementation can improve cerebral blood flow and cognition in middle aged adults.
5) Examine the effects of MC supplementation on $\dot{V}O_2$ kinetics and exercise performance.

2 Literature Review

2.1 Introduction

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

- Polyphenol structure and function with particular emphasis on those present in tart cherries.
- The bioavailability and the factors that affect the bioavailability of phenolic compounds in tart cherries.
- The potential mechanisms of action of polyphenols.
- The effects of tart cherry supplementation on various aspects of health and exercise.

Traditional medicines of ancient cultures like the Chinese, Egyptian, Asian, and Native Americans demonstrated an early knowledge of the use of plant constituents for preventing and curing diseases, and maintaining good health (Parkins, 2001; Higdon, 2007). In the past two to three decades there has been a growing awareness of the role of diet in the aetiology of chronic diseases, notably cardiovascular disease and cancer (World Health Organisation, 2016). A wide range of bioactive substances, including polyphenols have been identified in food and beverages, and it is likely that many more exist. Epidemiological studies have suggested that there is an inverse relationship between the consumption of foods rich in polyphenolic compounds, and the incidence of the aforementioned chronic diseases, indicating that these dietary polyphenols are involved in enhancing long term health (Scalbert et al., 2005).

2.2 Functional foods

The term “functional foods” was first introduced in Japan in the 1980s. The British Nutrition Foundation gave its own definition of functional foods in one of its expert reports. It states that functional foods deliver additional or enhanced benefits over and above their basic nutritional value (British Nutrition Foundation, 2016). Other respected agencies in the world, such as the Mayo Clinic in the United States, have similar definitions describing functional foods as food that are similar in appearance to a conventional food, consumed as part of the usual diet, with

demonstrated physiological benefits, and/or to reduce the risk of chronic disease beyond basic nutritional functions.

Research into functional foods supplementation in health and exercise science has gained momentum in recent years. This interest stems from the observation that functional foods contain a variety of naturally occurring polyphenols, (i.e.., flavonoids, anthocyanins) (Myburgh, 2014). These polyphenols appear to exhibit a broad range of beneficial physiological effects that include, but are not limited to, AOX and anti-inflammatory properties (Nikolaidis et al., 2012a; Sousa et al., 2013; Urso, 2013). Beetroot juice (Bailey et al., 2009b; 2010, Vanhatalo et al., 2010b, Lansley et al., 2011a; 2011b, Ferreira and Behnke, 2010, Clifford et al., 2016), purple sweet potatoes (Chang et al., 2007; 2011), blueberries (McAnulty et al., 2011, Sanchez-Moreno et al., 2008), pomegranate juice (Trombold et al., 2010; 2011), green tea (Jowko et al., 2011, Eichenberger et al., 2010), lychee extract (Kang et al., 2012, Nishizawa et al., 2011) and tart cherries (Connolly et al., 2006b, Howatson et al., 2010; 2012a; 2012b) Bowtell et al., 2011, Kuehl et al., 2010, Ducharme et al., 2009; Bell et al., 2015;2016) have received varying degrees of attention in relation to their purported applications in both health and exercise. The last of these, cherries, have provided several avenues for research because of the high levels of polyphenols present within them and compare favourably with other functional foods (Clifford, 2000, Table 1).

Tart cherries, also known as sour cherries, specifically the cultivar Montmorency tart cherry is a variety of cherry native to the United States, Canada and France that is very high in polyphenols. Montmorency cherries are widely available and are consumed in their raw state, cooked, dried, powdered, juiced and used as concentrated extracts. Montmorency tart cherry supplementation has received widespread attention for its application in both health and exercise, which will be discussed in more detail in sections 2.5 and 2.6 of this review. These exercise and health-related benefits have been postulated to arise from the high anthocyanin content (Kim et al., 2005). The major anthocyanin constituents of tart cherries are cyanidin 3-glucosylrutinoside, followed by cyanidin 3-rutinoside, cyanidin sophoroside, and peonidin 3-glucoside (Kirakosyan et al., 2009). However, tart cherries and their processed products have been shown to be high in numerous other polyphenolic compounds (Wang et al., 1999; Seeram et al., 2001). Amongst the 20 most commonly consumed fruits, cherries appear to have the fifth highest

total phenol content (Vinson et al., 2001). Data supports the presence of several other polyphenols in Montmorency tart cherries including the flavonoids isorhamnetin, kaempferol, quercetin, catechin, epicatechin and procyanidins (Kim et al., 2005; Blando et al., 2004) and non-flavonoid phenolic acids (Jakobek et al., 2007). The following section will attempt to classify these compounds with particular emphasis on those present in tart cherries.

Table 1 - Anthocyanin concentration of various fruit juices. Adapted from Clifford (2000).

Food	**Anthocyanin content ($mg.L^{-1}$)**
Montmorency Tart Cherry Juice	9117 (Biosciences, 2010)
Blackberry	1150
Blueberry	825-4200
Grape (Red)	300-7500
Sweet cherry	20-4500
Strawberry	150-350
Cranberry	600-2000

2.2 Polyphenols or secondary metabolites in plants

Plants synthesise a vast range of organic compounds that traditionally are classified as primary and secondary metabolites, although the precise boundaries between the two groups can, in some instances, be blurred. Primary metabolites are compounds that have essential roles in photosynthesis, respiration, growth and development (Heldt, 2005) that include acyl lipids, nucleotides and amino acids, amongst others. Secondary metabolites, which accumulate in large quantities in certain species, are diverse structurally. Although long ignored, the function of these compounds is now attracting widespread attention (Goldberg, 2003). Unlike the other nutrients i.e.. carbohydrates and protein, they appear not to be essential for short-term well-being, but there is increasing evidence that long-term intakes may have favourable impacts on various health markers.

Based on their biosynthetic origins, plant secondary metabolites can be divided into three major groups as seen below. Of the three, phenolic compounds are of most interest in this review as they and their subclasses are found in abundance in tart cherries.

1. Terpenoids
2. Nitrogen-containing alkaloids and sulphur containing compounds
3. Phenolic compounds

2.2.1 Terpenoids

Terpenoids represent the most structurally varied class of polyphenols with more than 30,000 compounds identified. The name terpenoid derives from the fact that the first members of the class were isolated from turpentine (Croteau et al., 2000). All terpenoids are derived by repetitive fusion of branched five-carbon units based on an isopentane skeleton. Carotenoids are an example of tetraterpenoids, a family of C_{40} compounds, which carry out essential functions in the life cycle of green plant. They also play a role in human health by virtue of their metabolism to vitamin A (retinol) which is involved in sight, growth and development process and its deficiency in humans can have serious consequences for long-term health (Beyer et al., 2002). Tart cherries have been shown to be high in beta carotene, containing roughly 770 µg (Ferretti et al., 2010), however terpenoids are not of particular interest in the current thesis.

2.2.2 Nitrogen-containing alkaloids and sulphur containing compounds

Alkaloid compounds have a 3000-year history of human use. They are nitrogen containing compounds synthesized principally, but not exclusively, from amino acids. Because of their various pharmacological activities, alkaloids have influenced human history profoundly, for both good and ill. Atropine, chloroquine, heroin, cocaine, caffeine, nicotine are some of the alkaloids that have influenced medicine, societies and even geopolitics (Zulak et al., 2006). The main sulphur compounds in the human diet are the glucosinolates found in cruciferous crops like cabbages, broccoli, watercress, and the allium compounds in Allium crops including garlic, onions and leeks (Mithen, 2006). Tart cherries have not been shown to possess any of these compounds.

2.2.3 Classification of phenolic compounds

Phenolics are secondary metabolites characterized by having at least one aromatic ring with one or more hydroxyl group attached. In excess of 800 phenolic structures have been reported and they are widely dispersed throughout the plant kingdom (Strack & Wray, 1994; Strack, 1997). Accounting for about 40% of organic carbon circulating in the biosphere, these phenolics are primarily derived from the phenylpropanoid and phenylpropanoid-acetate pathways in which p-coumaric acid is a key compound (Crozier et al., 2006). Phenolics range from simple, low molecular weight, single aromatic-ringed compounds to large and complex tannins and derived polyphenols. Phenolic compounds are concentrated in the skin and contribute to sensory and organoleptic qualities of fruits and vegetables, such as taste and astringency. They can be classified into two main groups: flavonoids, a structurally diverse group of C_{15} compounds arranged in a C_6-C_3-C_6 configuration and non-flavonoids which include condensed and hydrolysable tannins, stilbenes, phenolic acids and hydroxycinnamates. The term polyphenol is therefore very much an umbrella term for this abundance of compounds which can be subcategorised into smaller and more appropriate groups. Figure 1 provides a simplified diagram of these groups and some examples of dietary sources.

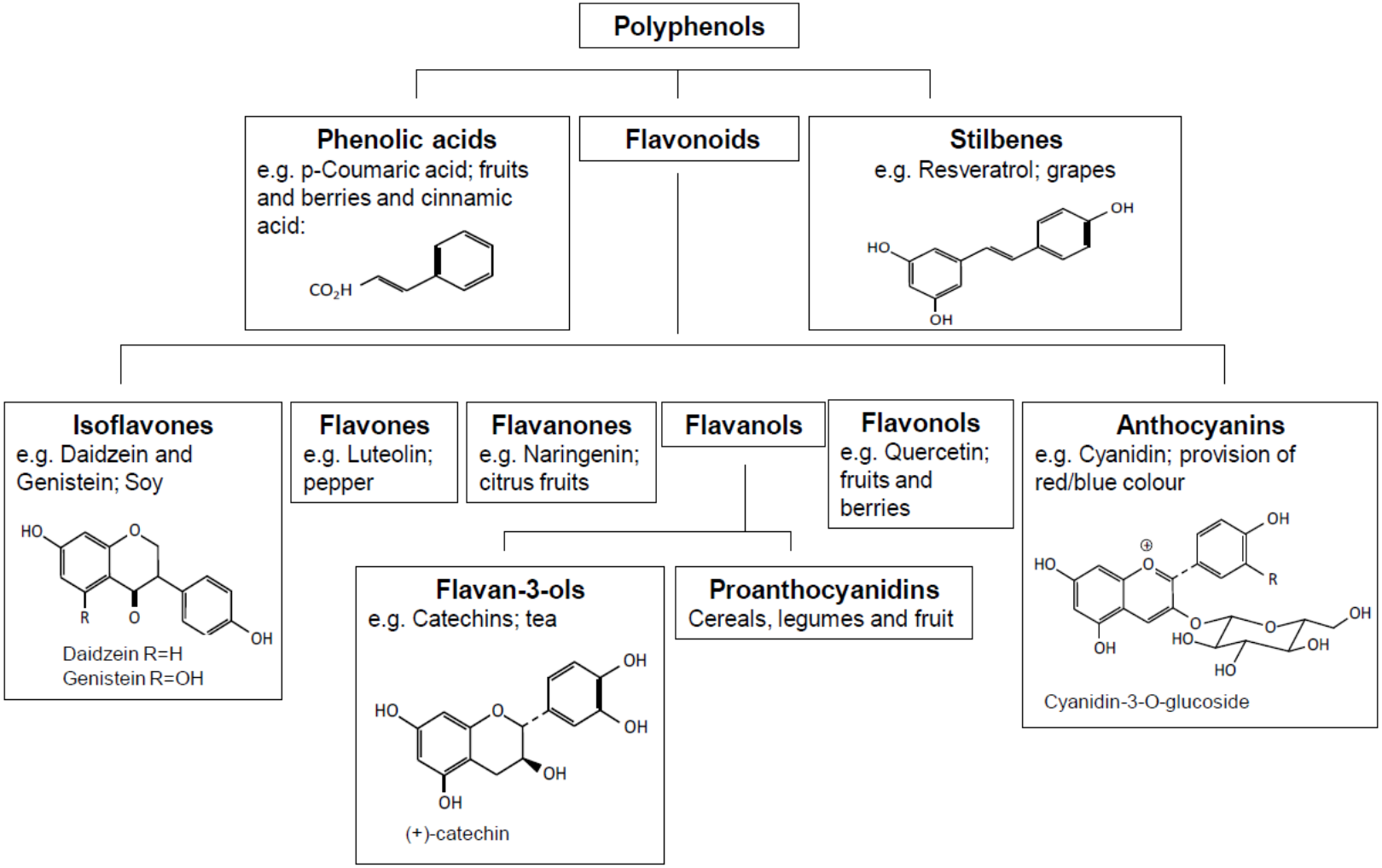


Figure 1- Main sub-groups of polyphenols and examples of dietary sources. Phenolic acids are included for completeness but are not technically 'poly'phenols due to the presence of only 1 phenol group. Chemical structures in diagram are adapted from Kennedy et al. (2010)

2.2.3.1 Flavonoids

Flavanoids are the most abundant of the phenolics and are found throughout the plant kingdom. The flavonoids are compounds based upon a C_6-C_3-C_6 structure. This structure is the product of two separate biosynthetic pathways formed by two aromatic rings, linked together by three carbon atoms forming oxygenated heterocycle. This basic flavonoid structure can have numerous substituents as a function of the type of heterocycle (the C ring), and as a results can be further divided into several sub-classes. It has previously been reported that tart cherries are very high in flavonoids (Blando et al., 2004) which is discussed in the following sub-sections**.** Figure 3 provides a general overview of the polyphenols that have been identified in tart cherries to date; each will be discussed in more detail in the forthcoming sections.

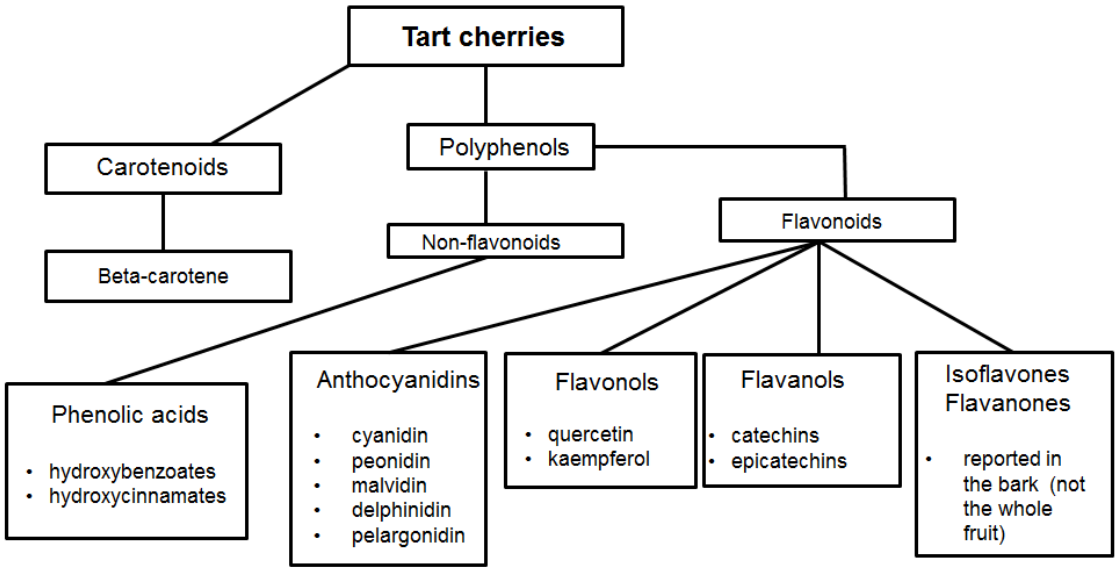


Figure 2 – What is in a tart cherry? A summary of the main polyphenols present in tart cherries

2.2.3.1.1 Flavonols

Flavonols are arguably the most widespread of the flavonoids. The distribution and structural variations of flavonols have been well documented (Williams & Harbone, 1992; Wollenweber, 1992). They have a double bond between C_2 and C_3, with a hydroxyl group in the C_3-position. Querectin is the most represented flavonol, alongside myricetin, isorhamnetin and kaempferol, all of which are

commonly found as *O*-glycosides. The main dietary sources are onions, curly kale, broccoli and blueberries (D'Archivio et al., 2007). With that said, various kaempferol and quercetin glucosides have been identified in Montmorency tart cherries (Shrikhande & Francis, 1973; Schaller & Von Elbe, 1970)

2.2.3.1.2 Flavones

In terms of their structure, flavones are very similar to flavonols, differing only in the absence of hydroxylation at the 3-position in the C-ring. However, flavones are far less distributed. Most flavones occur as 7-*O*-glycosides (Bohm, 1998). The only edible dietary sources include parsley and celery, with some polymethoxylated flavones contained in citrus species (Ooghe et al., 1994) and chrysin, a naturally occurring flavone, and tectochrysin, a chemical compound, have both been reported from the bark of tart cherries (Geibel et al., 1990), but not in the whole fruit.

2.2.3.1.3 Flavan-3-ols

Flavan-3-ols (also known as flavanols) are the most complex subclass of flavonoids as they exist as both the monomer (catechins) and the polymer (proanthocyanidins) form. They contain a saturated 3-carbon chain with a hydroxyl group in the C_3, however any further information on their structure is unknown. Unlike the majority of other flavonoid subclasses, flavan-3-ols are not glycosylated in foods. The main dietary sources of flavan-3-ols are fruit. Tart cherries belong to a group of fruits with the highest content of catechins (Czyzowska & Pogorzelski, 2004). Other common dietary sources of flavan-3-ols are tea (Arts et al., 2000) and red wine (Sun et al., 1999).

2.2.3.1.4 Anthocyanidins

Anthocyanidins are widely dispersed in the plant kingdom, especially in fruit and flower tissues where they are responsible for the red, blue and purple colours (Mazza, 2004). They have an important role for attracting insects to flowers to facilitate pollination and are involved in protecting the plant against UV light (Crozier, 2003). However, interest in anthocyanins has recently intensified because of their possible health benefits (Blando et al., 2004) discussed in section 2.6. They are found primarily as glycosides of their respective aglycones form, anthocyanins. Cyanidin, malvidin, peonidin, delphinidin, pelargonidin, petunidin

are the most common anthocyanidins. The main dietary sources are cereals, vegetables, with the highest concentrations present in fruits (D'Archivio et al., 2007). Tart cherry fruits and their relevant processed food products have already been shown to be a rich source of dietary anthocyanins with cyanidin and peonidin the two major contributors (Ou et al., 2012; Kirakosyan et al., 2009). However it is important to note that a large proportion of ingested anthocyanins may also be degraded into phenolic acids and aldehyde, discussed later. Yet there is still considerable uncertainty as to what occurs *in vivo*. In one paper, protocatechuic acid was reported to be a major metabolite of cyanidin glycoside in humans (Vitaglione et al., 2007), yet subsequent papers have failed to detect protocatechuic acid in rat blood plasma, stating protocatechuic acid is not a major intestinal metabolites of cyanidin glycosides (Ichiyanagi et al., 2007). Reasons for these discrepancies may be due to substantial degradation reactions within the small intestine and colon and further conjugation reactions occurring in the intestine, kidneys or liver which is discussed in later sections of this review.

2.2.3.1.5 Flavanones

A few decades ago, flavanones were considered as only minor flavonoids (Bohm, 1994) However, more recently the total number of known flavanones has increased to the point that they are now considered a major flavonoid class (Grayer and Veitch, 2006). Flavanones are first products of the flavonoid biosynthetic pathway and are characterized by the absence of the C_2-C_3 double bond and the presence of a chiral centre at C_2. These structures have been found to be highly reactive and undergo hydroxylation, glycosylation and *O*-methylation reactions. The main dietary sources of flavanones are citrus fruits, with the highest concentrations found in peel (Nogata et al., 2006). Flavanones such as naringenin, prunin and sakuranin have been reported from the bark of tart cherries (Geibel, 1995; Geibel & Feucht, 1991; Wang et al., 1999) but not yet in the whole fruit.

2.2.3.1.6 Isoflavone

Similar to flavanones, isoflavones are also derived from the flavonoid biosynthetic pathway. In terms of their structure, they have been likened to estrogen in that the hydroxyl groups are located in C_7 and C_4 positions, like estradiol (D'Archivio et al., 2007). As with the majority of the flavonoid family, they undergo several reactions

including hydroxylation, glycosylation and methylation (Dewick, 2002). Isoflavones are distributed throughout the plant kingdom but the main dietary sources are legumes i.e. soya bean (Eisen et al., 2003), however certain variations of genistein and prunetin have been found from the tart cherry bark (Geibel, 1995; Wang et al., 1999).

2.2.3.2 Non – flavonoids

The main non-flavonoids of dietary significance are subdivided into stilbenes, hydroxycinnamates, phenolic acids and other minor families. Both hydroxycinnamates and phenolic acids have been found in tart cherries and will be discussed in more detail in the forthcoming sections.

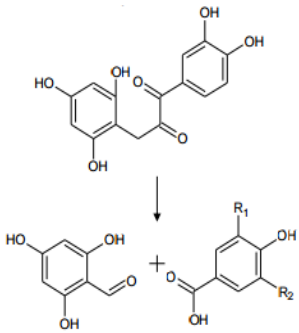
2.2.3.2.1 Stilbenes

Members of the stilbene family have a C_6-C_2-C_6 structure, and as a result are considered polyphenolic compounds. Stilbenes are produced in plants as a defence response to stress, microbial infection and UV irradiation (Fremont, 2000). Very low quantities are present in the human diet, mainly found in red grapes, wines and cranberries (Mulat et al., 2014), with resveratrol (3,5,4′-trihydroxystilbene) the most widely representative of the group. Resveratrol is a phytoalexin found in red wine that has received attention in recent years for its potential beneficial effects on human health (Smoliga, 2011). Salimi et al. (2014) reported that sour cherry seed kernel extract is composed of 1% stilbenes, however to date; stilbenes have not been identified in whole Montmorency tart cherries.

2.2.3.2.2 Phenolic acids

The C_6-C_1 phenolic acids (also commonly known as hydroxybenzoates) have gallic acid as their principal component and are produced in plants via shikimic acid through the phenylpropanoid pathway, as by-products of the monolignol pathway and as breakdown products of lignin and cell wall polymers in vascular plant (Harkin, 1973) These provide unique taste, flavour and health-promoting properties and are found in many vegetables and fruits (Tomas-Barberan & Espin, 2001). Tart cherries, for one have been shown to contain very small quantities of phenolic acids (Kim et al., 2005). Interestingly, as mentioned in Section 2.2.3.1.4, the concentrations of these compounds *in vivo* are often higher than the quantity

ingested, as the majority agree that they represent the major human metabolites of anthocyanins (Scalber and Williamson, 2000) (Figure 3). This has been verified with anthocyanins such as cyanidin-3-glucoside, the most represented anthocyanin in tart cherries (Vitaglione et al., 2007). This degradation occurs normally as a result of exposure to neutral pH, temperature or enzymes (β-glucosidase) i.e.. the warm, basic physiochemical conditions found in the gastrointestinal tract (Seeram et al., 2001; Kay et al., 2009; Woodward et al., 2009) and will be discussed in more detail in section 2.3 and 2.4 of this review. Figure 3 demonstrates the degradation of all primary anthocyanins into their respective phenolic acids.



Anthocyanidin	**Phenolic Acid**	**R_1**	**R_2**
Cyanidin	Protocatechuic acid	-OH	-H
Delphinidin	Gallic acid	-OH	-OH
Malvidin	Syringic acid	$-OCH_3$	$-OCH_3$
Pelargonidin	4-Hydroxybenzoic acid	-H	-H
Peonidin	Vanillic acid	$-OCH_3$	-H
Petunidin	3-Methylgallic acid	-OH	$-OCH_3$

Figure 3 - Degradation of anthocyanidins into phenolic acids and aldehyde. R_1 and R_2 represent functional groups. Adapted from (Keppler and Humpf, 2005; Fleschhut et al., 2006).

2.2.3.2.3 Hydroxycinnamates

Hydroxycinnamates are derivatives of cinnamic acid, a C_6-C_3 compound produced by phenylalanine ammonia lyase-catalysed deamination of the amino acid phenylalanine. The most common types are caffeic, p-coumaric, ferulic and sinapic acids. These are produced by the phenylpropanoid pathway via a series of hydroxylation and methylation reactions. Hydroxycinnamates are very rarely found in their free form, tending to accumulate as glycosylated conjugates in most plant tissues including pollen (Harborne, 1993). The main dietary sources are coffee, fruit and cereal grains (Sosulski et al., 1982). Chlorogenic and

neochlorogenic acid (Wang et al., 1999), p-coumaric (Kim et al., 2005) and isomers of caffeic and ferulic acid have all been identified in tart cherries (Schaller and Von Elbe, 1970)

To summarise, the structure and function of some of the polyphenols present in tart cherries, particularly in relation to anthocyanin content have been well documented (Blando et al., 2004; Kirakosyan et al., 2009; Ou et al., 2012 Bell et al., 2014). However, a detailed investigation of other phenolic compounds in tart cherries would be beneficial and although recent literature has shed some light on the downstream metabolites and bioavailability of these compounds, further research is needed.

2.3 Bioavailability of phenolics

When we consume a food or drink, the nutrients contained are released from the matrix, absorbed into the bloodstream and transported to their respective target tissues. However, not all nutrients can be utilised to the same extent. In other words, they differ in their bioavailability. This section will provide an overview of the bioavailability of polyphenols and describe the different steps of the metabolic pathway where changes in bioavailability may occur. Moreover, this section will examine the literature on Montmorency tart cherry bioavailability as well as highlighting key factors that affect the bioavailability of these compounds and as a result, their biological action.

For a food component to be considered beneficial for health it must be bioavailable *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 (Toutain & Bousquet-Melou, 2004). There are several definitions of bioavailability but the one that is most appropriate in nutrition research was described by Pandey and Rizvi (2009) as the proportion of the nutrient that is digested, absorbed and metabolised through normal pathways and distribution to target tissue. However, *in vivo* activity is largely dependent on the extent and manner of metabolism, with colonic microflora, the rate of excretion and the degree of retention in body tissues also playing a large part. As a result, Holst and Williamson, (2004) extended the classical pharmacological definition of bioavailability as a series of linked and integrated processes including liberation, absorption, distribution, metabolism and excretion.

The pharmacokinetic profile of a food or compound can be determined experimentally by the oral administration of a single dose of a phenolic-rich food or a pure compound to animals or humans. The peak plasma levels (c_{max}), the time to reach the peak plasma concentration (t_{max}) and the area under the curve (AUC) of plasma concentration vs time are then calculated. The chemical structure of polyphenols which was previously discussed in section 2.2.3, will inevitably determine their rate and extent of intestinal absorption and the nature of the metabolites circulating in the plasma (D'Archivio et al., 2007). A detailed review on the bioavailability of polyphenols in humans was published in 2005 (Manach et al 2005a) that showed large variability in polyphenol and flavonoid bioavailability (Figure 4). In this example, expressed by urinary excretions (as % of intake), ranging from 0% (epigallocatechin gallate; EGCG) to above 60% (daidzin).

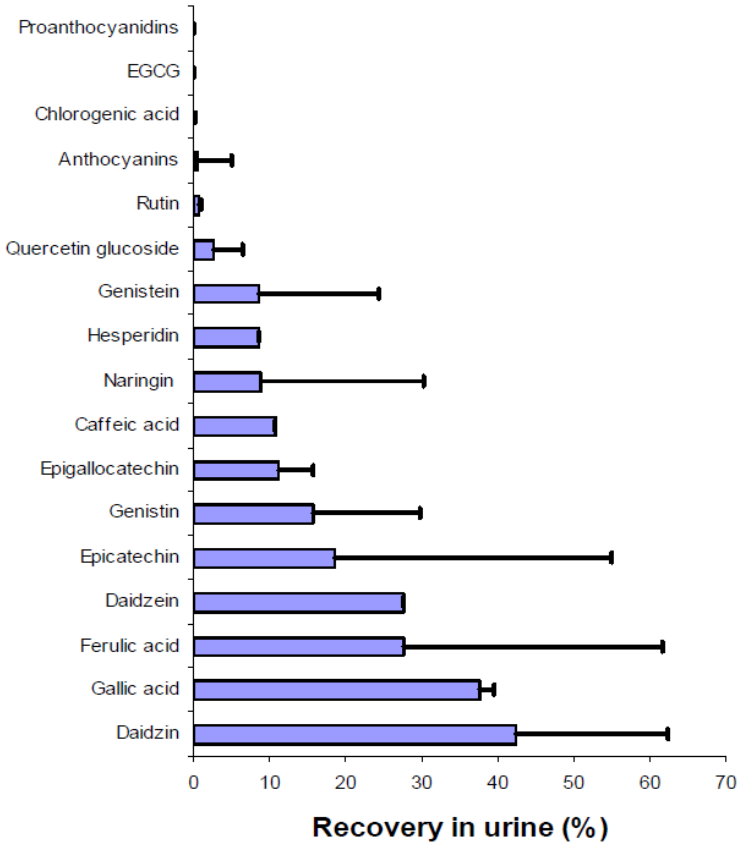


Figure 4 - Recoveries of different groups of phenolics in urine (Manach et al., 2005).

The route of absorption and metabolism of phenolics is shown in Figure 5. The polyphenol chemical transformation in the oral cavity has not yet been fully elucidated due to the limited number of published studies (Vacek et al., 2010). Based on the short interaction time, the impact of enzymatic digestion on

polyphenol release is assumed to be low (Laurent et al., 2007). Nevertheless, there is some existing evidence to suggest a partial participation in glycoside hydrolysis by the salivary enzymes (Ice and Wender, 1953), microbiota or oral epithelial cells (Walle et al., 2005). Furthermore, catechol containing compounds such as chlorogenic acid and (+)-catechin, have been shown to increase nitric oxide bioavailability when exposed to saliva (Peri et al., 2005). Dietary phenolics then pass through the stomach where they are reduced to even smaller particles thus enhancing the release of phenolic compounds from the food matrix (Scalbert et al., 2002). Polyphenols are extensively modified during the absorption period, with the hydrolysis of the glycosides continued by the intestinal enzyme systems [lactase phlorizin hydrolase (LPH) or β-glucoside (CBG) in the small intestine, however not all of ingested dietary polyphenols are metabolized in the stomach or small intestine and may reach the colon where they can be metabolized by the colonic bacteria, α-rhamnosidases, resulting in a conjugated derivatives (Lampe, 2003). This metabolic process of deglycosylation is named phase I metabolism. This phase also includes other chemical reactons namely oxidations, reductions and/or hydrolytic pathways adding a function group (e.g., OH, SH, NH_2) to the phenolic molecule (Parkinson, 1996).

During the course of absorption, these modifications means that the forms reaching the blood and tissues are different from those present in the food and it is very difficult to identify all the metabolites and evaulate their biological activity (Day et al., 2001). Certain compounds are never glycosylated i.e.. (-)-epicatechin, and are absorbed at enterocyte level without any deconjugation or hydrolysis. Once a final derivative has been absorbed, either in the small intestine or the colon, it undergoes some degree of phase II metabolism. On entering the circulatory system, they are transported to the liver via the portal vein where they may undero further phase II metabolism (conjugation reactions, including methylation, glucuronidation and/or sulphastion) which occurs in the brush border of the epithelial cells (Vacek et al., 2010) and transported to the bloodstream until they are secreted. Some of the liver conjugates are then excreted as bile components back to the small intestine (enterohepatic recirculation) and deconjugated compounds are regenerated by gut microbial enzymes before being reabsorbed again (Aura, 2008). The unabsorbed compounds and metabolites are excreted via faeces.

To summarise, the concept of bioavailability integrates several variables including intestinal absorption, distribution, microflora metabolism, nature of circulating metabolites and excretion. The evaluation of polyphenol bioavailability has recently been gaining increasing interest as the health benefits of polyphenols are largely dependent upon their bioavailability (Koli et al., 2010). Despite this sudden popularity and the amount of available data, definitive conclusions on bioavailability of most polyphenol compounds are difficult to obtain and further studies are necessary. There is no single approach recommended but techniques like radiolabelled compounds, microflora composition, *in vitro* fermentation or intervention studies with ileostomy volunteers could provide new insights into the metabolism of polyphenols. This is a fundamental part to fully understanding the potential role of polyphenols in human health and exercise performance.

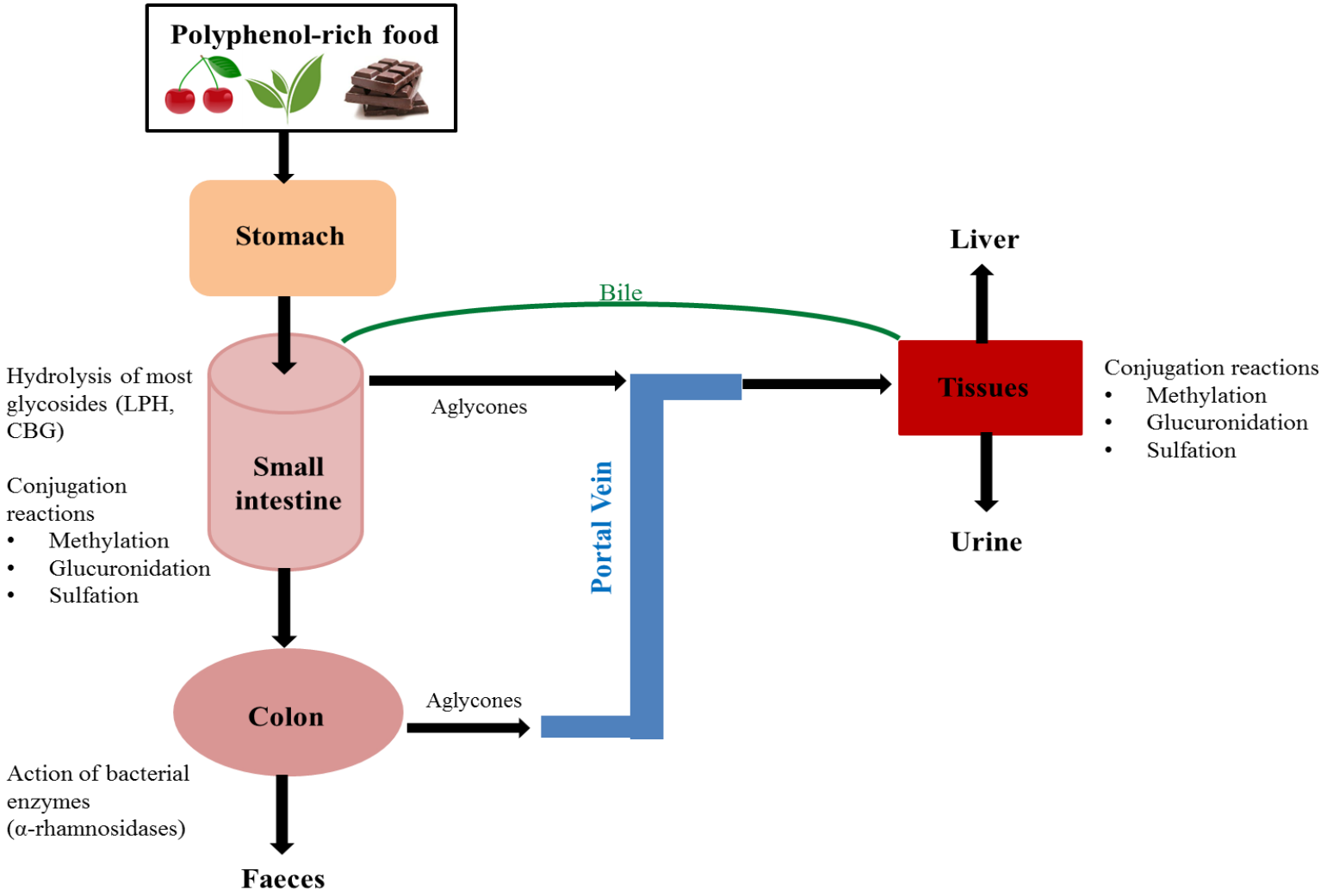


Figure 5 - Absorption and metabolism routes for dietary polyphenols and their derivatives in humans.

2.3.1 Bioavailability of tart cherries

It is critically important that any alleged health benefit of a food source be firstly verified with well-designed bioavailability studies that characterise the extent of its *in vivo* absorption (Rein et al., 2013). In recent years, there has been an attempt made to identify the bioavailability of polyphenolic compounds in tart cherries. A previous pharmacokinetic study on whole tart cherry intake in humans showed that the consumption of whole fruit tart cherries resulted in the appearance of two unmodified anthocyanins in plasma: cyanidin 3-glucosylrutinoside and cyanidin-3-rutinoside, and three modified anthocyanins in urine: cyanidin-xylosylxyloside, pelarogonidin-xylosylxyloside, and methylated peonidin-xylosyloside (Uhley et al., 2009). More recently, Seymour and colleagues (2014) investigated anthocyanin pharmacokinetics in 12 healthy humans following intake of either 45 or 90 cherries. Similiar to the finding reported previously, LC-MS/MS identified cyanidin-3-glucosylrutinoside and cyanidin-3-rutinoside in plasma and also cyanidin glycoside, peonidin glycoside or methylated cyanidin glycoside and a pelargonidin metabolite with an undetermined conjugate moiety in urine post tart cherry consumption. Interestingly, there were no significant differences between the low and high cherry dose, the only noticable difference was the times the compounds needed to achieve maximum plasma concentrations (t_{max}), this differed by compound detected (Figure 6).

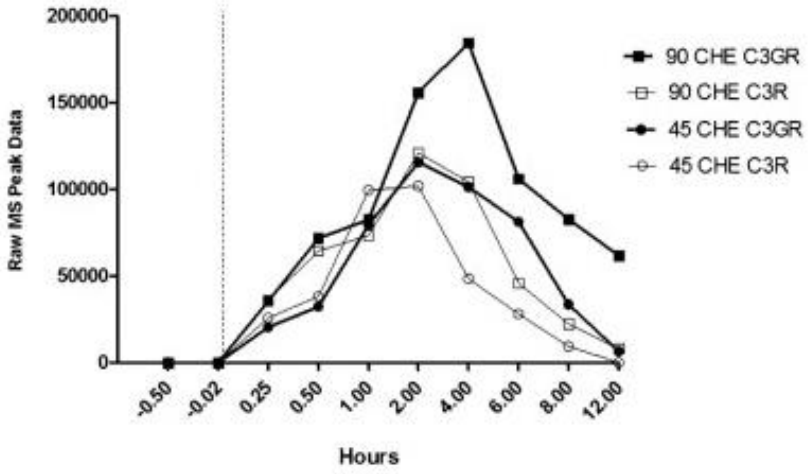


Figure 6 - Plasma anthocyanins cyanidin-3-glucosylrutinoside (C3GR) and cyanidin rutinoside (C3R) (representative plasma sample). 90 CHE = 90 cherries; 45 CHE = 45 cherries.

This same group also confirmed and identified the presence of tart cherry anthocyanins in several target tissues of healthy rats (Kirakosyan et al., 2015). They showed that four native anthocyanins were located in the bladder, kidney, liver, heart and brain of the rats post consumption. The detection of these native anthocyanins in the heart, brain and other tissues may correlate with some of the health-enhancing benefits of tart cherries particurily in relation to transcription factors and AOX and anti-inflammatory defence (Seymour et al., 2009; 2013). However, it is not yet known if tart cherry anthocyanins accummulate in skeletal or smooth muscle.

Taken together, the results of the aforementioned studies provide a good base of evidence that tart cherries are a bioavailable source of bioactive compounds (particularly anthocyanins) in humans and animals. With that said, anthocyanins have a very short half life which indiciates that their concentration in the body is decreased by half the original value very quickly and as a result future work should focus on the bioavailability of other polyphenolic compounds. Findings from these extended pharmacokinetic studies with tart cherries would reveal their breadth of bioavailability and potential health-enhancing properties**.**

2.3.2 Factors affecting bioavailability of tart cherries

There are several factors that affect the bioavailability of polyphenol compounds, these are summarised in Table 2. As previously mentioned, one of the major factors affecting bioavailability is the chemical structure of the compound, with the type of the sugar in the glycoside also determining the rate and extent of intestinal absorption. External factors or “pre-harvest” factors such as environmental ones i.e. rainfall, temperature, sun exposure and light intensity (Manach, 2004) can have a massive bearing on the polyphenol content and nutritional value of cherries. It has previously been shown that high temperature growing conditions (25/30ºC) significantly enhances the total phenolic content of cherries (Wang et al., 2006). Furthermore, the degree of ripeness can affect both the concentration and proportion of various polyphenols, more often than not phenolic acid concentrations decrease during ripening, whilst anthocyanin concentrations increase (Macheix et al., 1990). For all cherry cultivars, during this stage the ripening process advances, acidity decrease and colour intensity increases due to the elevation in anthocyanins (Ferretti et al., 2010).

“Post-harvest” factors such as food processing related factors, transport and storage can also affect the polyphenolic content of cherries and other foods. Thermal processing methods can reduce the total phenol content and, consequently, the AOX activity of foods (Xu & Chang, 2009). During canning, it has been reported that roughly half the anthocyanins and polyphenolics leach from the fruit into the syrup. Heat processing did not result in a loss of total anthocyanins, total phenolics, and AOX activity when the values for syrup and cherries were combined. In fact, samples show an apparent slight increase in total anthocyanin content with canning. This might be due to increased extraction efficiency in the softened fruits.

Additionally, cooking and the methods of food preparation can also have a remarkable effect on polyphenol content (Ferracan et al., 2008), with boiling, steaming and frying all having different effects (Miglio et al., 2008). A decrease in polyphenol content is typically associated with storage duration. More than 75% of anthocyanins in frozen Bing cherries were destroyed after 6 months of storage at -23°C (Chaovanalikit & Wrolstad, 2004). However, during postharvest storage the ripening process advances, acidity decreases and anthocyanins as well as colour intensity increases. During cold storage and subsequent shelf-life, a general increase (over 40 – 60% on average) in phenolic compounds was reported (Serrano et al., 2009; Goncalves et al., 2004). Direct interaction between polyphenols and other food components have been shown to effect bioavailability with the capacity of polyphenols and their metabolites to bind to proteins been often highlighted (Manach et al., 2004).

Effects of the harvest year and the harvest time on anthocyanin concentrations also have been reported (Poll et al., 2003). The highest levels of nutrients and bioactive food components were found in the year characterized by the highest temperature and greatest solar radiation exposure. The cyanidin-3-glucoside equivalent anthocyanin concentrations in the harvested cherry juice varied from as low as 500 mg/L to as high as 2300 mg/L. In fact, ultra violet light (UV-light) has reportedly increased anthocyanin concentrations of sweet cherries (Arakawa, 1993). Although tart cherries are a bit easier than sweet cherries to cultivate, as they are more tolerant of frost as well as humidity and precipitation, there are still big differences in annual crops which may have implications for the proposed health benefits of tart cherries.

Finally, host related factors can be subdivided into intestinal factors (arguably the most important) and systemic factors. Intestinal factors include variations in enzyme activity and colonic microflora, for examples; only 30-40% of the occidental people excrete significant quantities of equol after the consumption of isoflavones (Setchell et al., 2002), while the Japanese excrete up to 60% (Morton et al., 2002). Bioavailability can also depend on systemic factors including genetic characteristics, gender and age. The main factors that affect polyphenol bioavailability are summarised in Table 2.

Table 2 - Main factors affecting the bioavailability of dietary polyphenols in humans.

Polyphenol related factors	Chemical structure; concentration in food; amount administered
Food processing related factors	Thermal treatments; storage; homogenization, food preparation
Food related factors	Food matrix; presences of positive or negative effectors of absorption (i.e.., fat, fibre)
Interaction with other compounds	Bonds with proteins (i.e.., albumin) or with polyphenols with similar mechanism of absorption
Growing conditions	Weather, radiation exposure
Host related factors	Intestinal factors (i.e.., variations in enzyme activity; colonic microflora) Systemic factors (i.e.., gender; age; genetics; disorders)

Bioavailability studies are often difficult to accomplish due to several complexities involved in the biological system, 1) variation in food materials and the human subjects or surrogate models which are not always representative; 2) complex interactions amongst huge chemicals/food components during post-harvest, storage, processing, digestion, and absorption that may alter health benefits; and 3) mechanism pathways (Epriliati & Ginjom, 2012), the last of which will be discussed in the forthcoming section.

2.4 Mechanism of action of flavonoids and phenolic acids

Tart cherries, specifically polyphenols are receiving increasing interest from consumers and food manufacturers as epidemiological studies have suggested associations between the consumption of polyphenol-rich foods and the prevention of diseases including cancers and heart disease (Yang et al., 2001; Nobili et al., 2009). However, many studies often report biological effects of food polyphenols, without elucidating the underlying molecular, cellular, and physiological mechanisms due to their complex mechanistic nature. Despite difficulties in defining the mechanisms, previous work has shed light on more detailed underlying bioactive actions of polyphenols and thus, the potential protective and health-enhancing properties of tart cherries. Initially, the focal reason for this interest was the identification of the potential AOX properties associated with polyphenol compounds. This section will present the original AOX hypothesis and the more recently presented competing hypotheses that go beyond the AOX theory.

2.4.1 The AOX Hypothesis

The AOX hypothesis has its origins in 1954 when Harman published his free radical theory of aging (FRTA) and the simultaneous discovery of the free radicals in endogenous metabolic reactions. The FRTA considered reactive oxygen species to be one of the main reasons for the ageing process, including age related disorders such as cancers, diabetes and cardiovascular diseases. Consequently, it was felt there was an important need for the protection against these species by means of a high intake of exogenous AOX. The AOX properties of plant phenolics are due to the hydrogen of the phenoxyl groups that is prone to be donated to a radical, and by the ensuing structure that is chemically stabilized by resonance (Bors et al., 1997). As mentioned previously, most polyphenols are modified during absorption from the small intestine, through conjugation and metabolism, and by the large intestine, mainly through the action of colonic bacteria and by subsequent hepatic metabolism. Thus, flavonoid metabolites that reach the cells and tissues are chemically, biologically and probably functionally different from the ingested dietary form (Kroon et al., 2004). In terms of AOX activity, glucuronidation or sulphation of quercetin, for example, reduces its free radical scavenging activity (Morand et al., 1998).

Tart cherries have previously been shown to possess high amounts of AOX, and as a result reduce inflammation and oxidative stress (Howatson et al., 2010; Bell et al., 2013; 2014; 2016). The AOX hypothesis has been substantiated greatly by many investigations in the past fifty years, which have demonstrated that a high intake of AOX- and polyphenolic-rich fruit and vegetables have an inverse relationship with the incidence of many chronic disorders (Yang et al., 2001; Chong et al., 2010); therefore making it a plausible mechanism by which tart cherries could improve cardiovascular function.

However, accumulating evidence has shown that not all polyphenolics are readily bioavailable, reaching only low μM concentrations in plasma, despite a large intake. Additionally, most of these compounds are also extensively metabolized *in vivo* (see Section 2.3), and this can adversely affect their "natural" AOX activity (Lottito and Frei, 2006). As a result, it would now appear that the AOX concept is an oversimplified view of the polyphenol mode of action (Azzi et al., 2004).

2.4.2 Other mechanisms beyond the AOX hypothesis

As previously mentioned, the AOX view is being challenged by recent research and far more complex actions are under investigation (Chiva-Blanch and Visioli, 2012; Watson et al., 2014). In the past few years, research on polyphenols has expanded remarkably and is unveiling several nutrition pharma-biological activities of these compounds, most of which extend beyond AOX activity.

2.4.2.1 Cellular signalling pathways

The possibility that polyphenols may also modulate cellular signalling arose from the failure to explain the anti-inflammatory, antitumor, and anti-atherogenic abilities of polyphenols solely on the basis of their AOX properties. Polyphenols merely do not act as free radical scavengers, but may also modulate cellular signalling processes during inflammation or may themselves serves as signalling agents (Rahman et al., 2006). Cellular signal transduction refers to any process by which a cell convert one kind of signal or stimulus into another, most often involving ordered sequences of biochemical reactions inside the cell, that are carried out by enzymes and linked through second messengers resulting in what is known as a second messenger pathway. There is now evidence that polyphenols may play a role as modulators of cascade signalling mechanisms (Williams et al., 2004). It is

likely that cells respond to polyphenols mainly through direct interactions with receptors or enzymes involved in signal transduction, which may result in modification of the redox status of the cell and may trigger a series of redox-dependent reactions (Halliwell et al., 2005; Moskaug et al., 2005; Forman et al., 2002).

Some reports indicate that polyphenols can selectively regulate multiple signalling pathways at the level of transcription, especially those involving nitrogen-activated protein kinases (Frigo et al., 2002). Furthermore, polyphenols such as resveratrol, epigallocatechin gallate, gingerol, phytosterol, and myricetin directly influence various molecular signal transduction pathways like inflammation cascade, cell proliferation/migration, oxidative stress, and metabolic disorders, which are involved in the development of several noncommunicable diseases (Upadhyay & Dixit, 2015). Therefore, it is possible that the polyphenols present in tart cherries may have similar effects.

2.4.2.2 Modulation of gut microbiota

Polyphenols have the ability to modulate microbiota composition; however the mechanisms of modulation are largely unknown. In addition to the accepted beneficial effects of polyphenols on human physiology, it would appear the offer added health benefits via modulation of gut microecology (Parkar et al., 2008). Metabolites released into the lumen may influence the growth of the microbiota, which transformed them whilst affecting other neighbouring microflora species. A recent review suggested that polyphenol metabolites are able to enhance some beneficial probiotic species while inhibiting the growth of non-beneficial species. In support of this idea, green tea extracts have been reported to have general inhibitory effects on intestinal bacteria (Lee et al., 2006). Similar findings have been reported with cocoa and a by-product of pomegranate (Bialonska et al., 2010).

There are a range of additional mechanisms that have been proposed and studied to explain the decrease in CVD risk from consumption of polyphenols. These include anti-inflammatory, antithrombotic or anti-atherogenic action, inhibition or promotion of signalling pathways related to the maintenance of vascular health, and changes to the expression of genes related to those pathways (Quiñones et al. 2013; Tangney & Rasmussen 2013). However, linking the *in vitro* or animals experiments which investigate these mechanisms to the human studies that

demonstrate the health benefits of polyphenols is generally quite difficult. It must also be noted that recent speculation has suggested that the health effects associated with polyphenols may simply be because it limits other potentially noxious foods. Even though this is disputable, many believe the exclusion of other foods, namely those rich in animal protein and saturated fat that would increase the risk of disease, rather than plants and their polyphenols providing a protective effect (Chiva-Blanch & Visioli, 2012), although a combination is also conceivable.

2.4.2.3 Mechanism by which polyphenols could improve exercise performance

Polyphenols could potentially improve exercise performance either directly or indirectly. Indirect effects could include enhancement of training, reduction of physiological stressors that negatively impact on training (illness or the training response itself), or improvement in the ability to recover from training. Given that tart cherries have already been shown to attenuate exercise induced muscle damage (Howatson et al., 2010; Bell et al., 2013; 2014; 2016). This is a plausible mechanism.

Directly, polyphenols have been purported to improve performance by increasing mitochondrial biogenesis. Firstly, they stimulate stress-related cell signalling pathways that increase expression of genes encoding cytoprotective proteins such as nuclear respiratory factor 2 (Nrf2) (Eynon et al., 2010). Nrf2 plays an important role in mitochondrial biogenesis, and variants of the Nrf2 gene have been associated with endurance performance (Stevenson, 2012). Secondly, select polyphenols such as quercetin, resveratrol and curcumin, some of which are present in tart cherries, have been reported to modulate muscle function and mitochondrial biogenesis by activating sirtuins (SIRT1) and increasing peroxisome proliferator-activated receptor c coactivator (PGC-1a) activity (Malaguti et al., 2013). In addition, evidence shows that various polyphenols improve flow-mediated dilatation and endothelial function in humans by increasing endothelial nitric oxide synthesis (Nicholson et al., 2008; Ghosh & Scheepens, 2009). In sports where the rate of blood flow and maximum cardiac output are important determinants of cardiovascular performance, by acting on endothelial function, polyphenols could aid overall athletic performance.

It is pertinent to sound a word of caution concerning the interpretation of mechanistic studies in which purified compounds have been examined as putative

modifiers of pathophysiological processes. Many such studies have been undertaken by classical biochemical methods in which little attention has been paid to the physiological relevance of the concentrations at which the compounds have been examined. To summarise, the proposed beneficial health and exercise effects (see section 2.5 and 2.6) associated with tart cherries and general polyphenol intake are the results of a series of complex actions from polyphenols that likely extend beyond mere AOX actions. It would appear that polyphenols are important metabolic modulators and influence several cellular processes such as signalling, proliferation, apoptosis, redox balance and differentiation.

2.5 Tart cherries and exercise

While tart cherry supplementation has been shown to improve exercise recovery (Howatson et al., 2010; Bowtell et al., 2011; Bell et al., 2014), decrease markers of inflammation and oxidative stress (Wang et al., 1999; Bell et al., 2014; Howatson et al., 2010), studies investigating the effects of tart cherries on exercise performance are limited and equivocal. Therefore, this review will aim to critically evaluate these studies and also look at how other fruits with comparable polyphenol content and how some of the individual phenolic compounds in tart cherries affect exercise performance. Although exercise and tart cherry supplementation studies in animals are uncommon, the majority of the literature on polyphenols and exercise performance has been carried out in animal models. This chapter will also attempt to briefly summarise these findings.

2.5.1 Tart cherries and exercise recovery

Tart cherries have been shown on numerous occasions to be of benefit in exercise recovery. A series of studies have investigated the use of Montmorency cherries in influencing recovery from running (Howatson et al., 2010), heavy eccentric contractions (Bowtell et al., 2011) cycling (Bell et al., 2013; 2014) and prolonged intermittent exercise (Bell et al., 2016). Specifically, these studies have demonstrated that tart cherries can restore muscle function quicker following an exercise induced muscle damaging protocol. Bowtell and colleagues (2011) demonstrated faster recovery of isokinetic knee extensor force following tart Montmorency cherry juice consumption relative to an iso-energetic placebo. These results have been substantiated in recent years (Bell et al., 2013; 2014; 2016). Moreover, given the potential deleterious actions of reactive oxygen and

nitrogen species on recovery and performance, attenuating such indices has received attention in the literature (Gomez – Cabrera et al., 2006; Powers and Jackson, 2008). Tart cherries have been shown to alleviate indices of oxidative stress including thiobarbituric acid reactive substances (Howatson et al., 2010) and lipid hyperperoxides (Bell et al., 2016) in the days following intense exercise. Tart cherries, more specifically their anthocyanins were first shown to inhibit pain in the days following intense physical activity by Tall et al., (2004). Several of the studies already alluded to and others reported lower pain scores post tart cherry supplementation (Howatson et al., 2010; Kuehl et al., 2010; Bell et al., 2014; 2016). Collectively, there appears to be a great deal of literature that supports the use of tart cherry supplementation as an efficient and effective recovery method. All studies demonstrated that using a loading phase of 3-16 days is effective. However, it must be noted that one should be cautious when adaptation (rather than recovery) is the priority as high doses might diminish training benefits. Indeed, there is a growing concern that dampening exercise-induced oxidative stress and/or inflammation could actually mitigate or at least lessen some of the physiological adaptations evoked by training (Gross & Baum, 2015). To date, there has not been the same interest invested in the investigation of tart cherry supplementation and exercise performance.

2.5.2 Tart cherries and exercise performance

During highly intense exercise, the capacity for aerobic metabolism is a major factor in determining an individual's performance (Mortensen et al., 2008). During intense aerobic exercise and the resulting dramatic increases in oxygen consumption, production of free radicals can exceed the intrinsic AOX defence capacity, leading to both muscle fatigue and oxidative stress in the circulatory system, which is responsible for delivery of oxygen (Allen et al., 2008; Machefer et al., 2004; Davies et al., 1982). Many AOXs have been evaluated and shown to have some favourable effects alone or in combination in reducing oxidative stress generated during intense exercise (Baskin et al., 2000; Margaritis et al., 2003; Powers & Hamilton, 1999). More recently, several studies reported the significant beneficial AOX effects of natural polyphenols, in exercise-induced oxidative stress and performance (Panza et al., 2008; Morillas-Ruiz et al., 2006). Surprisingly, beside the possibility to partially prevent exercise-induced muscle damage and inflammation markers, very few studies (n=2) have been performed to analyse the

possibility that tart cherry supplementation could improve exercise performance in humans.

2.5.2.1 Tart cherries and exercise performance - in vivo human studies

Clifford and colleagues (2014) first examined the effect of tart cherries on exercise performance. This study examined the influence of 200 mg of CherryActive® capsules containing 216 mg of polyphenols on sub-maximal cycling and time trial performance. Participants supplemented 2 days before and on the day of each experimental trial. The experimental trials consisted of four 5 minute stages at 40%, 50%, 60%, and 70% maximal power output (Wmax), followed by a 20 km time trial (TT). Results showed that no significant differences between trials were found for heart rate, respiratory exchange ratio, gross mechanical efficiency, oxygen consumption, or blood lactate, at any of the intensities completed during the initial 20 minute phase of the trial ($P > 0.05$). Not surprisingly, final 20 km TT times were not different between trials ($P = 0.115$). However, this study had no control group and a low sample size which may have confounded the results, as VO_2, respiratory exchange ratio and gross mechanical efficiency data was only available for 6 participants. Contrastingly, a recent addition to the literature investigated the effects of powdered Montmorency tart cherry supplementation on acute endurance exercise performance in 27 aerobically trained individuals (Levers et al., 2016). Participants were randomly assigned, in a double- blind manner, to either capsules containing 480 mg of a rice flour placebo ($n = 16$) or powdered tart cherries [CherryPURE®] ($n = 11$) group. The authors speculated based of analysis carried out in 2012, that the 480 mg CherryPURE supplement contained 991 mg of phenolic compounds and 66 mg of anthocyanins. Results of this study revealed that short-term supplementation of Montmorency powdered tart cherries not only attenuated markers of muscle damage, but also increased performance in aerobically trained individuals. Participants in the tart cherry averaged 13 % faster half-marathon race finish times ($P = 0.001$) and tended to have smaller deviations from predicted race pace ($P = 0.091$) compared to their placebo counterparts. Although participants in this study were matched based on average reported race pace, fat free mass, body mass and age, differences in aerobic state of training beyond the study inclusion/exclusion criteria may have been a source of variability in study cohort.

The majority of studies demonstrating an advantageous effect of polyphenols on performance have utilized whole polyphenolic compounds. Quercetin has a relatively long half-life in circulation, and most attention has been paid to this polyphenol in exercise-related studies. A recent meta-analysis (Kressler et al., 2011) reported that on average, quercetin provided a statistically significant benefit in human endurance exercise capacity ($\dot{V}O_{2max}$ and endurance exercise performance). In the quercetin

Interestingly most of the studies examining quercetin use a dose of around or greater than the average intake of 688 $mgday^{-1}$. Achieving this intake of quercetin from the diet, would involve consuming either 2.4 kg of dark chocolate or 72 L of red wine daily, both unrealistic for an athlete, supporting the notion of specific quercetin supplementation. This increased performance benefit leads to two proposed theories: firstly that quercetin may have a greater performance increase compared with other polyphenols, and/or secondly that a greater performance improvement may be associated with increased daily polyphenol supplementation. Accordingly, further research needs to be conducted around varying doses of quercetin within a food matrix to determine if this is a specific polyphenol effect or a dose alone effect. Quercetin has also been reported in relatively high amounts in tart cherries as mentioned in earlier sections of this review (Shrikhande & Francis, 1973; Schaller & Von Elbe, 1970).

Although quercetin is the most studied flavonoid in relation to exercise, other molecules are under investigation for their ability to prevent exercise-induced muscle damage and to affect physical performance. Among them are catechins, which have also been identified in tart cherries (Czyzowska & Pogorzelski, 2004), have been shown to exert some positive effects at least in animal models, discussed in the next section (2.5.2.2). Nowadays, a considerable amount of data is available in humans. Dulloo and colleagues (1999) showed that green tea extract increased daily energy expenditure in humans. It has also been shown that an acute dose of green tea extracted improved fat oxidation and insulin sensitivity during moderate exercise in healthy males (Venables et al., 2008). Other recent findings reported that EGCG (primary polyphenol present in green tea) increased $\dot{V}O_{2max}$ in humans (Richards et al., 2010). In contrast, several studies have reported no significant effects following catechin or green tea consumption (Dean et al., 2009; Eichenberger et al., 2009; Jowko et al., 2012),

perhaps suggesting that catechins can improve physical performance particularly in terms of endurance capacity and $\dot{V}O_{2max}$ in untrained subjects, but the same results cannot be reached in physically active people and well-trained athletes.

Other studies examining whole foods have highlighted the performance-enhancing benefits of grape extract, chokeberries and blueberries (McAnulty et al., 2011; Pilaczynska – Szczesniak et al., 2005). In a randomized, double-blind, crossover design, Lafay et al (2009) supplemented with a grape extract containing several polyphenols (400 mg/day taken in capsule format), however the variation of sports in which the volunteers participated (handball, basketball, sprint and volleyball) resulted in large inter-individual variations for most parameters tested. Handball players (n=10), a homogenous sub group of the same study improved jump performance over 45 seconds following one month of grape extract supplementation when compared to the placebo. More recently, in another randomized, double-blind, crossover design, Cook et al. (2015) reported that following a seven-day intake of New Zealand blackcurrant extract, there was a significant improvement in cycling time-trial by 2.4% performance, coupled with an increase in fat oxidation when compared to the placebo. The authors of both studies speculated that this improvement may have been as a result of improved endothelial function and increased peripheral blood flow. Similar findings have been reported in other studies where polyphenolic content of a fruit – derived supplement is similar to tart cherries (Kang et al., 2012). Kang and colleagues reported that oligomerized lychee fruit extract significantly increased the anaerobic threshold by 7.4% (1.8, 13.0). Interestingly, these results suggest that a polyphenol-containing supplement and typical AOXs may have different mechanisms of action and that the endurance-promoting effect of oligomerized lychee fruit extract may not directly come from the scavenging of free radicals but may be attributed to other non-AOX properties of polyphenols, which were discussed in Section 2.4.2.

2.5.2.2 Tart cherries and exercise performance - in vivo animal studies

The majority of early studies investigated the effect of polyphenol supplementation on exercise performance in animal models. Previous literature has demonstrated the performance enhancing properties of polyphenols extracted from pomegranate peel (Swamy et al., 2011) green tea (Swamy et al., 2011) cocoa (Nogueira et al.,

2011) amongst others. Swamy and colleagues (2011) reported increased swimming time, reduced lipid peroxidation and lactic acid levels in tissues following polyphenol extract supplementation. Four groups of rats group 1 sedentary, group 2 sedentary but force fed with predetermined concentration of 25 mg polyphenol equivalent extract/day/rat, group 3 and 4 were subjected to force swimming but force fed water and with 25 mg equivalent extract of green tea, for 7 days after pre feeding the animals for 21 days with same dietary treatment. Concentration of polyphenols decreased in blood, liver and muscle suggesting the utilization of these polyphenols which enhanced the swimming time more than twice in group 4 than group 3, reduced lipid peroxidation and LDH activity and increased the activity of creatine phosphate kinase. Higher glycogen content was found in muscle and liver in group 4 than group 3 with no change in the fat content. Murase et al., (2005) have also demonstrated that green tea extract improves the time to exhaustion in a dose dependent manner, in mice undergoing a swimming test after 7 weeks of supplementation. Similar results were reported with pomegranate peel extract (Swamy et al., 2011). Both of these studies demonstrated the possible anti-fatigue role of pomegranate peel and green tea extract.

Additionally, 12 weeks of resveratrol supplementation has been shown to prevent the decline in running time to exhaustion, oxygen consumption and lipid oxidation in senescence-accelerated mice model. Data shown in this study demonstrate that the resveratrol induction of mitochondrial biogenesis is responsible for the prevention of ageing-related performance impairment (Murase et al., 2009). Accordingly, Dal-Ros et al. (2011) have recently demonstrated that chronic red wine polyphenols intake prevents aging-induced performance decline in rats. Moreover, dark-chocolate polyphenols have been ascribed to be responsible for some positive effects of dark-chocolate consumption during exercise. Results suggest that (–)-epicatechin increases the capacity for muscle aerobic metabolism, thereby delaying the onset of fatigue (Nogueira et al., 2011). Data showed that when mice were fed for 15 days with (–)-epicatechin (present in dark chocolate), they had improved exercise performance accompanied by: (1) an increased number of capillaries in the hind limb muscle; and (2) an increased amount of muscle mitochondria as well as signalling for mitochondrial biogenesis. Contrastingly, although dark-chocolate consumption was associated with lower oxidized low-density lipoprotein levels before and after exercise and with an

increase in free fatty acids levels during exercise, the time to exhaustion was not significantly affected (Allgrove et al., 2011).

Although it is clear that polyphenol supplementation in a variety of forms and doses is able to increase the capacity to quench free radicals, at least in the circulation, and enhance exercise performance in animal models, as the studies discussed differ in study design, subject population, supplementation regimen and testing protocols, there is insufficient evidence to conclude that polyphenol supplementation can improve endurance performance in humans. Interestingly, there are also insufficient negative data to discourage polyphenol supplementation use actively for purposes other than endurance performance (Myburgh, 2014). In a recent review (Somerville et al., 2017), the authors speculated that in relation to polyphenols and performance publication bias is also a potential issue. Very little studies are published showing a null or negative effect. This could either be due to polyphenols consistently having a performance benefit effect, or conversely that those papers were not published. Consequently, although based on the papers identified in the comprehensive search there is an overall positive effect, the authors acknowledge there is a potential that select papers may not have been published that could alter the overall improvement effect and/or size.

2.6 Tart cherries and health

Research on the effects of dietary polyphenols on human health has developed considerably in the past 10 years. It strongly supports a role for polyphenols in the prevention of several diseases. The literature on the health benefits of tart cherry has generally focused on their role in the prevention, treatment, and recovery of soft tissue injuries and pain remediation. The anti-inflammatory and anti-oxidative capacity of cherries has led to focus on supplementation for a number of pathologies. Inflammation and oxidative stress are commonly associated with several pathologies including cancer, arthritis (Fillippin et al., 2008), cardiovascular disease (Wu et al., 2014) and with acceleration of the aging process (Lau et al., 2005). Tart cherry supplementation has been previously shown to enhance sleep quality (Pigeon et al., 2010; Howatson et al., 2012), alleviate arthritic pain and gout (Jacob et al., 2003; Zhang et al., 2012) and inhibit intestinal tumor development in mice (Kang et al., 2003). This section will focus on the tart cherry supplementation and their effect on both cardiovascular and cognitive function.

2.6.4 Cardiovascular function

In Europe, CVD is the major cause of death in adults and is responsible for nearly half (48%) of all annual deaths (Allender et al., 2008; Centres for Disease Control and Prevention, 2015). Epidemiological studies have suggested that polyphenol-rich foods can exert positive cardiovascular health benefits (Joshipura et al., 1999; Bazzano et al., 2002; Hung et al., 2004). Oxidative stress and ROS have been vastly implicated in endothelial damage, progression to atherosclerosis, and injury in sustained myocardial infarction, as well as in ischemia reperfusion (Dhalla et al., 2000; Sugamura et al., 2011). Given the proposed relationship between tart cherries, inflammation and oxidative stress which has been well documented, and evidence demonstrating that cherry extracts exert a range of cardio-protective effects that include increasing nitric oxide production and AOX status, reducing lipid oxidation and inhibiting inflammatory pathways in cell and animal models (Wang et al., 1999; Seeram et al., 2001), it makes the rational expectation that Montmorency tart cherry consumption could exert positive cardiovascular health benefits. However, data from human trials are ambiguous.

2.6.4.1 Tart cherries and CV function - in vitro cell and in vivo animal studies

As previously mentioned, cherry extracts have been shown to exert a range of cardio-protective effects. Tart cherries as described in earlier sections are rich sources of anthocyanins (Chandra et al., 1992, 1993; Wang et al., 1997). These compounds, in particular cyanidin, have previously been reported to exhibit *in vitro* AOX and cyclooxygenase inhibitory activities comparable to those of commercial products (Wang et al., 1999). The best COX-I and COX-II inhibitory activities were observed in the anthocyanins from raspberries and sweet cherries, respectively. It has also been shown that the consumption of freeze dried tart cherries (1% w/w in the diet) by the Dahl-SS animals for 90 days, enhanced hepatic peroxisome proliferator-activated receptors (PPAR) and improve the condition of hyperlipidemia and hyperinsulinemia in rats (Seymour et al., 2008). The authors concluded that physiologically relevant tart cherry consumption had the ability to reduce several phenotypic risk factors associated with metabolic syndrome and type 2 diabetes mellitus.

Likewise, in 2009 a study examining the *in vivo* AOX efficacy, lipid peroxidation and anti-inflammatory properties of sour cherry juices reported similar findings (Šarić et al., 2009). Male CBA/Hr mice were randomly split into a control group (fed with commercial food pellets) and 2 experimental groups (fed with commercial food pellets with 10% or 50% of cherry juice added). Sour cherry juice supplementation resulted in increased SOD and GPx activity and decreased lipid peroxidation (LPO) concentrations. Similar to the studies mentioned earlier, this highlights cherry juice as a potent COX-2 inhibitor and might potentially be used as an AOX and anti-inflammatory product with beneficial health-promoting properties.

2.6.4.2 Tart cherries and CV function - in vivo human studies

The potential cardio-protective effects of cherries have not been investigated in humans to the same degree. Earlier observations reported indirect links between tart cherry supplementation and cardiovascular risk. Elevated levels of high-sensitivity C-reactive protein has been linked to increased cardiovascular disease risk (Koenig et al., 2013). Previous studies, which were discussed in earlier sections demonstrated the ability of tart cherry juice to attenuate inflammatory response to a series of exercise stresses (Howatson et al., 2010; Bell et al., 2014; 2016). Moreover, Kelley and colleagues (2006) reported that 280 g/day of sweet cherries (supplying approx. 100 mg/day anthocyanins) reduced CRP by 8 % after two weeks of supplementation and by 25 % after four weeks of supplementation.

In 2008, a pilot study investigated the effects of sour cherry juice on blood glucose and some cardiovascular risk factors improvements in diabetic women (Ataie-Jafari et al., 2008). In this study, 19 diabetic women were asked to consume 40g of concentrated sour cherry juice daily for 6 weeks. Results found that significant reductions in body weight, blood pressure (Table 3a) and hbA1c were observed post supplementation period. In addition, total cholesterol and LDL-C decreased in a sub group (n=12), with LDL-C less than 100mg/dl. The authors attributed these improvements in blood pressure and blood lipid profiles to the high anthocyanin content of sour cherries. In particular, endothelial nitric oxide synthase (eNOS) plays an important role in maintaining blood pressure homeostasis and vascular integrity. Cyanidin-3-glucoside, the most abundant anthocyanin in cherries, has been shown to induce eNOS expression and escalate NO production in the vascular endothelial cells (Xu et al., 2004). One big limitation

of this study is that there was no control group included and therefore results should be interpreted with a certain degree of caution. Despite this, these findings are in accordance with many similar studies on hypocholesterolemic effects of polyphenol rich foods (Reshef et al., 2005; Hobbs et al., 2012).

Contrastingly, a more recent study (Lynn et al., 2014) examining the effect of tart cherry juice supplementation on arterial stiffness and inflammation in healthy adults reported no significant differences in arterial stiffness, BP, CRP or risk markers for cardiovascular disease following supplementation (Table 3b), but evokes a minor increase in AOX status. In this study, 47 healthy adults consumed 30 ml of cherry concentrate diluted to a volume of 250 ml with water or the same volume of an energy matched control drink daily for six weeks, the same duration as the previous study. At the end of the intervention, there was no significant differences in arterial stiffness ($P = 0.218$), CRP ($P = 0.220$), systolic blood pressure ($P = 0.163$), diastolic blood pressure ($P = 0.121$), total cholesterol ($P = 0.342$) and high density lipoprotein cholesterol ($P = 0.127$). However, the authors speculated that this was due to numerous limitations including their sample size, blood sampling technique and population. Therefore, it cannot rule out the possibility that cherry juice may be more effective in individuals with existing hypertension or cardio-metabolic disease, as demonstrated by the obvious higher baseline blood pressure values and blood lipid profile between the two set of participants highlighted below.

Table 3 - Change in cardiovascular outcome variables following tart cherry juice consumption.

(a)

	Cherry Juice		
	Baseline	*End*	P value
SBP (mmHg)	129 ± 16	123 ± 13	P < 0.01
DBP (mmHg)	82 ± 8	76 ± 9	P < 0.01
Cholesterol (mmol/L)	11.9 ± 0.50	10.7 ± 0.22	P < 0.05

Adapted from Ataie-Jafari et al. (2008)

(b)

	Cherry Juice		Placebo		
	Baseline	*End*	*Baseline*	*End*	P value
SBP (mmHg)	111 ± 14	110 ± 13	110 ± 12	113 ± 12	P = 0.163
DBP (mmHg)	70 ± 10	69 ± 10	67 ± 8	70 ± 8	P = 0.121
Cholesterol (mmol/L)	4.25 ± 0.5	4.22 ± 0.8	3.76 ± 0.7	4.12 ± 0.7	P = 0.342

Adapted from Lynn et al. (2014)

In addition, polyphenols have garnered attention for their ability to modulate blood flow in both clinical and non-clinical populations. Grapes, cocoa, beetroot and blueberries have all been shown to modulate peripheral blood flow using techniques such as flow mediated dilation and laser Doppler imaging. In one such study (Stein et al., 1999), patients with coronary artery disease were supplemented with 4 ml/kg/body weight of purple grape juice twice daily for 14 days. Flow-mediated vasodilation was increased by 6.4% following supplementation indicating an overall improvement in endothelium dependent vasodilation. This observation was also reported with healthy participants (Rodriguez-Mateos et al., 2013) whereby following 766 mg of blueberry supplementation, a significant increase in endothelium-dependent brachial artery vasodilation was observed relative to the placebo group. Similarly, the

incremental area under the curve (0–6 h post ingestion) for endothelium dependent vasodilation as assessed by laser Doppler was greater (P = 0.017) after beetroot bread ingestion compared to the control trial. These effects occurred in conjunction with increases in plasma and urinary nitrate (P < 0.0001) and nitrite (P < 0.001) (Hobbs et al., 2013). To date, no research has looked at the effect of tart cherry juice supplementation on blood flow using these methods.

2.6.5 Cerebral haemodynamics and cognitive function

Aging is associated with deficits in motor function, which include decreases in balance, muscle strength, coordination, and cognitive function, especially in tasks that require the use of spatial learning and memory. This has been suggested to be caused by a concurrent decline in cerebral blood volume and metabolism of oxygen which also occurs as a result of aging (Machal et al., 1992). These decrements have been reported in numerous studies in both animals (Joseph et al. 1983; Shukitt-Hale et al., 1998) and humans (Brayne et al. 1995; Hofer et al. 2003). A large number of dietary interventions using polyphenol-rich foods or beverages, in particular those using tea (Chan et al., 2006), Gingko Biloba (Birks & Evans, 2009), cocoa (Scholey et al., 2010) and blueberry (Shukitt-Hale et al., 2008), have demonstrated beneficial effects on memory and learning in both animal and human models (See Lamport et al., 2012 for full review). Although it is not clear whether tart cherries decreases the risk of neurodegenerative aging or diseases such as Parkinson's and Alzheimer in humans, studies with animal models are more positive and suggest that the phenolic compounds found in tart cherries, may exert their beneficial effects through their ability to lower oxidative stress and anti-inflammatory properties or by altering directly the signalling involved in neuronal communication, calcium buffering ability, stress signalling pathways among others (Shukitt-Hale et al., 2008; Shukitt-Hale et al., 2006). The foregoing literature provides a brief synopsis of how tart cherries and their isolated components influence cognitive function in both animal and human models.

2.6.5.1 Cerebral haemodynamics and cognitive function - in vivo animal studies

As previously alluded to, the majority of literature supporting the use of tart cherries to improve various aspects of cognition has been demonstrated in animal

models. Seymour et al. (2013) showed that intake of 1% tart cherry diet significantly reduced stroke-related phenotypes in rats. Tart cherry intake also reduced brain NFκB activity and the related pro-inflammatory transcripts. Interestingly in 2015, Kirakosyan and colleagues confirmed that tart cherry anthocyanins cross the blood-brain barrier (Kirakosyan et al., 2015). A more recent addition to the literature examined thirty 19-month-old male Fischer 344 rats who received either a control diet or a diet supplemented with 2% tart cherry for six weeks. Results showed that although there were no changes on motor performance, tart cherry supplementation significantly improved working memory of aged rats (Thangthaeng et al., 2016). The authors attributed this finding to the high polyphenols content of tart cherries and the subsequent reduction in inflammatory markers (GFAP, NOX-2, and COX-2) and improvement in autophagy function (phosphorylated mTOR, Beclin 1, and p62/SQSTM).

This is not the first study to demonstrate that fruits high in polyphenols can exert positive influences on cognitive function and memory. Ramirez and colleagues (2005) supplemented twenty 12-month-old male Wistar rats with a dried blueberry extract per day for a month. Results demonstrated an increase in short-term memory, improvements in working memory and an overall reduction in anxiety following blueberry supplementation. Moreover, in another study twenty-eight 19-month-old rats were supplemented with a reconstituted plum juice beverage or water for eight weeks. Results showed improvements in spatial working memory. Rendeiro and colleagues (2013) supplemented eight 18-month-old male Wistar rats with one of four diets for six weeks. The diets included a control, a 2% blueberry diet, a high anthocyanin diet and a flavanol-enriched. They too, reported that spatial working memory scores increased in all conditions with the exception of the control diet. This improvement was coupled with increased levels of BDNF mRNA expression in the hippocampus. In addition, a study examining the effects of cyanidin on cognition reported that cyanidin 3-O-glucoside (Cy3G) (one of the main anthocyanins in tart cherries) is protective against the Aβ-induced impairment of learning and memory, but has no effect on normal learning and memory. Moreover, they found that Cy3G attenuated the Aβ-induced tau hyperphosphorylation and GSK-3β hyperactivation observed in Alzheimer's disease. Taken together, these results demonstrated that Cy3G can rescue the cognitive impairments that are induced by Aβ via the modulation of GSK-3β/tau, suggesting a potential therapeutic role of Cy3G in Alzheimer's disease.

2.6.5.2 Cerebral haemodynamics and cognitive function - in vivo human studies

Following the recent finding by Kirakosyan and colleagues (2015) where it was shown that tart cherry anthocyanins accumulated in the brain of young rats following 3 weeks of supplementation with either 1 or 10 % tart cherry diets, there has been more literature focusing on the use of tart cherries to improve cognitive function with mixed findings. Caldwell et al., (2015) previously demonstrated that regardless of dose, cherry juice had no acute impact on cognitive function in young people, older people or dementia patients. They concluded that although cherry juice may have an acute impact on cardiovascular function, there was no change in cognitive performance 6 h post consumption. Contrastingly, a chronic supplementation study (Kent et al., 2015) reported that the daily consumption of sweet cherries 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. However, the former study used sweet cherries as an intervention, it has been speculated that sweet cherries are not as rich in polyphenol compounds as tart/sour cherries (Kim et al., 2005). These inconsistent findings have also been reported with other functional foods where Kelly et al., (2013) and Thompson et al., (2014) showed that after acute beetroot supplementation, there were no changes in cognitive performance for concentration, memory, attention or information processing ability. However, when older type 2 diabetics were supplemented with beetroot juice for 14 days, they experienced a significant improvement in simple reaction time compared to a control group (Gilchrist et al., 2004). It would seem that the cerebrovascular response required to elicit measurable changes in cognitive function can only be achieved with longer term dosing strategies that have the potential to induce sustained modifications to cerebrovascular function (Clifford et al., 2015).

Although there have only been a handful of studies that investigated the relationship between tart cherry intake and cognitive function, far more studies have looked at individual phenolic compounds and other fruits and their impact on various aspects of cognition with positive results. It has been shown that an increased intake of catechins, flavonols and phenolic acids, all present in tart

cherries, was associated with better verbal memory in a 13-y assessment of middle aged adults (Kesse-Guyot et al., 2012). Moreover, grapes (Krikorian et al., 2010a), blueberries (Krikorian et al., 2010b) and blackcurrants (Watson et al., 2015) have all been reported to positively impact cognition function in young and old populations. In a 2012 review, Lamport and colleagues concluded that overall findings suggest that polyphenol consumption has the potential to benefit cognition both acutely and chronically.

Polyphenol-rich foods have also been reported to improve cerebral haemodynamics assessed by near infrared spectroscopy (NIRS) and functional magnetic resonance imaging (fMRI). Wightman and colleagues (2012) assessed the effect of EGCG on cerebral blood flow using NIRS in healthy adults. Results suggested that 135 mg of EGCG caused a reduction in total haemoglobin, a proxy for cerebral blood flow during cognitive tasks relative to the placebo. Changes in cerebral blood flow has also been demonstrated following resveratrol (Kennedy et al., 2010), beetroot (Wightman et al., 2015). Krikorian et al., (2012) used fMRI to examine the effect of Concord grape juice on neurocognitive function. Sixteen adults aged >68 y with mild age-related memory decline were supplemented with either a grape juice (444 ml average) containing on average, 209mg of polyphenols, or a sugar matched placebo for 16 weeks. Results found that after 16 weeks, there were reductions in semantic interference on memory tasks and relatively greater activation in anterior and posterior regions of the right hemisphere in the grape juice treated group. Similarly, people with mild memory complaints, who drank pomegranate juice daily, performed better on memory task compared to a placebo and displayed an increase in brain activation measured by fMRI (Bookheimer et al., 2013). Whilst these studies have assessed the impact of polyphenol supplementation on cerebral haemodynamics, no attempt has been made to examine the haemodynamic response to tart cherries.

Figure 7 summarises the proposed protective effects of cherry polyphenols and their implication in chronic disease management In conclusion, research using animal models has shown that supplementation of tart cherries or tart cherry compounds can positively affect working memory and protect against cognitive decline (Qin et al., 2013; Thangthaeng et al., 2016). However, research involving humans is not as straight-forward and future research is needed to fully

understand the relationship between tart cherry supplementation, cerebral blood flow and cognitive function.

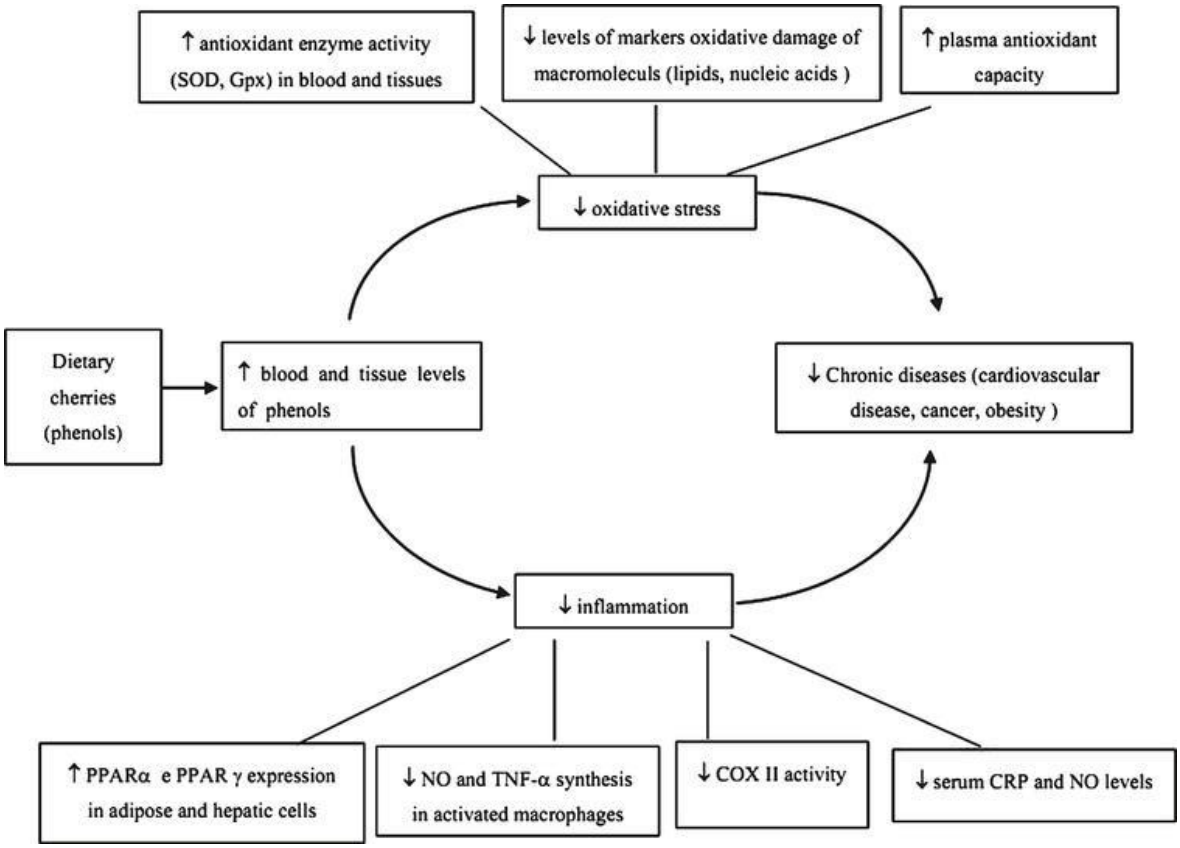


Figure 7 - The proposed protective effects of cherry polyphenols and their implication in chronic disease management (Adapted from Ferretti et al., 2010).

2.7 Conclusion

The effects of polyphenolic rich foods in various health and exercise paradigms have received a great deal of attention in the past 10-20 years. It is generally well accepted that a diet high in polyphenols is associated with decreased risk of a range of diseases including cardiovascular disease (CVD), specific forms of cancer and neurodegenerative disease. Along with this, reasonable understandings of the bioavailability of polyphenols and the mechanisms by which they exert such benefits *in vivo* have been determined. These mechanisms are now believed to involve interactions with a number of cellular signalling pathways, which are important in the normal functioning of cells. Such interactions appear to modulate these pathways in a way that acts to control various pathogenic processes relevant to chronic disease progression.

From an exercise perspective, the available literature seems to suggest that polyphenols, can provide protection against exercise-induced muscle damage and oxidative stress thanks to their AOX and anti-inflammatory properties. However, the possibility to improve the exercise performances remains unclear. Tart cherries are a functional food that has received substantial attention in recent years for its potential benefits for both general health and exercise recovery. Recent studies have provided evidence that tart cherries and its constituents may afford beneficial effects for disorders characterize by inflammation, oxidative stress, and aberrant vascular function.

The overall aim of this thesis is to investigate the bioavailability of MC and the influence on vascular function in high risk populations. Furthermore, this thesis aims to investigate the effect of MC on exercise performance. The paucity of tart cherry research in these particular areas meant that this review necessarily had to begin by providing evidence of the cardiovascular and exercise effects of other fruits with similar polyphenol content, and whole polyphenol compounds present in tart cherries. Research reveals that the supplementations of these fruits / compounds can improve aspects of vascular and cognitive function and exercise performance. This review of literature has identified a number of areas that require further study and consequently some of these will be addressed in the subsequent experimental chapters by raising the following questions:

1) Establish the polyphenol content of a commercially available Montmorency tart cherry juice, and the plasma bioavailability of principal downstream metabolites.

2) Examine the effects of phenolic acids on vascular smooth muscle cells in vitro.

3) Examine whether MC supplementation can improve indices of vascular function in males with early hypertension.

4) Examine whether MC supplementation can improve cerebral blood flow and cognition in middle aged adults.

5) Examine the effects of MC supplementation on $\dot{V}O_2$ kinetics and exercise performance.

3 Polyphenol uptake following human consumption of Montmorency tart cherry and influence of phenolic acids on vascular smooth muscle cells *in vitro*

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3.1 Introduction

Epidemiologic studies have shown that consumption of food and beverages containing polyphenols is associated with reduced cardiovascular morbidity and mortality (Habauzit & Morand, 2012; Arranz et al., 2012). Amongst the 20 most commonly consumed fruits, cherries appear to have the fifth highest total phenol content (Vinson et al., 2001). Tart cherries and their processed products are a functional food of growing interest and have been shown to be high in numerous polyphenols (Wang et al., 1999; Seeram et al., 2001). Data support the presence of several polyphenols in tart cherries including the flavonoids isorhamnetin, kaempferol, quercetin, catechin, epicatechin, procyanidins and anthocyanins (Kirakosyan et al., 2009).

These polyphenols might be capable of exerting beneficial physiological effects and could be used as an effective intervention in health maintenance and exercise recovery (Bell et al., 2013). It has been previously shown that tart cherries attenuate circulating inflammatory markers (Wang et al., 1999; Howatson et al., 2010; Bell et al., 2014), improve recovery following exercise (Howatson et al., 2010; Bell et al., 2016) and improve sleep quality (Pigeon et al., 2010; Howatson et al., 2012). Despite previous studies in cell culture and animal models, where cherry extracts have been shown to exert a range of cardio-protective effects (Wang et al., 1999; Seeram et al., 2001), there has been only two published studies illustrating the pharmacokinetics of tart cherry polyphenols and concurrent evidence of a biological effect (Bell et al., 2014; Seymour et al., 2014). The health-related benefits have been postulated to arise from the high anthocyanin content of tart cherries; however, the biological effectiveness of tart cherries might be due to polyphenol interactions, which accomplish complementary effects. Such synergies occur when combinations of bioactive substances exert effects at target sites that are greater than the sum of individual components (Lila & Raskin, 2004).

Furthermore, it has been noted that anthocyanins may not be stable during processing or storage (Patras et al., 2010). Thus, it is not surprising that tart cherry secondary metabolites could be biologically more active than individual ‘whole’ components in cherries. The main non-flavonoid polyphenols of dietary significance are the C_6–C_1 phenolic acids; these provide unique taste, flavour and health-promoting properties and are found in many vegetables and fruits (Tomas-Barberan & Espin, 2001). A human study, feeding blood orange juice, suggested

that the phenolic acid degradation product, protocatechuic acid (PCA), was a major metabolite of anthocyanins (Vitaglione et al., 2007). In addition, a range of phenolic acids, including vanillic acid (VA), syringic acid, caffeic acid and ferulic acid, has been identified within human serum, following the consumption of anthocyanin-rich berries (Nurmi et al., 2009; de Ferrars et al., 2014), but data on the bioavailability of these phenolic acids are very scarce.

Vascular smooth muscle cells (VSMCs) are responsible for the provision of vascular tone in normal, healthy blood vessels, and their behaviour is critical in the development of atherosclerotic Plaques (Ross, 1993). The VSMC can be found in two states—healthy blood vessels are surrounded by differentiated VSMC in a contractile, quiescent state (Owens, 1995). Part of the response to vascular injury and the cascade of events that lead to the build-up of an atherosclerotic Plaque cause VSMC to de-differentiate into a phenotype that proliferates and migrates (Owens, 1995). These de-differentiated cells migrate through the intima and form part of the fibrous cap on an atherosclerotic plaque. De-differentiated VSMC in these fibrous caps comes into direct contact with metabolites in the circulating blood and therefore makes them a biologically relevant cell type to test whether metabolites could impact on their behaviour.

The presence of polyphenol compounds in Montmorency tart cherries that might improve vascular health has yet to be fully elucidated, and importantly if these compounds can be absorbed and potentially exert a physiological effect. Furthermore, metabolites from ingestion of cherries that alter the behaviour of VSMC would be of interest to ascertain further applications of this functional food. Based on the previous literature, it was hypothesised that Montmorency tart cherries would contain vasoactive compounds that would be absorbed and detectable in plasma and that these compounds would alter VSMC behaviour *in vitro*. Therefore, this study addresses the specific aim of this thesis, which were (1) the time course of selected phenolic compounds following ingestion of a Montmorency cherry concentrate and (2) exposure of VSMC to selected phenolic compounds would influence cell behaviour *in vitro*.

3.2 Methods

3.2.1 Participants

Twelve non-smoking males were recruited to take part in the study; the mean ± SD age, stature, mass and BMI were 26 ± 3 years, 178.5 ± 7.6 cm, 85.2 ± 11.7 kg

and 26.7 ± 3.2 kg/m^2, respectively. All participants were in apparent good health as assessed by a health-screening questionnaire. Exclusion criteria for the study were as follows: food allergy (as discussed with research team), history of gastrointestinal, renal or cardiovascular disease and current use of any food supplementations. The study was conducted in accordance with the Helsinki Declaration and ratified by the University's Research Ethics Committee. All enrolled participants provided written informed consent. This study was registered as a clinical trial with clinicaltrials.gov (NCT01825070).

3.2.2 Study design

As a first step in the current study, we analysed three analogues of tart Montmorency cherries (frozen, dried and concentrated) in order to identify which was superior in terms of the total anthocyanin and phenolic content as well as total AOX capacity and used in subsequent studies. The second part of the study utilised a double-blind, two-phase (separated by at least 10 days), randomised, crossover, but counterbalanced design in order to identify the bioavailability of specific phenolic acids and their influence on cell behaviour following the ingestion of two different doses of Montmorency tart cherry concentrate (MC). Each visit was at the same time of day and preceded by an overnight fast (≥10 h). On arrival to the laboratory, participants provided a baseline venous blood sample. As previously described (Bell et al., 2014), subsequent blood samples were taken at 1, 2, 3, 5 and 8 h post-MC consumption. No additional food or fluid was provided during the study period except for low-nitrate mineral water.

3.2.3 Treatments and dietary control

The MC concentrate (CherryActive, Sunbury, UK) was stored at 4 °C prior to use. Participants consumed either 30 or 60 mL of MC concentrate diluted with 100 mL of water in a double-blind crossover manner. According to the manufacturer's information, a 30 mL dose of concentrate was equivalent to approximately 90 whole cherries. Participants were instructed to follow a low-phenol diet for 48 h prior to each arm of the trial by avoiding fruits and its equivalents (i.e.. juices), vegetables, tea, coffee, alcohol, chocolate, cereals, wholemeal bread, grains and spices and were asked to refrain from strenuous exercise. Compliance with the dietary restrictions was examined with a self-completed standardised 2-day dietary record.

3.2.4 Montmorency tart cherry analysis

3.2.4.1Total anthocyanins (TACN)

The monomeric anthocyanin pigment content of the MC concentrate and aqueous Montmorency cherry fruit extracts (whole frozen and dried) was determined using the pH differential method (Buckow et al., 2010). The MC concentrate was diluted 1:20 in 25 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 (Ultraspec 2000 UV/Vis spectrophotometer, Pharmacia Biotech, Sweden). The absorbance difference A was calculated as A = (A510–A700) pH 1.0 − (A510–A700) pH 4.5. The TACN concentration C (mg/L) was expressed as mg cyanidin-3-glucoside equivalents according to the following equation: $C = A \cdot MW \cdot DF \cdot 1000/(\varepsilon \cdot l)$, where MW was the molar mass for cyanidin-3-glucoside (449.2 g/mol); DF was the dilution factor; 1000 was the conversion from g to mg; ε was the molar extinction coefficient for cyanidin-3-glucoside (26,900 L/mol); and l was the path length (1 cm).

3.2.4.2 Total phenolic content (TPC)

Total phenolic content was measured using a modified Folin–Ciocalteu colorimetric method (Shahidi, 2007). Samples were diluted in deionised water (1:10 or 1:100), and 50 µL of the diluted extract, 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 (Greiner Bio-One, Monroe, USA). Absorbance readings were taken at 725 nm, at 5-min intervals, over a 30-min period at 25 °C (BioTek Synergy HT Multi-Mode MicroPlate Reader, Winooski, USA). A stock solution of gallic acid (5.8 mM) was prepared in aqueous methanol (80 % (v/v), and quantification was performed on the basis of a standard curve in the range 0–50 mg/mL ($R^2 = 0.99$). The analysed samples were measured versus a blank sample. All values are expressed as means of gallic acid equivalents per gram of sample ±SE for six replications.

3.2.4.3 Trolox equivalent AOX capacity (TEAC)

A modified DPPH assay used for AOX activity measurements was adjusted for use in the present study (Brand-Williams, 1995). The DPPH solution was prepared freshly before analysis, by dissolving the DPPH reagent (2.4 mg) in 80 % methanol (100 mL). Then, 10 µL of extract, 40 µL of deionised water and 200 µL of DPPH solution were added into each well of the 96-well Plate (CELLSTAR,

Greiner Bio-One, Monroe, USA). Absorbance readings were taken at 515 nm, at 3-min intervals over a 30-min period at 37 °C, using a Multi-Mode MicroPlate Reader (BioTek synergy HT, Winooski, USA). A calibration curve using Trolox (0–500 μM, R2 = 0.99) was plotted. Final values are expressed as means of Trolox equivalents per milligram of sample ±SE for six replications.

3.2.4.4 Individual phenolic analysis

The levels of individual phenolics (CHL, PCA and VA) were determined by HPLC and diode array detection (DAD), using the methods described by Bell and colleagues (Bell et al., 2014). These phenolic acids were preferentially selected as they are the most abundant degradation products of cyanidin and peonidin, the two major anthocyanins detected in the Montmorency whole cherry (Kirakosyan et al., 2009) and concentrate (Bell et al., 2014). A 1 % solution of MC juice was prepared in 1:1, 0.1 % formic acid: 2 % HCl in methanol (MeOH) filtered with a 0.2-μm polytetrafluoroethylene (PTFE) filter and analysed by HPLC–DAD using a Phenomenex Luna C_{18}, (250 × 2.0 mm × 5 μm). To characterise the major phenolics present, a sample of MC concentrate was analysed by LC–MS. All of the phenolic acids were identified in the MC concentrate after applying the extractive procedure and chromatographic method. The MS chromatogram of CHL in MC concentrate is presented in Figure 8 (A: 1-2).

3.2.5 Blood sampling

Fasting whole blood samples were collected in a 10 mL EDTA Vacutainer system (Becton, Dickinson and Company, Plymouth, New Zealand), inverted to mix the anticoagulant and immediately centrifuged at 3000×g for 10 min at 4 °C. Plasma was aspirated and pipetted into ~1 mL aliquots and then immediately stored at −80 °C for later analysis.

3.2.6 HPLC analysis

Under the selected chromatographic conditions, calibration graphs were obtained by preparing standard samples of each compound in triplicate, with increasing concentration of each analyte. From calibration graphs, the limit of detection and linearity were calculated (Table 5). The HPLC– DAD was used to identify plasma concentrations of phenolics for the acute phase of the study (pre-supplementation through to 8 h post-supplementation). Plasma samples were extracted using a solid-phase extraction procedure. Briefly, 1 mL of plasma was mixed with 4 mL oxalic acid (10 mM) and 0.1 mL HCl (12.06 M) in 15 mL Falcon tubes and

centrifuged at 826×g for 5 min. The supernatant was absorbed on to a primed solid-phase extraction cartridge (Waters Sep-Pak C_{18} plus short cartridge, 360 mg sorbent per cartridge, 55–105 µm), conditioned with MeOH with 0.2 % trifluoroacetic acid (TFA) followed by 2 × 5 mL of water. The sample was eluted with 3 mL of MeOH with 0.2 % TFA, dried under N2 at 45 °C. Samples were then reconstituted in 400 µL of dilution solvent (0.1 % formic acid in water: 2 % HCl in MeOH) and filtered through a 0.2-µm PTFE filter prior to HPLC analysis. The method's recovery was assessed by analysing separate aqueous solutions of each of the AOXs at 1, 25 and 50 µL/mL, as well as blank plasma samples with added AOXs at the same three concentrations. The recovery for all sample preparations was 99.1–101.4 %.

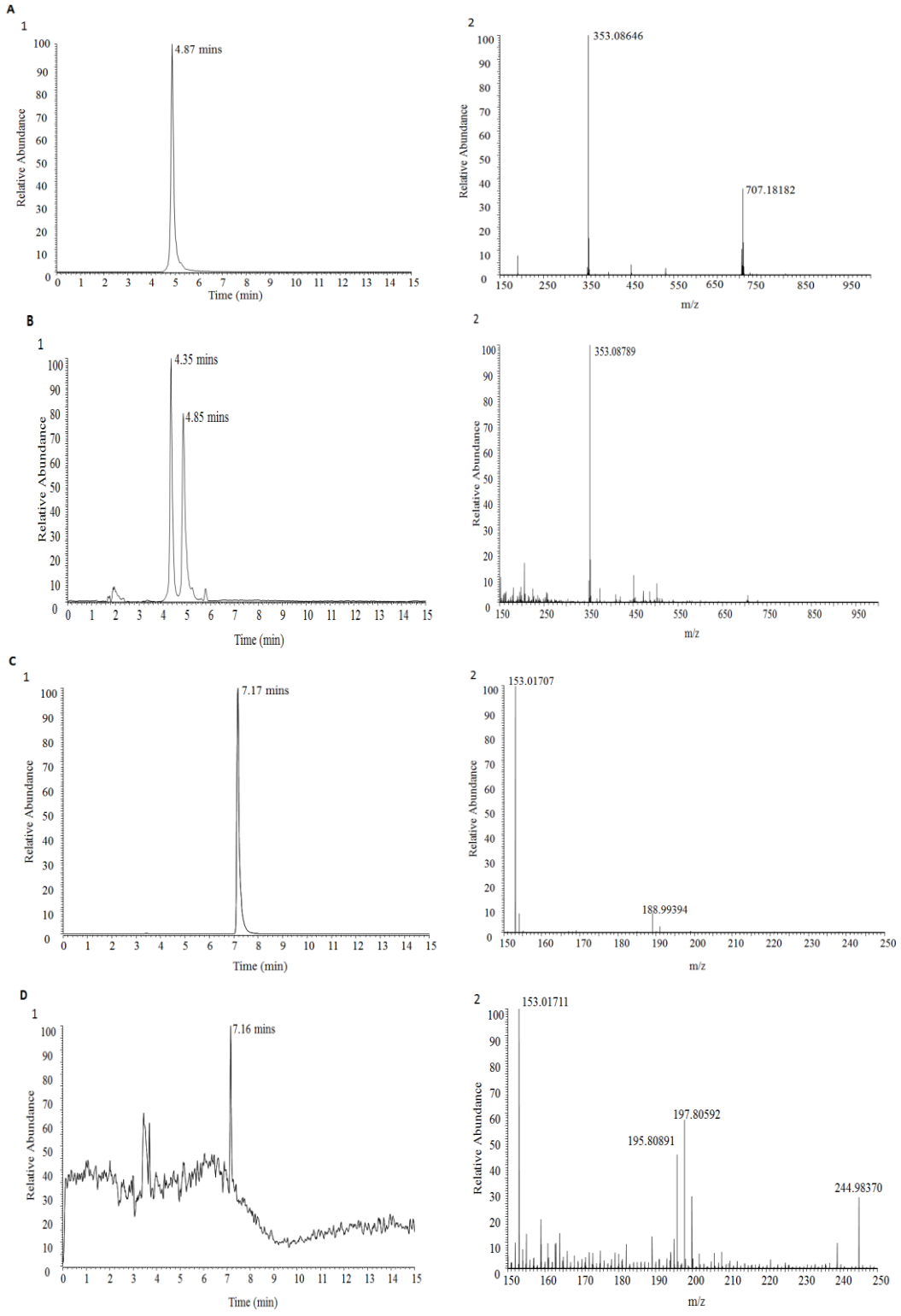

Figure 8 - A (1) LCMS Chromatograms of CHL, extracted ion mode range m/z 352-256 (RT=4.87 min) (2) MS output of CHL, @ RT = 4.87 min (the m/z at 707 could be a CHL dimer, formed in the MS) B (1) MC Juice, extracted ion, range m/z 352-356. The two distinct peaks in the juice (RT=4.37 and 4.87 min) have similar MS spectra (m/z 353 main ion) which could indicate the presence of another isomer i.e.. crypto- or neo- CHL (2) MC Juice @ RT = 4.87 min C (1) PCA, extracted ion range m/z 150-155 (RT=7.17 min) (2) PCA @ RT = 7.17 min D (1) Plasma sample (S1-B-Z), extracted ion, range m/z 150-155 (RT=7.17 min) (2) Plasma sample (S1-B-Z), @ RT=7.17 min.

HPLC–DAD analysis of phenolics was carried out using HPLC equipped with a pump, autosampler and UV– Vis detector (UltiMate 3000 HPLC system, Dionex, Camberly, UK). Known volumes of model system solutions (0.1–0.3 mL), were transferred into an autosampler vial, and deionised water was added to afford a final volume of 1.5 mL. Sample aliquots (10 µL) of plasma were injected on a 2.1 cm × 150 mm i.d., 3-µm particle size reverse-phase column Phenomenex Luna C_{18}(2) (250 × 2.0 mm, 5 µm particle size) that was thermostatically regulated at 30 °C. The mobile phase consisted of water with 1 % acetic acid (solvent A) and acetonitrile with 1 % acetic acid (solvent B). After a 5-min equilibration with 20 % B, the elution program was as follows: 0–20 min, 10–100 % B, (0.2 mL/min) followed by a washing stage (100 % B, 20–28 min, 0.2 mL/min) and re-equilibration at the initial conditions for 5 min. Detection was performed at the following wavelengths: λ = 260 nm for PCA and VA and λ = 326 nm for CHL. The polyphenolic content of plasma extracts was calculated by interpolation from the calibration graph and expressed as micrograms per millilitre (µg/mL).

The total anthocyanin, total phenolic and total AOX capacities for each analogue of tart Montmorency cherry are presented in Table 4. Additionally, the individual phenolic acid quantities in the concentrate are also provided.

Table 4 - Total anthocyanin, phenolics and AOX activity in pitted, frozen, whole, dried and concentrated Montmorency tart cherry.

	TACN	TPC	TEAC	Total CHL*	Total PCA*	Total VA*
30 mL MC Concentrate	31.24 ± 0.16	71.37± 0.11	0.30±0.01	0.205±0.24	0.020±0.11	0.253±0.84
60 mL MC Concentrate	62.47 ± 0.31	142.73 ±0.22	0.60±0.03	0.410±0.48	0.040±0.22	0.506±1.68
Frozen cherries	0.03 ± 0.0009	0.005± 0.0004	0.002± 0.0002	-	-	-
Dried cherries	0.008 ± 0.0003	0.006± 0.0005	0.002± 0.0001	-	-	-

Values are presented as Mean ± SEM, n = 6 sample preparations per analysis, (*n=3). TACN, total anthocyanin content, MC = mg cyanidin-3-glucoside /L, Whole Food = mg cyanidin-3-glucoside/100 g of cherries; TPC, total phenolic content, MC = mean gallic acid equiv/L, Whole Food = mean gallic acid equiv/g of cherries; TEAC, trolox equivalent AOX capacity, MC = mean Trolox equiv /L, Whole Food: mean Trolox equiv/g of cherries; CHL, chlorogenic acid; PCA, protocatechuic acid; VA, vanillic acid, MC = µg/m

Table 5 - Retention times (min) and selected UV-Vis wavelengths for quantitation of phenolics by HPLC-UV/Vis

Compound	UV/Vis wavelength (nm)	Retention time (min)	LOD (µg/mL)	Range of linearity(µg/mL)
PCA	260	9.263	<0.05	0.5-80
CHL	326	10.140	<0.04	0.4-80
VA	260	11.326	<0.04	0.5-100

LOD, limit of detection

3.2.7 LC–MS analysis

A liquid chromatography–mass spectrometry (LC–MS) method, utilising the same chromatographic conditions as the HPLC–DAD analyses, was used for the identification of individual compounds in the plasma and juice samples. Briefly, LC–MS analyses were carried out on a Dionex UltiMate 3000 RSLC HPLC System (Dionex, Camberly, UK) equipped with an UltiMate 3000 RS pump, an UltiMate 3000 RS autosampler and a QExactive Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, Waltham, USA). Electrospray ionisation at both negative and positive ion modes was performed with a spray voltage of 2.00 kV and capillary temperature of 280 °C. The total ion current (TIC) with a range of 100–1500 m/z and 70,000 resolution was measured. Sample aliquots (2 µL) were injected on an Phenomenex Luna C_{18}(2) (250 × 2.0 mm, 5 µm particle size) reverse-phase column thermostatically regulated at 40 °C. The mobile phase consisted of water with 1 % acetic acid (solvent A) and acetonitrile with 1 % acetic acid (solvent B). The same method as applied for the HPLC analysis was carried out on the LCMS. The identification of phenolics in the MC concentrate was verified by retention time and spectral data comparison with the corresponding reference compounds.

3.2.8 Vascular smooth muscle cell culture and migration

Primary human aortic smooth muscle cells (VSMC; Life Technologies, Paisley, UK) were cultured in 231 medium supplemented with smooth muscle cell growth supplement (Life Technologies, Paisley, UK) including 5 % foetal bovine serum. The VSMC cultures were maintained in 75-cm^2 tissue culture flasks in a humidified incubator at 37 °C. For all experiments, VSMC was used between passages 3 and 8. Migration of VSMC in response to metabolites PCA and VA was determined using xCelligence realtime cell analyser. PCA and VA were dissolved in 100 % ethanol, to a concentration of 100 mM. VSMCs were serum-starved for 24 h and then detached from the flask with trypsin. The cells were then incubated with PCA at a molarity concentration of 32 µM and VA at 4 µM or ethanol only (<0.04 % (v/v)) control for 1 h at 37 °C. These concentrations were based on the plasma bioavailability concentrations ascertained by the *in vivo* part of this work and fall within the range of values we observed following consumption of the cherry juice. The VSMC was Plated onto an xCelligence cell invasion and migration (CIM) Plate containing serum-free medium in the top and bottom chambers. The VSMC was added to the top chamber at a density of 8000 cells/chamber. Migration of VSMCs

was determined by measuring impedance, which is created as cells move from the top chamber, through a microporous membrane to the bottom chamber and attach to a gold electrode on the underside of the top chamber. Measurements were taken every 15 min over a 24-h period. Measurements were then converted to cell index values, which were used as a relative measure of cell migration.

3.2.9 Vascular smooth muscle cell culture and proliferation

Kinetic proliferation assay—an xCelligence real-time cell analyser—was used to monitor cell proliferation in real time (Acea Biosciences Inc, CA, USA). Primary VSMCs were seeded onto an xCelligence E Plate at 6000 cells/ well, with metabolites or ethanol only as control in normal VSMC growth media. E Plates consist of a gold microelectrode, and growth of cells is determined by measuring relative electrical impedance across the cell monolayer. Impedance measurements were taken every 15 min for up to 72 h to determine cell proliferation.

3.3 Statistical analysis

Statistical analysis was performed using PASW statistics 21.0 for Windows (SPSS, Inc., Chicago, IL.). Descriptive statistics are reported as mean ± SEM. All dependant variables were analysed by using a treatment (30 vs. 60 mL) by time (baseline, 1, 2, 3, 5 and 8 h) mixed model analysis of variance (ANOVA). Mauchly's test of sphericity was used to check homogeneity of variance for all variables; where necessary, any violations of the assumption were corrected using the Greenhouse–Geisser adjustment. Significant interaction effects were followed up using LSD *post hoc* analysis. Further analysis was conducted to identify maximum plasma concentrations (c_{max}) and times to achieve maximum plasma concentrations (t_{max}), which were directly obtained from the plasma concentration–time profiles. As a measure of overall Plasma bioavailability of individual phenolic acids, the area under the plasma concentration– time curve (AUC0–8h) was estimated by using the linear trapezoidal rule. For the cellular experiments, significance was judged using Student's t test. Results are reported as mean ± SEM. The alpha level for statistical significance was set at 0.05 *a priori*.

3.4 Results

3.4.1 Protocatechuic acid (PCA), vanillic acid (VA) and chlorogenic (CHL) acid

Firstly, all participants (n = 12) complied with the low polyphenolic experimental diet according to the food diaries. The MS chromatogram of PCA in plasma is presented in Figure 8 (C: 1-2). The PCA (Figure 9) revealed no significant treatment effect (F = 0.59, P = 0.810) or treatment by time interaction effect (F = 0.405, P = 0.845). Following supplementation, there was a significant time effect on PCA plasma levels (F = 2.956, P = 0.015). The PCA levels in plasma were significantly higher 1 h following consumption of the low (30 mL) and the high dose (60 mL) of MC when compared to baseline (P = 0.014 and 0.05, respectively). For both the 30 and 60 mL MC dose, the tmax was 1 h for PCA. The C_{max} values for PCA were not different between the 60 mL (2.75 ± 0.13 μg h/mL^{-1}) and the 30 mL (2.76 ± 0.10 μg h/mL^{-1}) dose. Furthermore, $AUC_{0–8h}$ values for PCA were not different between the 30 and 60 mL doses, 102.4 ± 0.9 and 106.4 ± 0.1 μg h/mL^{-1}, respectively. The presence of PCA was confirmed in plasma by comparison of the experimentally determined monoisotopic molecular weights to literature value, in which all were within ±1.5 ppm (Table 6).

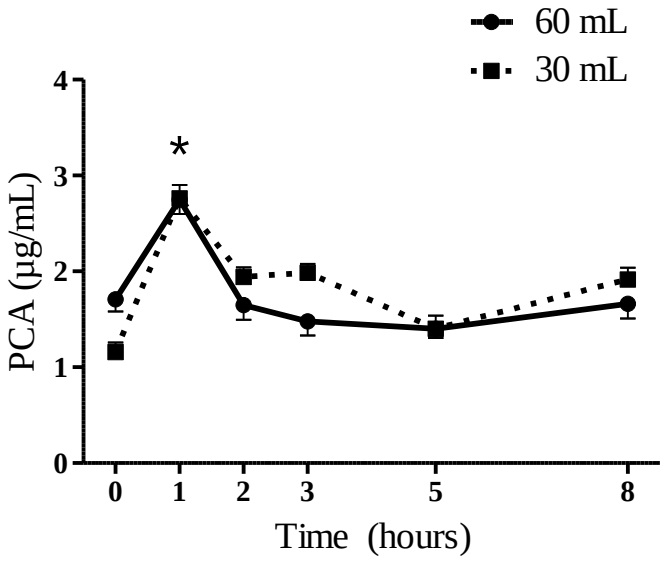

Figure 9 - PCA responses from baseline to 30 mL and 60 mL Montmorency cherry concentrate (MC). Absolute baseline values were 1.16 ± 0.326 and 1.70 ± 0.435 ug/mL for 30 mL and 60 mL, respectively. * indicates a significant time effect (P < 0.05) (30 mL and 60 mL dose); data presented as mean ± SEM.

Plasma VA (Figure 10) revealed no treatment effect (F = 0.004, P = 0.951) or treatment by time interaction effect (F = 1.583, P = 0.195). However, following supplementation, there was a time effect (F = 3.329, P = 0.008). VA levels were higher 1 h after consumption of the higher dose (60 mL) when compared to baseline (P < 0.05). Pairwise comparisons revealed increases in VA from 1 to 5 and 8 h post- 60 mL MC juice consumption (P < 0.05) compared to baseline. However, no significant time effects were observed with the lower dose. For VA, the t_{max} differed depending on the dose administered, occurring at 1 h with the 60 mL dose and 2 h in the 30 mL dose. C_{max} values for VA were not significantly different between the 60 mL (0.29 ± 0.03 μg h/mL^{-1}) and the 30 mL (0.30 ± 0.01 μg h/mL^{-1}) dose. Furthermore, AUC0– 8h values for VA were not different between the 30 and 60 mL doses, 10.7 ± 0.1 and 11.8 ± 0.1 μg h/mL^{-1}, respectively. The presence of VA was confirmed in plasma by comparison of the experimentally determined monoisotopic molecular weights to literature value, in which all were within ±1.5 ppm (Table 6). The current study did not detect CHL in the plasma post-MC consumption (F = 0.148, p=0.704).

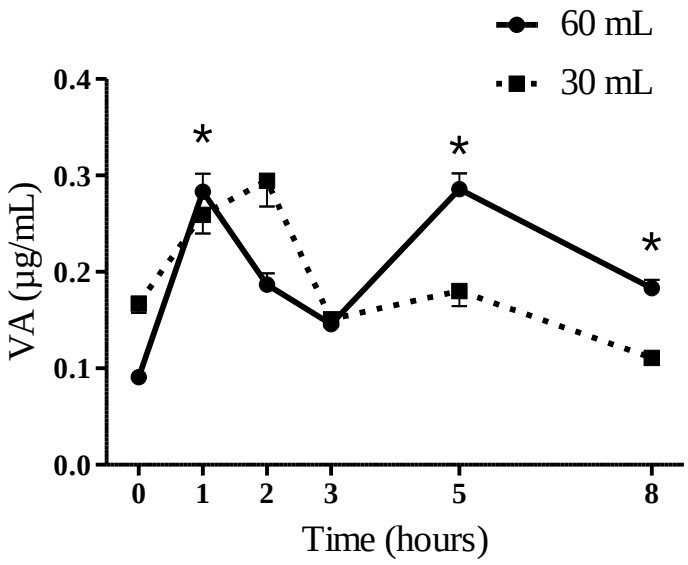

Figure 10 - VA responses from baseline to 30 mL and 60 mL Montmorency cherry concentrate (MC). Absolute baseline values were 0.158 ± 0.031 and 0.093 ± 0.024 ug/mL for 30 mL and 60 mL, respectively. * indicates a significant time effect (P < 0.05) (60 mL dose only); data presented as mean ± SEM.

Table 6 - LCMS characterization of phenolic peaks.

Polyphenol	Formula	Found	Monoisotopic Mass	
			Ionisation Mode	Literature Value[1]
PCA	$C_7H_6O_4$	154.034	Negative	154.026611
CHL	$C_{16}H_{18}O_9$	354.102	Negative	354.095093
VA	$C_8H_8O_4$	168.049	Negative	168.042252

[1]Royal Society of Chemistry, (2013), Chemspider chemical database. Found at: http://www.chemspider.com/. ND, Not Detected.

3.4.2 Cell behaviour

The maximum mean concentration of PCA measured in plasma was 2.5 µg/mL (range 0.33–6.65 µg/mL) (Figure 9) and VA was 0.3 µg/mL (range 0.1–1.32 µg/mL) (Figure 10); these correspond to molar concentrations of 16 and 2 µM, respectively. The concentrations were doubled to 32 µM PCA and 4 µM VA for the cell experiments to fall within the maximum range of concentrations we observed *in vivo*. Both PCA and VA were applied to VSMC in isolation and showed no significant increase in migration behaviour in comparison with a control. When PCA and VA were combined and added to the culture, there was an increase ($P = 0.038$) in migration of VSMC by 36 ± 12 % when compared to the control (Figure 11 and 12). Finally, there was no effect of the metabolites on VSMC proliferation (Figure 13).

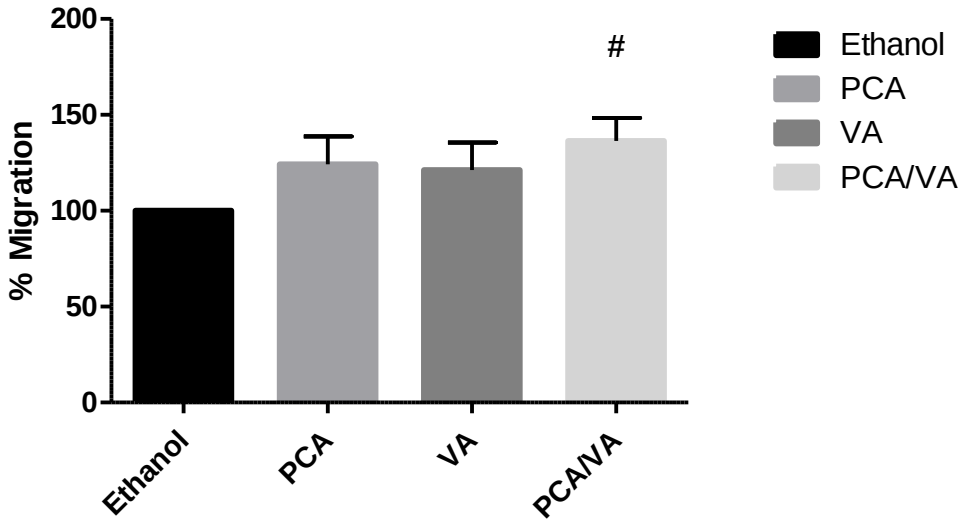

Figure 11 - % Migration of human vascular smooth muscle cells *in vitro* in response to metabolites PCA (32µM) and VA (4µM) compared to ethanol only control, over 24hours. Combined data from three separate experiments. # indicates a significant difference between PCA/VA and ethanol (control) condition ($P < 0.05$); data presented as mean ±SEM.

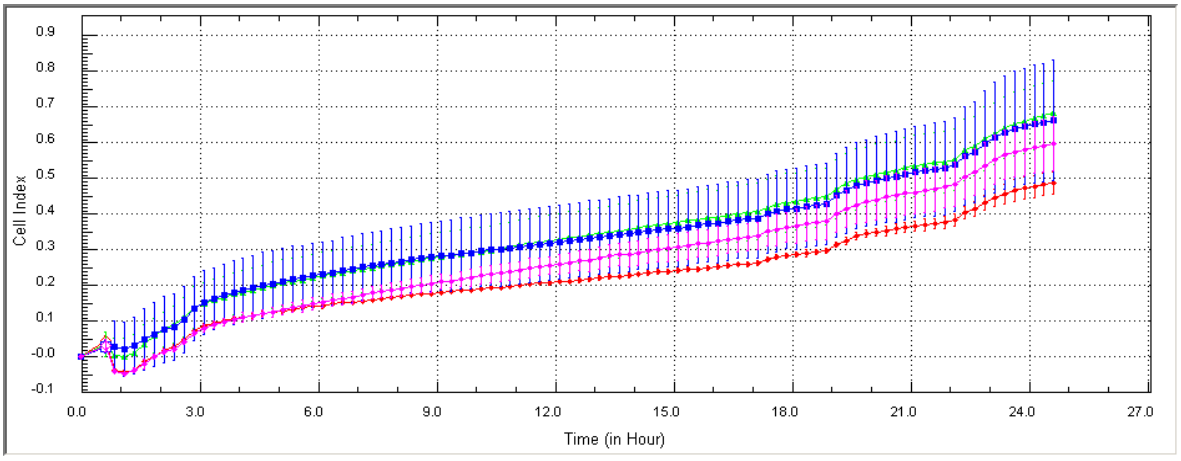

Figure 12 - Cell migration over time. Red = ethanol only (control), green = PCA only, blue = VA only and pink = PCA and VA combined.

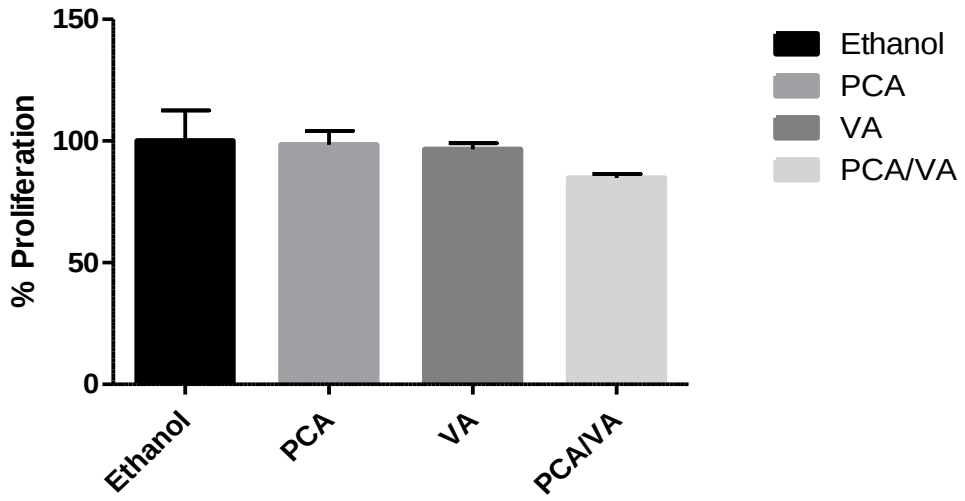

Figure 13 - % Proliferation of human vascular smooth muscle cells *in vitro* in response to metabolites PCA (32µM) and VA (4µM) compared to ethanol only control, over 24hours. Combined data from four separate experiments; data presented as mean ± SEM.

3.5 Discussion

Following the identification of which Montmorency cherry analogue (frozen, dried and concentrated) had the greatest AOX activity, total anthocyanin and phenolic content, MC concentrate was used to investigate the plasma kinetics of selected phenolic acids and their subsequent effect on cell behaviour *in vitro*. This investigation presents new information on the appearance and time course of phenolic compounds in plasma following consumption of a lower and higher dose of MC concentrate. The hydroxybenzoic acid content of edible plants is generally very low, with the exception of certain red fruits, black radish and onions (Brand-Williams et al., 1995). As a result, very little is known about the metabolism and absorption of these compounds. However, this study showed that both PCA and VA are most bioavailable in plasma 1–2 h post-MC consumption, whilst other hydroxycinnamic acids (CHL) were not present in the plasma. Furthermore, the combination of PCA and VA increased cell migration, but had no effect on the proliferation of VSMC.

High concentrations of VA *in vivo* can be attributed to the large quantity of anthocyanins in fruits and vegetables (Nurmi et al., 2009). Ou et al. (2012) previously established that the anthocyanins (in order of decreasing prevalence) in processed tart cherry products were cyanidin-3-glucosylrutinoside, cyanidin-3-rutinoside and peonidin-3-rutinoside. These findings concur with those data from Balaton cherries (Wang et al., 1997) and Montmorency cherries (Kirakosyan et al.,

2009). VA is the major degradation product of the parent compound peonidin, and this could explain the detection of VA in plasma 1–2 h post-MC consumption in the current study. Interestingly, it has been proposed that VA possesses chemopreventive properties due to its AOX activity and its ability to scavenge free radicals (Kamat et al., 2000). VA has also been shown to exhibit immunostimulatory effects in enhancing interferon gamma (IFN-γ) secretion and stimulating proliferation of human peripheral blood mononuclear cells (Chiang et al., 2003).

Similar to VA, high concentrations of PCA *in vivo* are likely to be as a direct result of the original anthocyanin content of both fruits and vegetables (Fang, 2014). Cyanidin is the main anthocyanin in tart cherry products (Kirakosyan et al., 2009); this was recently clarified in the MC concentrate (3.346 mg/mL) where cyanidin accounted for the overwhelming majority of anthocyanins with far smaller amounts of peonidin and malvidin (Bell et al., 2014). Cyanidin-3-glucoside has been shown to readily degrade to cyanidin and then further metabolised to PCA. Vitaglione et al. (2007) reported that PCA accounts for almost 73 % of cyanidin ingested. They concluded that a high concentration of PCA could explain the short-term increase in plasma AOX activity observed after intake of cyanidin-rich food. PCA has also been shown to remain in biological tissues for longer periods of time than the parent anthocyanin (Azzini et al., 2010). A recent addition to the literature (Kirakosyan et al., 2015) examined the tissue bioavailability of cherry phenolic compounds in rats following 3 weeks of supplementation. The work showed some tissues preferentially store these phenolic compounds, but importantly when examined with the data from the current study the transient increase in compounds seen in plasma might be the first step to increase tissue bioavailability and hence a potential pathway to the proposed health-enhancing benefits of cherry phenolics. Consequently, more longitudinal supplementation studies should investigate tissue bioavailability in humans to ascertain whether increased tissue concentrations of these compounds are possible in a human model. The detection of high concentrations of PCA in human plasma 1–2 h post- MC ingestion has the potential to exert some physiological potential; for example, previous research has shown that PCA possesses antibacterial, AOX, antidiabetic, anticancer, antiulcer, antiaging, antiviral, anti-inflammatory,anti-atherosclerotic properties (Kakker & Bais, 2014).

Despite there being high quantities of CHL in the MC concentrate, it was not detected in plasma. Other food and beverage studies administered much higher concentrations of CHL; for example, Stalmach et al. (2014) gave participants a single serving of a coffee beverage fortified with CHL, the serving consisted of low (412 µmol), medium (635 µmol) and high (795 µmol) quantities of CHL.Although 412 µmol/L represented the low dose, this is still far greater than the amounts identified in the MC concentrate in the current study, where 6.8 µg/mL which equates to ~22.1 µmol/L. Unlike the previous work, this study utilised an ecologically valid quantity of CHL that was found the MC concentrate that represents a sensible portion to consume, rather than an artificially derived concentration used previously (Stalmach et al., 2009; 2014). In support of this, a previous study using comparable concentrations of CHL also failed to detect its presence *in vivo* (rodent plasma) following CHL ingestion (Azuma et al., 2000). Given that CHL was present in the MC concentrate, it is quite plausible that CHL was metabolised quickly to the downstream metabolites caffeic, quinic and ferulic acid. This provides some explanation for the non-detection in plasma; however, the possibility remains that CHL become degraded during sample treatment process (Farah & Donangelo, 2006). A study (Farah et al., 2008) evaluating the pharmacokinetic profile and bioavailability of CHL in plasma and urine of 10 healthy participants showed a great deal of inter-individual variation in CHL absorption, metabolism and kinetics with uptake values ranged from 7.8 to 72.1 % amongst participants. Large inter-individual variations in the plasma concentrations of all compounds in the current study are not unexpected because of the multifaceted factors such as metabolism and genetic disposition to gut microbial composition (Mountzouris et al., 2007).

The effects of the metabolites PCA and VA on the migration of VSMC *in vitro* were also assessed. Migration increased when the cells were treated in concert with both metabolites, demonstrating that these metabolites, at a similar level to that seen in plasma, can alter VSMC function. Migration of de-differentiated VSMC is required for vessel remodelling which occurs from exercise and vascular injury. The VSMC migration in advanced atherosclerotic plaques is often considered to be protective as it increases stability, protecting against plaque rupture and ensuing vascular trauma such as myocardial infarction or stroke (Louis & Zahradka, 2010). By increasing VSMC migration, the metabolites may potentially be beneficial for blood vessel remodelling, although this would require further

investigation. Elsewhere there are conflicting reports of the effects of PCA on cell migration; for example, PCA has been shown to increase the migration of adipose tissue-derived stromal cells (Wang et al., 2008) and to inhibit the migration of gastric cancer cells (Lin et al., 2011), the mechanism of which are thought to involve alterations in matrix metalloprotease activity. These conflicting reports are likely attributable to the different cell culture models used in each study. It is interesting to note that the concentration of PCA used in this study was similar to physiological concentration observed *in vivo*, whereas previous studies required between 15 and 47 times greater concentrations to observe an effect on cell behaviour (Wang et al., 2008; Lin et al., 2011). VA is less studied, but has been reported to have a small effect on lung cancer cell migration in comparison with controls, at a concentration 1000 times greater than used in the current investigation (Lirdprapamongkol et al., 2009). This is the first study where PCA and VA have been examined in concert and show that VSMC migration can be influenced at physiologically relevant levels that can be consumed from MC. Importantly, the work suggests that the examination of phenolic acids (or other polyphenols) in isolation may be of limited value, particularly when whole foods and their analogues are far more complex.

An acknowledged limitation of the current study is that the analysis was not exhaustive and so not every polyphenol was analysed; instead, the focus was on the degradation products of two of the main anthocyanidins reported in the MC juice that could exert a positive effect on vascular function. In addition, we did not investigate compounds, for instance procyanidins, which appear to have poor bioavailability due to instability, large molecular weight or are quickly excreted. Conceivably, these compounds might also contribute to any potential physiological effects exerted by MC and cannot be excluded. Furthermore, enterohepatic metabolism could predict that the absorption of polyphenols and their metabolites are not limited to few hours after intake (Seymour et al., 2014; de Ferrars et al., 2014). As a result, the timeframe of the analysis in the current study may be regarded as a potential limitation.

In conclusion, these data provide new information on the presence of phenolic acids in plasma following MC concentrate consumption in humans. The time course of metabolite absorption peaks at 1–2 h post-consumption, and this information could inform future *in vivo* work that examines the health-related

benefits associated with Montmorency tart cherries. Lastly, MC concentrate provides a bioavailable source of polyphenols that could be helpful in modulating vascular function (Broncel et al., 2010; Mudnic et al., 2012) and influencing cell behaviour.

3.6 Perspectives

This Chapter aimed to identify the polyphenol uptake of polyphenolic compounds following human consumption of Montmorency tart cherry and the subsequent influence, if any, of these compounds on cell behaviour. To date, the majority of work involving Montmorency tart cherry supplementation focuses on the absorption of primary anthocyanins. Given that the bioavailability of these compounds is poor, exploring the absorption time of their primary downstream metabolites may be more appropriate when trying to determine the compounds eliciting positive health benefits. This current study is of importance as it is the first study to identify both PCA and VA in plasma 1-2 h post MC consumption. These two dihydroxybenzoic acids are the main degradation metabolites of cyanidin and peonidin, the most abundant anthocyanins present in MC concentrate. The physiological concentrations of these phenolic acids at peak time points (1-2 h) altered VMSC behaviour, specifically cell migration. By increasing VSMC migration, the metabolites may potentially be beneficial for blood vessel remodelling, although this would require further investigation.

Importantly, this work addresses the first and second aim of the thesis investigating if the downstream metabolites of the principal anthocyanins in MC concentrate are detectable in plasma post consumption and whether these would have an effect on cell behaviour. This work suggests that the examination of phenolic acids (or other polyphenols) in isolation may be of limited value, particularly when whole foods and their analogues are far more complex. The information provided in this study could inform future *in vivo* work that examines the health-related benefits associated with Montmorency tart cherries. Future chapters will attempt to explore this premise.

4 Effects of Montmorency tart cherry consumption on vascular function in men with early hypertension

Publication arising from this Chapter: Keane, K.M., George, T.W., Constantinou, C.L., Brown, M.A., Clifford, T. and Howatson, G. (2016) Effects of Montmorency tart cherry (*Prunus Cerasus L.)* consumption on vascular function in men with early hypertension. *The American Journal of Clinical Nutrition,* 1-9.

4.1 Introduction

The previous Chapter of this thesis provided new information on the appearance and time course of phenolic compounds in plasma post Montmorency tart cherry consumption, and highlighted the ability of these compounds to modulate vascular smooth muscle cell behaviour at physiologically relevant concentrations. To date, the majority of work focusing on the health effects of polyphenols has examined phenolic acids (or other polyphenols) in isolation. Whole foods and their analogues are far more complex, the previous chapter has shown that MC concentrate consumption leads to the presence of phenolic acids in the plasma at concentrations that can physiologically modulate vascular smooth muscle cell behaviour. Therefore, the following study will attempt to further explore the cardiovascular benefits of tart cherries in humans.

Cardiovascular disease (CVD) is the primary cause of global mortality (Naghavi, 2015). In the United States, one in four deaths can be attributed to a cardiovascular related event, equating to roughly 610,000 people per annum (Centers for Disease Control and Prevention, 2015). In Europe, CVD is the major cause of death in adults and is responsible for nearly half (48%) of all annual deaths (Allender et al., 2008; Centers for Disease Control and Prevention, 2015). Epidemiological studies have suggested that polyphenol-rich foods can exert positive cardiovascular health benefits (Joshipura et al., 1999; Bazzano et al., 2002; Hung et al., 2004) on blood pressure (Taubert et al., 2007), insulin resistance (Grassi et al., 2005), cholesterol concentrations (Davies et al., 2002) and Platelet activity (Del Rio et al., 2013), which are thought to be attributable to the high polyphenol content in fruits and vegetables (Liu, 2003). Several studies have investigated these cardiovascular health benefits of polyphenolic-rich foods; for example, George and colleagues (George et al., 2009; 2012a; 2012b) examined the acute and chronic effects of a fruit and vegetable puree-based drink on vascular function and other CVD risk factors and showed that endothelium-dependent vasodilation was greatest at 3 h post consumption. Additionally, Dohadwala and colleagues demonstrated improvements in arterial stiffness and brachial artery flow mediated dilation 4 h post cranberry juice consumption (Dohadwala et al., 2011).

Tart cherries and their derivatives are high in numerous polyphenols (Wang et al., 1999; Seeram et al., 2001; Seymour et al., 2014; Bell et al., 2014) that include the

flavonoids isorhamnetin, kaempferol, quercetin, catechin, epicatechin, procyanidins, and anthocyanins (Kim et al., 2005; Kirakosyan et al., 2009). It has been previously shown that tart cherries attenuate inflammation (Wang et al., 1999), oxidative stress (Howatson et al., 2010; Bell et al., 2014) and accelerate exercise recovery (Howatson et al., 2010; Bowtell et al., 2011; Bell et al., 2014; 2016). Furthermore, cherry extracts have been shown, in cell and animal models, to exert a range of cardioprotective effects that include increasing nitric oxide production and AOX status, reducing lipid oxidation and inhibiting inflammatory pathways (Wang et al., 1999; Seeram et al., 2001). However, data from human trials are not clear; recently, it was postulated in the previous Chapter that the health-related benefits associated with Montmorency tart cherry consumption might, at least in part, be due to the downstream metabolites of the principle anthocyanins present in the fruit, as alluded to in Chapter 3. Specifically, this work demonstrated an increase in plasma phenolic acids (vanillic and protocatechuic acid) following Montmorency tart cherry (MC) consumption in humans. These specific phenolic acids are known to improve vascular function (Kamat et al., 2000; Kakker & Bais, 2014) and were shown in the preceding Chapter of this thesis to modulate vascular smooth muscle cell behaviour *in vitro*; however it is unclear if Montmorency cherry consumption influences *in vivo* cardiovascular function. In a previous study investigating this premise, Lynn and colleagues (Lynn et al., 2014) examined the effect of a tart cherry juice supplement on arterial stiffness and inflammation in healthy adults and showed no positive response. The authors speculated that this was due to numerous limitations including their sample size and blood sampling technique.

Although the potential for dietary polyphenols to improve cardiovascular health is encouraging, there is a clear need for randomized, placebo-controlled trials with appropriate experimental controls to ascertain the role that polyphenols from whole foods and their analogues might exert in health maintenance. Given that tart cherries are high in numerous polyphenols, such as anthocyanins, that increase the bioavailability of phenolic acids *in vivo*, it makes the expectation tenable they would positively modulate vascular function. Therefore, this study addresses the specific aim of this thesis, which was to examine the acute effects of the consumption of Montmorency tart cherries on arterial stiffness, blood pressure, microvascular vasodilation in males with early hypertension.

4.2 Methods

4.2.1 Participants

Sixteen non-smoking males with early hypertension (with SBP ≥130 mmHg, DBP ≥80 mmHg, or both) volunteered to participate; baseline characteristics are presented in Table 7. For inclusion, the criteria was a resting SBP >130 mmHg. A total of 56 participants were screened prior to study commencement. SBP >120 mmHg is indicative of early hypertension and reflects increased systemic vascular resistance and values above 120 mmHg are associated with increased risk of coronary heart disease and stroke (Lewington, 2003). Resting BP was measured using an automated sphygmomanometer (Omron 70CP, Tokyo, Japan), according to British Hypertension Society guidelines following a 10 min rest. Exclusion criteria were; food allergy (as discussed with the research team), history of gastrointestinal, renal or cardiovascular disease, BP-lowering or anticoagulant medication, current use of any food supplements and other risk factors that make them eligible for drug treatment of raised BP according to the British Hypertension Society guidelines. All participants were otherwise, in apparent good health as assessed by a health-screening questionnaire. The study was conducted in accordance with the Helsinki Declaration 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 clincialtrials.gov (NCT02234648).

Table 7 - Baseline characteristics of the study participants (n=15)[1]

Characteristic	Values
Age (years)	31 ± 9
Height (cm)	182.4 ± 7.3
Mass (kg)	89.7 ± 13.3
BMI (kg/m^2)	27.0 ± 3.8
SBP (mm Hg)	137 ± 11
DBP (mm Hg)	82 ± 11
MAP (mm Hg)	98 ± 11
HR (bpm)	63 ± 10

[1] Values are presented as means ± SD. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; HR, heart rate.

4.2.2 Study design

This study employed a randomised, but counter-balanced, placebo-controlled, blinded, cross-over, Latin square design with two experimental arms and a

washout period of at least 14 days; participants were randomized to receive either Montmorency tart cherry concentration (MC) followed by placebo (Pla), or Pla followed by MC. A washout of at least 14 days was chosen based on previous literature that suggests these phenolic compounds are quickly absorbed and/or excreted, as demonstrated in the previous Chapter. Each visit was at the same time of day and was preceded by an overnight fast (≥10 h). Participants reported to the lab at 8am and provided a baseline venous blood sample. This was followed by baseline microvascular vasodilation by laser Doppler imaging (LDI) with iontophoresis, arterial stiffness by pulse wave analysis (PWA) and pulse wave velocity (PWV), digital volume pulse (DVP) and blood pressure (BP) were performed with the participant in the supine position. Participants then consumed the intervention beverage and subsequent blood samples, LDI, PWV, PWA, DVP and heart rate (HR) measures were taken 1, 2, 3, 5 and 8 h post consumption; BP was performed every hour. No food or fluid was provided during the study period except for low-nitrate mineral water. The total amount of water consumed on the first study day ad libitum was noted and participants consumed the same quantity on the subsequent visit.

4.2.3 Treatments and dietary control

The MC concentrate (CherryActive, Sunbury, UK) was stored at 4ºC prior to use. Participants consumed either 60 mL of MC concentrate (which according to the manufacturer is estimated to be equivalent to ~180 whole cherries) or fruit-flavoured cordial in a single blind cross-over manner. The decision to use 60 mL was based on previous work that showed a greater uptake of anthocyanin and phenolic acids *in vivo* post-consumption, this was also shown in Chapter 3 of this thesis (Bell et al., 2014). The concentrate was diluted with 100 mL of water prior to consumption. The Pla supplement consisted of a commercially available, low fruit mixed berry (<1%) cordial (Kia Ora, Coca Cola Enterprises, Uxbridge, UK) with anthocyanins used only for colour, mixed with water, whey protein isolate (Arla Foods Ltd., Leeds, UK) and maltodextrin (MyProtein Ltd., Northwich, UK), to match the MC concentrate for volume and macronutrient content (Energy = 204 kcal, volume = 60 mL, carbohydrates = 49 g, protein = 2.2 g and fat = 0 g). The Pla also contained small amounts of antioxidants (ascorbic acid). Prior to study commencement, it was explained to participants that the aim of the study was to investigate the effect of a fruit juice on vascular function; therefore they were unaware which beverage was the experimental drink. Participants were instructed

to follow a low phenolic diet for 48 h prior to each arm of the trial by avoiding fruits, vegetables, tea, coffee, alcohol, chocolate, cereals, wholemeal bread, grains and spices and were asked to refrain from strenuous exercise. Compliance with the dietary restrictions was assessed with a self-reported standardized 2-day dietary record.

4.2.4 Blood sampling

Fasting whole blood samples were collected at baseline (before supplementation), 1, 2, 3, 5 and 8 h in a 10 mL ethylenediaminetetraacetic acid vacutainer system (Becton, Dickinson and Company, Plymouth, New Zealand), which was inverted to mix the anticoagulant and immediately centrifuged at 3000 × g for 10 minutes at 4°C. Plasma was aspirated into aliquots and then immediately stored at -80°C for later analysis.

4.2.5 Laser Doppler imaging

Subjects were placed supine in a quiet, temperature controlled room, where the ambient temperature was 23±1ºC for all measures. Two perspex chambers (ION6, Moor Instruments Limited, UK) with an internal platinum wire electrode were placed on the volar aspect of the forearm and attached to the skin using adhesive discs (MIC-1AD; Moor Instruments Limited, UK) and connected to the iontophoresis controller (MIC2, Moor Instruments Limited, UK). Acetylcholine chloride (2.5 ml, 1%; Sigma-Aldrich, UK) in 0.5% NaCl solution was placed in the anodal chamber and 2.5 ml of 1% sodium nitroprusside (Sigma-Aldrich, UK) in 0.5% NaCl solution was placed in the cathodal chamber. Circular glass coverslips were placed over each chamber to prevent loss of solutions. Current delivery was controlled by a laser Doppler imager Windows software v.5.1 (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 area under the flux vs. time curve over the twenty scans was calculated as a measure of microvascular response to acetylcholine (ACh; endothelium-dependent vasodilation) and sodium nitroprusside (SNP; endothelium-independent vasodilation).

4.2.6 Pulse wave velocity / analysis

PWV and PWA were determined by using Arterial Tonometry (SphygmoCor CPV system, ScanMed Medical, UK). There is strong association of PWV and PWA with incident cardiovascular disease, independently of traditional risk factors (Cruickshank et al., 2002; Choi et al., 2007; Buckow et al., 2010). The aortic pulse waveform and augmentation index (AIx) were derived at the radial artery; PWV was determined between carotid and femoral sites. A pencil-type probe was used for all measurements and was held at the base of the neck over the carotid artery and at the inguinal crease over the right femoral artery. Recordings were taken when a reproducible signal was obtained with a high amplitude excursion. 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.

4.2.7 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 first and the second wave peaks, and it is usually correlated with the stiffness of large arteries. The DVP-RI is the relative height of the second peak compared with the first and is associated with smaller artery stiffness. Collectively, these variables provide an indication of the stiffness of the arterial stiffness for an individual.

4.2.8 Blood pressure

Blood pressure was measured using a non-invasive digital automatic BP monitor (M10-IT Omron Healthcare, UK). The BP cuff was fitted by the same individual at each of the nine time points. All vascular measurements took place on the non-cannulated arm.

4.2.9 Juice analysis

4.2.9.1 Total anthocyanins (TACN)

The monomeric anthocyanin pigment content of the MC concentrate and the placebo was determined using the pH-differential method (Buckow et al., 2010).

The MC concentrate was diluted 1:20 in 25 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 (Ultraspec 2000UV/Vis spectrophotometer, Pharmacia Biotech, Sweden). The absorbance difference A was calculated as A = (A510-A700) pH1.0 – (A510-A700) pH 4.5. The TACN concentration C (mg/L) was expressed as mg cyanidin-3-glucoside equivalents according to the following equation: C=A.MW.DF.1000/(Ɛ.l), where MW was the molar mass for cyanidin-3-glucoside (449.2 g/mol); DF was the dilution factor; 1000 was the conversion from g to mg; ε was the molar extinction coefficient for cyanidin-3-glucoside (26900 L/mol); and l was the path length (1 cm). The inter- and intra-assay %CV for this method were <5%.

4.2.9.2 Total phenolic content (TPC)

Total phenolic content was measured using a modified Folin-Ciocalteu colorimetric method (Shahidi, 2007). Samples were diluted in deionised water (1:10 or 1:100) and 50 µl of the diluted extract, 50 µl of Folin-Ciocaltea 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 (Greiner Bio – One, Monroe, USA). Absorbance readings were taken at 725 nm, at 5 minute intervals, over a 30 minute period at 25°C (BioTek Synergy HT Multi-Mode MicroPlate Reader, Winooski, USA). A stock solution of gallic acid (5.8 mM) was prepared in aqueous methanol (80% (v/v) and quantification was performed on the basis of a standard curve in the range 0-50 mg/mL (R^2 = 0.99). The analysed samples were measured versus a blank sample. All values are expressed as means of gallic acid equivalents per gram of sample ± SE for 6 replications. The inter- and intra-assay %CV for this method were <4%.

4.2.9.3 Trolox equivalent AOX capacity (TEAC)

A modified DPPH assay used for AOX activity measurements was adjusted for use in the present study (Brand-Williams, 1995). The DPPH solution was prepared freshly before analysis, by dissolving the DPPH reagent (2.4 mg) in 80% methanol (100 mL). Then 10 µl of extract, 40 µl of deioned water and 200 µl of DPPH solution were added into each well of the 96 well Plate (CELLSTAR, Greiner Bio-One, Monroe, USA). Absorbance readings were taken at 515 nm, at 3 minute intervals over a 30 minute period at 37°C, using a Multi-Mode MicroPlate Reader (BioTek synergy HT, Winooski, USA). A calibration curve using Trolox (0-500 µM, R2 = 0.99) was plotted. Final values are expressed as means of Trolox

equivalents per milligram of sample ± SE for 6 replications. The inter- and intra-assay %CV for this method were <3%. The total anthocyanin, total phenolic and total AOX capacities for the Montmorency cherry concentrate and placebo are presented in Table 8.

Table 8 - Total anthocyanin, phenolics and AOX activity in 60mL MC concentrate and PLA

	TACN (mg cyanidin-3-glucoside /L)	TPC (mean gallic acid equiv/L)	TEAC (mean Trolox equiv/L)
60 mL MC Concentrate	73.50 ± 0.20	178.75 ± 0.87	0.58 ± 0.01
Pla	ND	10.36 ±0.13	0.01 ± 0.01

Values are presented as Mean ± SEM, n = 6 per analysis. TACN, total anthocyanin content; TPC, total phenolic content; TEAC, trolox equivalent AOX capacity.

Table 9 - Retention times (min) and selected UV-Vis wavelengths for quantitation of phenolics by HPLC-UV/Vis

Compound	λex/ λem (nm)	Retention time (min)	LOD (µg/mL)	Range of linearity (µg/mL)
Protocatechuic acid	278 / 360	7.07	<0.01	0.05-50
Chlorogenic acid	260 / 422	7.65	<0.05	0.25-50
Vanillic acid	260 / 422	9.09	<0.01	0.05-50
Propyl gallate	278 / 360	11.91		

λex/ λem, fluorescence wavelengths; LOD, limits of detection

4.2.10 Plasma analysis

4.2.10.1 High Performance Liquid Chromatography (HPLC) Analysis

Under the selected chromatographic conditions, calibration graphs were obtained by preparing standard samples of each compound in triplicate, with increasing concentration of each analyte. From calibration graphs the limit of detection and linearity were calculated (Table 9). The HPLC-diode array detector (DAD) was used to identify plasma concentrations of phenolics for the acute phase of the study (pre-supplementation through to 8 h post-supplementation). A method previously described by Bell et al., (2014) was adapted for the extraction of phenolic compounds from the plasma. 1 mL of plasma and 0.5 mL of propyl gallate (internal standard, 50 µg, 100 µL/mL) was mixed with 4 mL oxalic acid (10 nM) and 0.1 mL HCl (12.6 M) in 15 mL falcon tubes and centrifuged at 3000 rpm for 15 minutes. The supernatant was absorbed on to a primed (washed with 5 mL methanol with 0.2% trifluoroacetic acid (TFA) followed by 2 × 5 mL of water) solid phase extraction cartridge (Waters Sep-Pak c17, 360 mg sorbent per cartridge, 55-105 µm). The sample was eluted with 3 mL of MeOH + 0.2% TFA and dried under N^2 at 45°C. Samples were then reconstituted in 400 µl of 0.1% formic acid in water: 2% HCl in methanol and filtered through a 0.2µm polytetrafluoroethylene filter prior to HPLC analysis.

A high pressure liquid chromatography-fluorescence (FLD) method for the detection and quantitation of selected phenolic compounds in the plasma samples and juice was carried out using a Dionex UltiMate 3000 HPLC System (Dionex, Camberly, UK) equipped with an UltiMate 3000 RS pump, an UltiMate 3000 autosampler and a 3000 RS Fluorescence Detector. The filtered samples (20 µL) were injected on an Phenomenex Luna C_{18}(2) (250 x 2.0 mm, 5um particle size) reverse-phase column thermostat controlled at 30 °C. The mobile phase consisted of water with 1% acetic acid (solvent A), and acetonitrile with 1% acetic acid (solvent B). After a 5-minute equilibration with 20% A, the elution programme was as follows: 0-15 min, 20-100% B, (0.2 mL/min) followed by a washing stage (100% B, 15-18 min, 1.0 mL/min) and return at the initial conditions within 2 minutes. Detection was performed at the following excitation/emission wavelengths: λex = 278 nm and λem = 360 nm for protocatechuic acid (PCA) and propyl gallate (PG), and λex = 260 nm and λem = 422 nm for chlorogenic acid (CHL) and vanillic acid (VA), respectively. The identification and quantitation of PCA, CHL and VA content of plasma samples was based on a combination of retention time and

spectral matching of reference standards (Table 9). Samples were analysed on a batch basis, where each batch included standards prepared in 0.1% formic acid in water: 2% HCl in methanol, blank control plasma samples, and fortified plasma samples at 1 (low), 10 (mid) and 25 (high) ug/mL. The recovery ranges were 88.73-94.98%, 87.31-103.78% and 89.16-105.98% for low, mid- and high fortified levels, respectively. The final results were collected for recovery at the low fortification level. Calibration curves were prepared for all AOX compounds and final results are expressed as micrograms per milligram (µg/mL).

4.2.10.2 Liquid Chromatography-Mass Spectrometry (LC-MS) Analysis

A LC-MS method, utilising the same chromatographic conditions as the HPLC-DAD analyses, was used for the confirmation of individual compounds in the plasma and juice samples. Briefly, LC-MS analyses were carried out on a Dionex UltiMate 3000 RSLC HPLC System (Dionex, Camberley, UK) equipped with an UltiMate 3000 RS pump, an UltiMate 3000 RS autosampler and a QExactive Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, Waltham, USA). Electrospray ionization at both negative and positive ion modes was performed with a spray voltage of 2.00 kV and capillary temperature of 280°C. The total ion current (TIC) with a range of 100-1500 m/z and 70000 resolution was measured. Sample aliquots (2 µL) were injected on a Phenomenex Luna C_{18}(2) (250 x 2.0 mm, 5um particle size) reverse-phase column thermostatically regulated at 40°C. The mobile phase consisted of water with 1% acetic acid (solvent A), and acetonitrile with 1% acetic acid (solvent B). The same method as applied for the HPLC analysis was carried out on the LCMS. The identification of phenolics in the MC concentrate was verified by retention time and spectral data comparison with the corresponding reference compounds.

4.2.10.3 Total plasma nitrate/nitrite measurements

Plasma nitrate/nitrite (NOx) measurements were carried out on plasma samples using a nitric oxide (NO) quantification kit (Catalogue No. KGE001, R&D Systems Europe, UK). All reagents, standard dilutions and samples were prepared according to manufacturer's instructions. Total plasma NOx determination was used as a surrogate marker of systemic NO production. The inter- and intra-assay %CV for this method were <5%. It should be noted that this measurement is not a reliable index of NO bioactivity *in vivo*, but may be used as an indicator of NO production.

4.2.11 Sample size calculation

Power calculations were performed for the primary outcome: systolic blood pressure. At 80% power and 5% significance, the minimum number of participants required to allow detection of a difference of 5 mmHg (clinically relevant outcome) between the responses to the two intervention drinks was estimated to be 12. A total of 16 participants were recruited to allow for a 25% drop – out rate.

4.2.12 Reliability

Six subjects volunteered to participate; the mean ± SD age, stature, mass and BMI was 24 ± 1 years, 180 ± 8.7 cm, 79.6 ± 5.48 kg and 24.8 ± 3.1 kg/m2, respectively. Both intra trial reliability (repeat measures made within the same data collection session) and inter trial reliability (repeat measures made within three days of each other) measures were taken in order to comprehensively test the reliability of the blood pressure, digital volume pulse and pulse wave measures. Reliability for these measures was obtained using variance estimates obtained through ANOVA, from which an interclass correlation coefficient (ICC) was calculated. The range of an ICC may be between 0.0 – 1.0. Coefficient of variation was calculated for these measures. Reliability for all methods were found to be excellent with the lowest ICC reported as 0.934 (>0.75 (Fleiss, 1986)), when the ICC value is high, there is little variation expressed. Coefficient of variation (CoV) was also found to be very low <5% for all measures. See Table 10 for full reliability analysis.

Table 10 - Inter and inter - reliability data for vascular measures

	CoV (%)		**ICC**	
	Intra	Inter	Intra	Inter
Systolic BP	4	2	0.954	0.923
Diastolic BP	3	3	0.963	0.934
DVP-SI	1	4	0.988	0.975
DVP-RI	2	4	0.965	0.944
PWV	3	4	0.942	0.933
PWA	2	4	0.934	0.937

CoV, Coefficient of Variation; ICC, Interclass Correlation Coefficient.

4.2.13 Statistical Analysis

Statistical analysis was performed using PASW Statistics 21.0 for Windows (SPSS, Inc., Chicago, IL.). All group characteristics were reported as means ± standard errors, unless otherwise stated. All dependent variables were analysed using a within subject, crossover design; treatment, 2 [cherry juice vs placebo] by time, 6 [pre–supplement, 1, 2, 3, 5 and 8 h post supplement] mixed model analysis of variance (ANOVA). Mauchy's Test of Sphericity was used to check homogeneity of variance for all ANOVA analyses; where necessary, violations of the assumption were corrected using the Greenhouse–Geisser adjustment. Significant main effects were followed up using LSD *post hoc* analysis. As a secondary analysis, the AUC was calculated by the trapezium rule, which was subtracted from the fasting value to derive incremental AUC (iAUC). Maximum plasma concentrations (c_{max}) and times to achieve maximum plasma concentrations (t_{max}), were directly obtained from the plasma concentration-time profiles. A correlation analysis was performed by using Pearson's correlation coefficient to examine the relationships between indices of vascular function and appearance of phenolic metabolites in plasma.

4.3 Results

Sixteen hypertensive men volunteered to take part in the study, but 1 participant voluntarily withdrew after the first study day. There were no adverse events reported in response to the intervention products. All participants (n = 15) complied with the low-polyphenolic experimental diet according to the food diaries. The washout period of ≥ 14 d appeared to be sufficient given that the active compounds of interest were similar at baseline for both visits.

4.3.1 Microvascular vasodilation by LDI with iontophoresis

There was no time, treatment, or treatment × time interaction effect observed for acetylcholine (endothelium-dependent vasodilation) or SNP (endothelium-independent vasodilation). The incremental AUC (1–8 h after ingestion) for microvascular vasodilation was not significantly different between groups ($P > 0.05$) (Figure 14). Values are presented in Table 11.

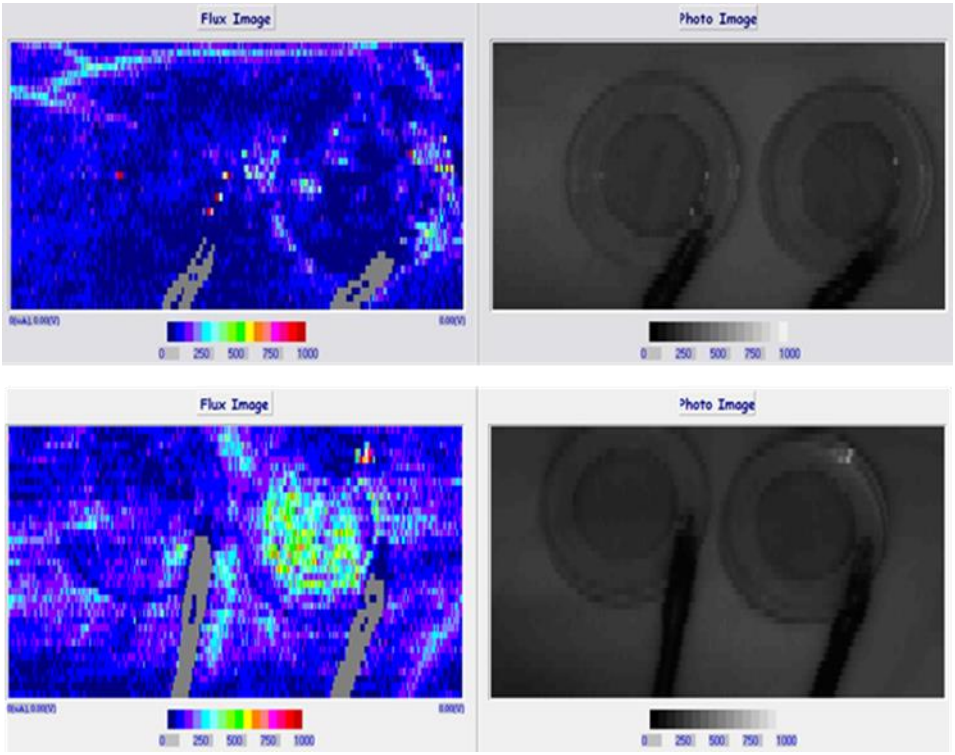

Figure 14 - Representative laser Doppler scan showing both the SNP (left) acetylcholine (right) response before (top) and 1 h post (bottom) MC consumption in a single subject. The illuminated regions indicate areas of increased blood flow.

4.3.2 Blood pressure

SBP exhibited a significant time ($F = 7.885$, $P = 0.001$) and treatment × time interaction effect $F = 3.478$, $P = 0.003$) with the MC concentrate trial. A *post hoc* least significance difference test indicated that this difference occurred at 1, 2, and 3 h after supplementation in the MC group, with peak reductions of 7 ± 3 mmHg at 2 h after MC consumption relative to the placebo. Individual responses to 60 mL MC consumption and placebo at the relevant time points are illustrated in Figure 15 (A and B). Absolute values are presented in Table 12. DBP showed a significant time effect ($F = 6.060$, $P = 0.01$) but no treatment or treatment × time interaction effects. Mean arterial pressure demonstrated a significant time ($F = 10.389$, $P = 0.001$) and treatment × time interaction effect ($F = 2.500$, $P = 0.01$).

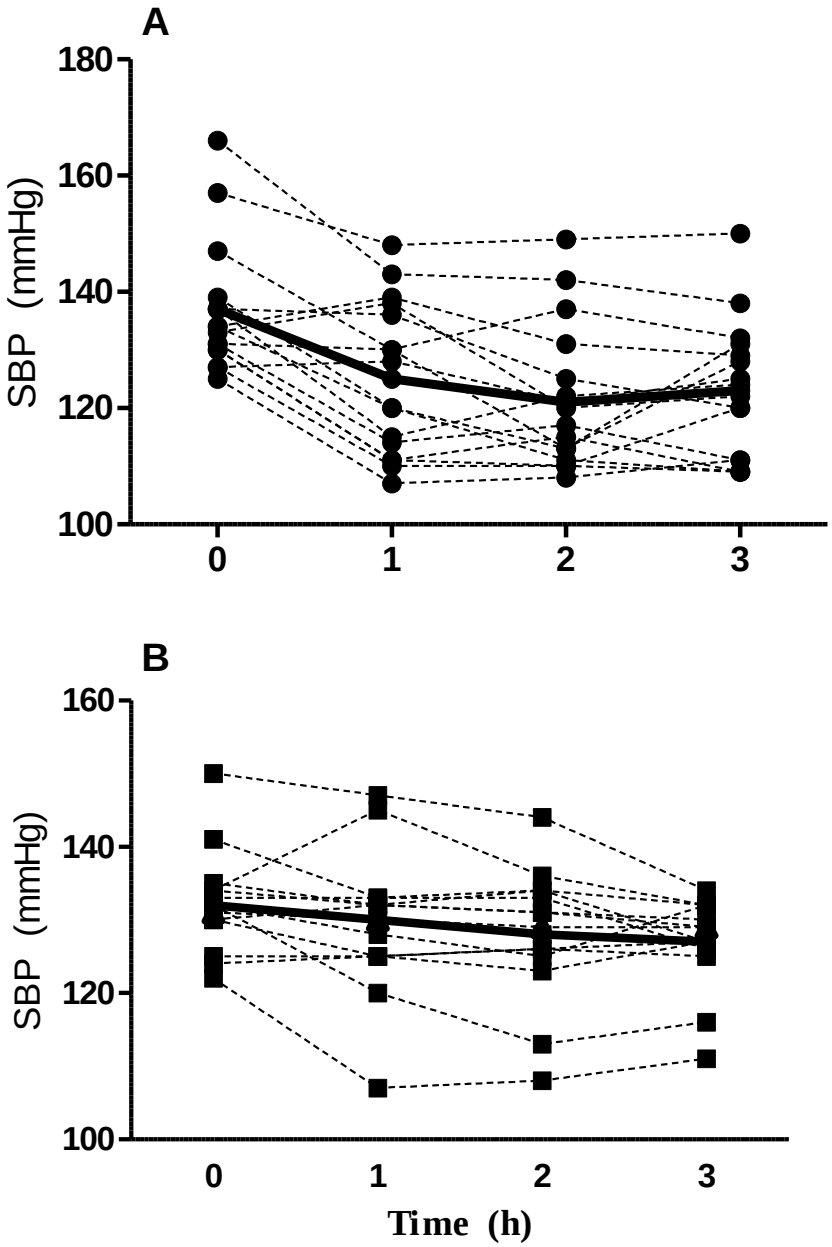

Figure 15 - Individual responses to 60 mL MC concentrate (A) and placebo (B) consumption at relevant time points. The mean individual response is highlighted in bold. MC, Montmorency tart cherry; SBP, systolic blood pressure.

No other vascular variables (HR, DVP-SI, DVP-RI, PWV, AIx, and AIx corrected for HR at 75 beats/min) were altered after consumption of the MC concentrate compared with the placebo treatment. The absolute values for all variables are presented in Tables 11 and 12.

Table 11 - Acute effects of tart Montmorency cherry juice polyphenols on vascular function

	Baseline	1 h	2 h	3 h	5 h	8 h	ANOVA		iAUC (0-8 h)
							Effect	*P*	
LDI ACh (PU)									
60mL MC	1409 ± 183	2017 ± 356	1397 ± 226	1373 ± 232	1442 ± 189	1231 ± 157	T	0.060	7459 ± 348
Pla	1671 ± 275	1647 ± 207	1178 ± 187	1242 ± 171	1168 ± 115	1180 ± 133	T × T	0.530	6415 ± 237
LDI-SNP (PU)									
60mL MC	1666 ± 206	1616 ± 283	_	1453 ± 206	1559 ± 241	1424 ± 178	T	0.240	8543 ± 209
Pla	1891 ± 228	1847 ± 233		1552 ± 221	1636 ± 140	1690 ± 187	T × T	0.976	8475 ± 202
PWV (m/s)									
60mL MC	6.0 ± 0.2	5.7 ± 0.2	5.9 ± 0.2	5.9 ± 0.2	5.8 ± 0.2	5.9 ± 0.2	T	0.029	_
Pla	5.9 ± 0.2	5.8 ± 0.2	5.9 ± 0.3	6.2 ± 0.2	6.1 ± 0.2	6.2 ± 0.2	T × T	0.211	
AIx (%)									
60mL MC	11.0 ± 1.7	8.4 ± 1.9	9.7 ± 1.8	8.6 ± 1.9	8.4 ± 1.9	9.5 ± 2.1	T	0.582	_
Pla	10.8 ± 2.2	10.8 ± 2.0	10.0 ± 1.8	10.4 ± 2.0	9.0 ± 2.0	10.9 ± 2.6	T × T	0.182	
DVP–SI (m/s)									
60mL MC	5.7 ± 0.2	5.7 ± 0.2	5.8 ± 0.2	6.0 ± 0.3	5.9 ± 0.3	6.1 ± 0.3	T	0.068	_
Pla	6.1 ± 0.3	6.0 ± 0.2	6.2 ± 0.3	6.3 ± 0.2	6.2 ± 0.3	6.3 ± 0.2	T × T	0.957	
DVP–RI (%)									
60mL MC	47.2 ± 2.8	50.1 ± 2.8	54.6 ± 3.2	55.5 ± 3.6	53.1 ± 2.9	59.0 ± 3.7	T	0.001	_
Pla	50.6 ± 3.5	51.7 ± 3.0	58.2 ± 4.3	57.9 ± 3.7	52.9 ± 3.5	63.4 ± 3.5	T × T	0.938	

All values are means ± SEM (n=15). There were no significant difference between Placebo and cherry concentrate treatment. LDI, laser Doppler imaging; PWV, pulse wave velocity; AIx, augmentation index; DVP-SI, digital volume pulse stiffness index; DVP-RI, digital volume pulse reflection index; MC, Montmorency cherry concentrate; Pla, Placebo; T, time effect; T × T, time × treatment interaction effect.

Table 12 - Acute effects of tart Montmorency cherry juice on blood pressure and heart rate

	Baseline	1 h	2 h	3 h	4 h	5 h	6 h	7 h	8 h	ANOVA	
										Effect	**P**
PSBP (mmHg)											
60mL MC	137 ± 3	125 ± 3[b,y]	121 ± 3[b,y]	123 ± 3[b,y]	124 ± 3[b]	125 ± 4[b]	128 ± 3[b]	128 ± 3[b]	131 ± 3[b]	T	0.001
Pla	134 ± 2	130 ± 3[a]	128 ± 3[b]	127 ± 2[b]	127 ± 2[b]	128 ± 2[b]	129 ± 1[a]	129 ± 2[a]	131 ± 3	T × T	0.003
PDBP (mm Hg)											
60mL MC	82 ± 3	76 ± 3	75 ± 3	76 ± 2	76 ± 2	76 ± 3	78 ± 2	78 ± 2	80 ± 2	T	0.010
Pla	79 ± 3	76 ± 3	76 ± 2	75 ± 2	76 ± 2	75 ± 2	76 ± 2	76 ± 3	81 ± 3	T × T	0.779
MAP (mm Hg)											
60 mL MC	101 ± 3	92 ± 3[b]	91 ± 3[b]	91 ± 3[b]	92 ± 2[b]	93 ± 3[b]	95 ± 2[a]	95 ± 2[a]	97 ± 2	T	0.001
Pla	97 ± 2	94 ± 2[b]	93 ± 2[a]	93 ± 2[b]	93 ± 2[b]	93 ± 2	94 ± 2	94 ± 2	97 ± 2	T × T	0.014
Heart Rate (bpm)											
60mL MC	63 ± 3	61. ± 2	60 ± 3	62 ± 3	_	62 ± 3	_	_	61 ± 2	T	0.702
Pla	59 ± 2	60 ± 2	58 ± 2	58 ± 2		60 ± 2			61 ± 3	T × T	0.184

All values are means ± SEM (n=15). [a,b] Significant difference between baseline and post intervention (1, 2, 3, 5 or 8 h) (repeated – measures ANOVA): [a] $P < 0.05$, [b] $P < 0.01$. [y] Significant difference between Placebo and cherry concentrate treatment (2-factor repeated measures ANOVA): y $P < 0.05$. PSBP, peripheral systolic blood pressure; PDBP, peripheral diastolic blood pressure; MAP, mean arterial pressure; MC, Montmorency cherry concentrate; Pla, Placebo; T, time effect; T × T, time × treatment interaction effect.

4.3.3 Plasma nitrite and nitrate

Because of a sampling error, blood was analyzed in 13 participants. There was no time, treatment, or time × treatment interaction effect for plasma nitrate or nitrite ($P > 0.05$).

4.3.4 PCA, VA, and CHL

Plasma PCA (Figure 16 A) revealed a time ($F = 30.595$, $P < 0.001$), treatment ($F = 444.637$, $P < 0.001$), and treatment × time interaction effect ($F = 29.814$, $P < 0.001$). PCA in plasma was higher after MC consumption across all time points relative to the placebo ($P < 0.001$). For the 60-mL MC dose, the t_{max} was 1 h after consumption, yielding a c_{max} value of 2.35 ± 0.08 µg/mL. $AUC_{0–8\ h}$ values for PCA were different between the 60-mL dose and the placebo (93.7 ± 2.3 µg · h · mL^{-1} and 4.2 ± 0.3 µg · h · mL^{-1}, respectively; $P = 0.005$). The presence of PCA was confirmed in plasma by comparing the experimentally determined monoisotopic molecular weights to the literature values, all of which were within ±1.5 parts per million. Plasma VA (Figure 16 B) revealed a significant time ($F = 8.575$, $P = 0.001$), treatment ($F = 24.610$, $P = 0.001$), and treatment × time interaction effect ($F = 11.561$, $P = 0.001$). VA was markedly higher in plasma for ≤ 5 h after MC consumption than after the placebo trial. Similar to PCA, the t_{max} for VA was 1 h after consumption, yielding a c_{max} value of 0.20 ± 0.01 µg/mL. $AUC_{0–8\ h}$ values for VA were statistically significant between the 60-mL dose and the placebo (39.6 ± 2.5 µg · h · mL^{-1} and 0.5 ± 0.1 µg · h · mL^{-1}, respectively; $P = 0.026$). The presence of VA was confirmed in plasma by comparing the experimentally determined monoisotopic molecular weights to literature values, all of which were within ±1.5 parts per million. The CHL concentrations in plasma after consumption were below the limits of detection in this study. Peak plasma PCA (1 h) negatively correlated with SBP at 1 and 2 h after MC consumption ($r = -0.182$ and -0.131, respectively). Peak VA in the plasma (1 h) negatively correlated with SBP at 2 h after MC consumption ($r = -0.095$). However, these correlations were not statistically significant. There were no negative correlations, significant or otherwise, observed in the placebo trial.

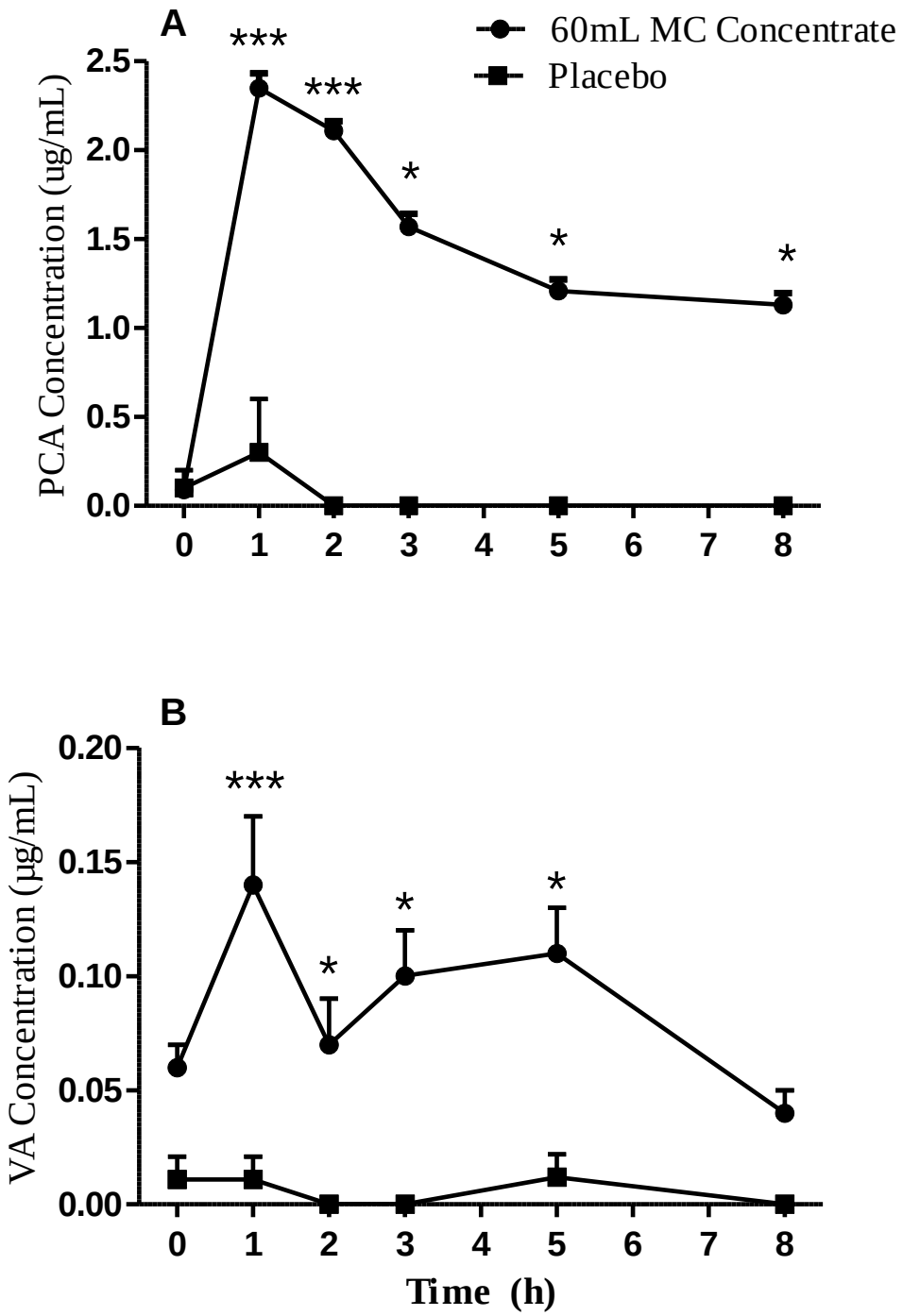

Figure 16 A: Time course of protocatechuic acid and B: vanillic acid (mean ± SEM) response after consumption of MC concentrate- and macronutrient – matched control (n=15). Data was analysed by using a 2-factor repeated – measures ANOVA with time and treatment as the 2 factors [significant effects of time (P = < 0.001), treatment (P < 0.001), and the interaction between time and treatment (P < 0.001) were observed for both variables]. Significantly different from the Placebo drink: * P < 0.05 *** P < 0.001.

4.4 Discussion

To our knowledge, this study is the first to investigate the acute effects of MC consumption on arterial stiffness, BP, and microvascular vasodilation in men with early hypertension. In support of our hypothesis, this study presents new information that consuming 60 mL MC reduced SBP ≤ 3 h post-prandially. This improvement in BP occurred at the same time points as peak increases in plasma phenolic acid uptake.

BP is a modifiable but nonetheless major risk factor for CVD (Lewington et al., 2002), and diet is believed to play an important contributing factor in the advent of hypertension. Relatively small reductions (2–5 mm Hg) in BP have been reported to have an important impact on cardiovascular mortality (Collins et al., 1990). To our knowledge, this is the first study to report a positive modulation of SBP after MC consumption. Previous studies have demonstrated that other polyphenol-rich foods such as cocoa, beetroot, and grape extract can have a positive effect on BP (Grassi et al., 2005; Hobbs et al., 2013; Draijer et al., 2015). This study is particularly noteworthy because data from prospective observational studies have shown a reduction in mean SBP of 5–6 mm Hg over a 5-y period was associated with 38% and 23% reduction risk of stroke and coronary artery disease, respectively (Collins et al., 1990). Herein, we reported peak reductions in postprandial SBP of 7 ± 3 mm Hg relative to the placebo. Previously, Lynn et al., (2014) did not detect any changes in BP after tart cherry consumption in normotensive participants (~111/70 mm Hg). This discrepancy might be attributable to suggestions that the magnitude of change in the BP response is directly related to baseline BP (Kapil et al., 2010), making it possible that a higher baseline BP will likely experience a greater change after an intervention. In addition, Lynn et al., (2014) had little control over the supplement timing in free-living participants. It is therefore conceivable that any vaso-modulatory effects from the cherries were missed. This is especially evident given that measures of vascular function were assessed after an overnight fast; this work clearly demonstrates that any positive effects are transient and return to baseline after 4 h. The magnitude of BP-lowering effects observed in this study is comparable to those achieved by a single antihypertensive drug in mildly hypertensive patients (MRC, 1985) and highlights the potential importance of MC supplementation as an adjuvant in the management of hypertension. In this study, the greatest improvements in SBP occurred in association with peak plasma PCA and VA,

indicating that both of these metabolites could be partly responsible for the effects observed, particularly given that these hydroxybenzoic acids were shown in the previous Chapter to modulate vascular smooth muscle cell behaviour *in vitro*.

To our knowledge, only one study has investigated the effect of a tart cherry juice supplement on arterial stiffness (Lynn et al., 2014). PWV is emerging as an important measure of vascular function that relates to CVD risk and has been shown to be a stronger predictor of arterial stiffness than AIx and central pulse pressure (Mitchell et al., 2004). We did not observe any significant differences in PWV between the MC concentrate and placebo across the 8-h trial. These findings are in agreement with Lynn et al., (2014), in which no effect was observed on arterial stiffness in healthy subjects. Notwithstanding the aforementioned limitations of that study, there are several possible explanations for these results that could be applicable to our findings. Lynn et al., (2014) speculated that arterial stiffness is less responsive to a short-term increase in polyphenols in healthy populations. Similar to BP, it might be the case that the magnitude of change is directly related to PWV at baseline, although this is speculative. In this study, the mean value at baseline was 5.9 m/s, which, according to the reference values for arterial stiffness collaboration (Mitchell, 2009), is well within the normal ranges for this population.

The pivotal role vascular dysfunction Plays in the progression of atherosclerosis has been increasingly recognized; therefore, the vasculature has emerged as an important target for dietary therapies (Armah et al., 2008). We assessed microvascular vasodilation with the use of LDI, which measured the response to cutaneous perfusion of the forearm with acetylcholine and SNP (Turner et al., 2008). Contrary to our expectations, we did not observe any change in microvascular vasodilation after the intervention. It was somewhat surprising that no change was evident given that an increase in endothelium-dependent microvascular reactivity after high flavonoid (Macready et al., 2014) and fruit and vegetable intake (McCall et al., 2011) has been previously observed. However, these data are not in isolation; a study by Jin et al., (2011) showed no change in acetylcholine or SNP response after blackcurrant consumption. However, it is particularly noteworthy that in this study certain individuals had a far higher endothelium-dependent vasodilator response than others in the same cohort. Perhaps similar to what George et al., (2012) previously demonstrated, there is a

differential response to the MC concentrate that is potentially based on genotype. They showed that acute consumption of a flavonoid-rich drink resulted in a considerable increase in dilation of the microcirculation in the forearm in response to acetylcholine after 180 min in GG individuals alone. However, there was no effect of the same beverage on endothelium-dependent vasodilation in the GT genotype or on endothelium-independent vasodilation in response to SNP in either genotype. However, this analysis was outside the remit of this study. These variables might be modulated after chronic supplementation and are certainly worth future investigation. In addition, the assessment of endothelial function by LDI is specific to the region examined and therefore may not provide a fuller picture of global vascular function.

Peripheral PWA is frequently used to measure the AIx, an index of arterial stiffness (Wilkinson et al., 1998). Increased arterial stiffness results in a faster propagation of the forward pulse wave as well as a more rapid reflected wave. Therefore, a high AIx has been previously linked with greater arterial stiffness and has been shown to be a predictor of adverse cardiovascular events in a variety of patient populations (Shimizu, 2008). In this study, there were no noteworthy changes in AIx or AIx when corrected for an HR of 75 beats/min after acute ingestion of MC concentrate. Similarly, Hobbs et al., (2013) failed to detect any meaningful changes in AIx after beetroot bread ingestion. Although we reported marked improvements in SBP following the consumption of MC concentrate, there were no changes in microvascular vasodilation, arterial stiffness, DVP, and HR; it has previously been reported that concurrent improvements in all measures of vascular function are not always observed (Hobbs et al., 2013).

There are 2 proposed mechanisms by which tart cherries are thought to improve indexes of cardiovascular function. The first is via the NO pathway, which increases the bioavailability of NO via its potential to inhibit NADPH oxidase. Cyanidin-3-glucoside, an anthocyanin found in abundance in tart cherry products (Kirakosyan et al., 2009; Bell et al., 2014), has also been shown to increase endothelial NO synthase (eNOS) expression (Xu et al., 2004) and decrease inducible NO synthase expression (Wang et al., 2008). Such changes in the balance between eNOS and inducible NO synthase expression/abundance/activity would favour the bioavailability of the vasoactive NO. However, this study measured plasma total NOx concentration, often used as a surrogate marker of

eNOS activity (Moncada & Higgs, 1991), and found no changes in the plasma NOx. The second and perhaps more likely idea by which MCs improve factors associated with CVD is based on the uptake of polyphenols that possess cardio-protective properties. An analysis of the MC juice revealed larger amounts of total anthocyanins and phenolic content than in the placebo. To investigate a cause-and-effect relation between improvements in cardiovascular function and the intake of cherry polyphenols, we examined plasma concentrations of anthocyanin metabolite profiles after consumption. In line with BP, we observed a peak increase in plasma phenolic metabolites at 1 h after MC consumption. This also supports previous observations in the earlier Chapter that showed that PCA and VA peaked between 1 and 2 h after MC consumption in healthy men. These phenolic acids have previously been observed in plasma after blueberry, cranberry, and blackcurrant consumption (Rechner et al., 2002; Zhang et al., 2004; Stalmach et al., 2009) and may be responsible for driving the beneficial vascular response observed. Both compounds have been shown to modulate vascular function in isolation (Kamat et al., 2000; Kakker & Bais, 2014) and in concert can influence vascular smooth muscle cell behaviour *in vitro* as demonstrated in the previous Chapter. Concentrations of these acids gradually decreased after peaking at 1 h, indicating further chemical or microbial degradation (Keppler & Humpf, 2005), excretion, or tissue uptake (Kirakosyan et al., 2015) of these compounds.

In conclusion, these data provide the first evidence to our knowledge that circulating phenolic metabolites derived from MC juice are at least partly responsible for acute improvements in SBP in men with early hypertension. This study provides further evidence that diets containing polyphenolic-rich foods have the potential to exert positive effects on vascular function. In particular, tart cherries, which contain a high concentration of bioactive polyphenols, can modulate human physiological function. This study also provides information on a new application of tart cherries in health maintenance, particularly in positively modulating SBP.

4.5 Perspectives

Following on from Chapter 3 and the significant effects tart cherries imposed on vascular smooth muscle cell behaviour, this Chapter aimed to explore the cardiovascular benefits of tart cherries in humans. The results from this Chapter add further application to the findings reported earlier, demonstrating that tart

cherries may be an adjuvant in the management of hypertension. In males with early hypertension (SBP ≥130 mmHg, DBP ≥80 mmHg, or both), SBP was reduced by up to 7 ± 3 mmHg relative to the placebo in the hours following consumption. In the UK, about half of people aged over 65, and about 1 in 4 middle-aged adults, have high blood pressure. Raised blood pressure is the greatest risk factor for cardiovascular disease and even small reductions in blood pressure can have a big impact on mortality rates. Therefore, the findings from this Chapter potentially extend the application of MC supplementation to a much wider group of individuals. Furthermore, this Chapter demonstrated that the circulating phenolic metabolites (VA and PCA) derived from Montmorency tart juice are, at least in part, responsible for this modulation of SBP. Importantly, regardless of the mechanism, this improvement in blood pressure is of most interest to clinical nutritionists, dieticians, clinicians, researchers, applied nutritionists and also the wider population in furthering our understanding of the health benefits associated with tart cherries.

Conceptually, in light of the vaso-modulatory effects of polyphenol-rich foods, and the findings of this Chapter which highlight the ability of MC juice to modulate aspects of vascular function, it is possible that Montmorency cherries might also be capable of conferring a positive vascular effect by improving blood flow and human performance. Resultantly, the following Chapters of this thesis will investigate the effects of MC supplementation on 1) cerebral blood flow, cognitive function and mood in middle-aged adults, and 2) on exercise performance in trained cyclists.

5 Tart Montmorency cherries modulate vascular function acutely, in the absence of improvement in cognitive performance

Publication arising from this Chapter: Keane, K.M., Haskell-Ramsay, C.F., Veasey, R.C. and Howatson, G. (2016) Montmorency Tart cherries (*Prunus Cerasus L*.) modulate vascular function acutely, in the absence of improvement in cognitive performance. *British Journal of Nutrition*, 1-10.

5.1 Introduction

Tart cherries and their derivatives are a functional food that are high in numerous polyphenols (Wang et al., 1999; Seeram et al., 2001; Seymour et al., 2014; Bell et al., 2014) that include the flavonoids isorhamnetin, kaempferol, quercetin, catechin, epicatechin, procyanidins, and anthocyanins (Kim et al., 2005; Kirakosyan et al., 2009). It has been previously shown that tart cherries attenuate inflammation (Wang et al., 1999), oxidative stress (Howatson et al., 2010; Bell et al., 2014) and improve aspects of vascular function as demonstrated in Chapter 4. One property underlying the potential vascular effects of tart cherries is an ability to modulate blood flow parameters. Cherry extracts have been shown, in cell and animal models, to exert a range of cardio-protective effects that include increasing nitric oxide production and AOX status, reducing lipid oxidation and inhibiting inflammatory pathways (Wang et al., 1999; Seeram et al., 2001). In Chapter 2 of this thesis, an increase in plasma phenolic acids (vanillic and protocatechuic) was observed following Montmorency tart cherry consumption in humans; these compounds were also shown to modulate vascular smooth muscle cell behaviour *in vitro*. Moreover, in Chapter 4, it was demonstrated that circulating phenolic metabolites derived from Montmorency tart cherry juice are, at least in part, responsible for an acute reduction in systolic blood pressure in men with early hypertension.

Aging is associated with deficits in motor function, which include decreases in balance, muscle strength, coordination, and cognitive function, especially in tasks that require the use of spatial learning and memory. This has been suggested to be caused by a concurrent decline in cerebral blood volume and metabolism of oxygen which also occurs as a result of aging (Marchal et al.,1992). These decrements have been reported in numerous studies in both animals (Joseph et al., 1983; Shukitt-Hale et al., 1998) and humans (Ajmani et al., 2000; Hofer et al., 2003). A large number of dietary interventions using polyphenol-rich foods or beverages, in particular those using tea (Chan et al., 2006), Gingko Biloba (Birks & Evans, 2009), cocoa (Scholey et al., 2010) and blueberry (Shukitt-Hale et al., 2008), have demonstrated beneficial effects on memory and learning in both animals and humans. Although it is not clear whether tart cherries can decrease the risk of neurodegenerative aging or diseases such as Parkinson's and Alzheimer in humans, studies with animal models are more positive and suggest that the phenolic compounds found in tart cherries, may exert their beneficial

effects through their ability to lower oxidative stress and anti-inflammatory properties or by altering directly the signalling involved in neuronal communication, calcium buffering ability, stress signalling pathways among others (Shukitt-Hale et al., 2006; 2008).

Seymour et al., (2013) showed that intake of 1% tart cherry diet significantly reduced stroke-related phenotypes in rats. Tart cherry intake also reduced brain NFκB activity and the related pro-inflammatory transcripts. Interestingly in 2015, Kirakosyan and colleagues (Kirakosyan et al., 2015) confirmed that tart cherry anthocyanins cross the blood-brain barrier. In a more recent addition to the literature, thirty 19-month-old male Fischer 344 rats who received either a control diet or a diet supplemented with 2% tart cherry for six weeks were examined. Results showed that although there were no changes on motor performance, tart cherry supplementation significantly improved working memory of aged rats (Thangthaeng et al., 2016). However, there is a paucity of data from human trials to extrapolate these findings to hominids.

Caldwell et al., (2015) previously demonstrated that regardless of dose, cherry juice had no acute impact on cognitive function in young people, older people or dementia patients. Oxygen radial absorbance capacity of the cherry juice was 58.99 µmol Trolox equivalent/g and the anthocyanin content was 18.6 mg/100 ml, there was no mention of the total phenolic or flavonoid content of the juice. They concluded that although cherry juice may have an acute impact on cardiovascular function, there was no change in cognitive performance 6 h post consumption. Contrastingly, a chronic supplementation study (Kent et al., 2015) reported that the daily consumption of sweet cherries 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. It would therefore appear that the cerebrovascular response required to elicit measurable changes in cognitive function can only be achieved with longer term dosing strategies (Clifford et al., 2015). Contrary to this theory, two recent additions to the literature suggest that acute supplementation has the ability to improve aspects of cognitive function. Acute blackcurrant supplementation was shown to improve both digit vigilance and rapid visual information processing in healthy younger humans (Watson et al.,

2015). Similarly, acute wild blueberry supplementation was shown to improve final immediate recall, delayed word recognition, and accuracy on cognitively demanding incongruent trials in the interference task in in children (Whyte et al., 2016). Therefore, it is possible that Caldwell and colleagues reported no impact of cherry supplementation on cognitive function as they used sweet cherries as an intervention. It has previously been speculated that sweet cherries are not as rich in polyphenol compounds as tart cherries (Kim et al., 2005).

Polyphenol-rich foods have also been reported to improve cerebral haemodynamics assessed by near infrared spectroscopy (NIRS) and function magnetic resonance imaging (fMRI). Wightman and colleagues (Wightman et al., 2012) assessed the effect of EGCG on cerebral blood flow using NIRS in healthy adults. Results suggested that 135 mg of EGCG caused a reduction in total haemoglobin, a proxy for cerebral blood flow during cognitive tasks relative to the placebo. Changes in cerebral blood flow has also been demonstrated following resveratrol (Kennedy et al., 2010) and beetroot supplementation (Wightman et al., 2015). Krikorian et al., (2012) used fMRI to examine the effect of Concord grape juice on neurocognitive function. Sixteen adults aged >68 y with mild age-related memory decline were supplemented with either a grape juice (444 ml average) containing on average, 209mg of polyphenols, or a sugar matched placebo for 16 weeks. Results found that after 16 weeks, there were reductions in semantic interference on memory tasks and relatively greater activation in anterior and posterior regions of the right hemisphere in the grape juice treated group. Similarly, people with mild memory complaints, who drank pomegranate juice daily for four weeks, performed better on memory task compared to a placebo and displayed an increase in brain activation measured by fMRI (Bookheimer et al., 2013). In a recent addition to the literature, Lamport et al., (2017) investigated the effects of flavanone – rich citrus juice consumption on cognitive function and cerebral blood flow using fMRI. They reported that the citrus juice was associated with significantly increased regional perfusion in the inferior and middle right frontal gyrus at 2 h relative to baseline and the control drink. Furthermore, the citrus juice was associated with significant improvements in cognition, namely the digit symbol substitution test at 2 h relative to baseline and the control drink.

Very little has been reported on the effect of acute polyphenol supplementation on cerebral haemodynamics, with the majority of this work carried out with flavanol-

rich cocoa (Francis et al., 2006; Sorond et al., 2008). At present, no attempt been made to examine the haemodynamic response to acute tart cherry supplementation.

Notwithstanding, given that Montmorency tart cherries are capable of modulating human vascular function (particularly in relation to blood pressure and vascular smooth muscle behaviour), we hypothesised that cerebral blood flow could also be acutely modulated and consequently improve cognitive performance in humans. Therefore, this study addresses the specific aim of this thesis, which was to assess the impact of Montmorency tart cherry juice consumption on pre-frontal cortical haemodynamics, cognitive function and blood pressure in middle aged adults.

5.2 Methods

5.2.1 Participants

Thirty healthy middle aged (defined as 45-60 years) adults (10 female, 20 male, 28 right-handed, 2 left-handed) were recruited to take part in the study; the mean ± SD age, stature, mass and BMI were 50 ± 6 years, 170.7 ± 9.1 cm, 76.0 ± 16.0 kg and 26.1 ± 4.9 kg/m^2, respectively. All participants were in apparent good health as assessed by a health-screening questionnaire. This questionnaire was administered to highlight any contraindications to taking part in the study. Exclusion criteria included those who had suffered a head injury, neurological disorder or neuro-developmental disorder. In addition, those who had any relevant food allergies or intolerances, smoked tobacco, drank excessive amounts of caffeine [>6 cups coffee/d (>450 mg caffeine/d)], or took illicit social drugs were also identified as contraindications to participation. All exclusion criteria were self-reported. The study was conducted in accordance with the Helsinki Declaration and ratified by the University's Research Ethics Committee. All enrolled participants provided written informed consent. This study was registered as a clinical trial with clinicaltrials.gov (NCT02381860).

5.2.2 Study Design

This study employed a placebo-controlled, double blinded, cross-over, randomised Latin square design with two experimental arms and a washout period of at least 14 days (mean ± SD, 15 ± 2 days); participants were randomly allocated to receive a 60 mL dose of a Montmorency cherry (MC) concentrate or a placebo (PLA).

Fourteen participants received the MC concentrate on the first visit, with the remainder receiving the PLA. A washout of at least 14 days was chosen based on findings from Chapters 3 and 4 as well as previous literature that suggests these phenolic compounds are quickly absorbed and/or excreted (Manach et al., 2005). Each participant was required to attend the laboratory on three separate occasions. Each visit was at the same time of day (within participant) and was preceded by an overnight fast (≥10 h). The first visit was an initial screening and familiarisation visit during which, participants were screened with regards to the study exclusion/inclusion criteria, briefed with regards to compliance requirements, provided written informed consent and given full training and familiarisation on the cognitive tasks. On the subsequent experimental days, participants reported to the lab between 7 and 9am and a baseline blood pressure (BP) reading was taken. This was followed by a baseline cognitive assessment and cerebral blood flow measures by near infrared spectroscopy (NIRS) and transcranial Doppler (TCD). Participants then consumed the intervention beverage (either MC or PLA), following which, they sat quietly, watching one of a selection of non-arousing DVDs, during a 1 hour "absorption period". Subsequent cognitive assessments and blood flow measures were taken 1, 2, 3, and 5 h post consumption; BP was performed hourly. Between cognitive test sessions, participants continued to watch a selection of non-arousing DVDs. No additional food or fluid was provided during the study period except for low-nitrate mineral water, which was consumed *ad libitum*. The total volume of water consumed on the first experimental day was recorded and participants consumed the same volume on the second visit. The reason for this was to accurately examine the efficacy of the intervention.

5.2.3 Treatments and dietary control

A MC concentrate (CherryActive, Sunbury, UK) was stored at 4º C prior to use. Participants consumed either 60 mL of MC concentrate (which according to the manufacturer is estimated to be equivalent to ~180 whole cherries) or fruit-flavoured cordial in a double blind, cross-over manner. This estimate is based on the brix value of sucrose in 100 g of solution. The decision to use 60 mL was based on previous literature and Chapter 3 of this thesis that showed a greater uptake of anthocyanin and phenolic acids *in vivo* post-consumption when compared to a 30 mL dose (Bell et al., 2014). Additionally, Chapter 3 identified that of the three Montmorency cherry analogue studied (frozen, dried and concentrated), the MC concentrate had the greatest AOX activity, total

anthocyanin and phenolic content. The MC concentrate was examined for total anthocyanins, total phenolic content and Trolox Equivalent AOX Capacity using techniques previously described in Chapter 4. The MC concentrate was found to contain 68.0 ±0.26 mg cyanidin-3-glucoside /L, 160.75 ± 0.55 mean gallic acid equiv/L and 0.59 ± 0.02 mean Trolox equiv/L, respectively. The concentrate was diluted with 100 mL of water prior to consumption.

The Pla supplement consisted of a commercially available, low fruit (<1%) cordial (Kia Ora, Coca Cola Enterprises, Uxbridge, UK) mixed with water, whey protein isolate (Arla Foods Ltd., Leeds, UK) and maltodextrin (MyProtein Ltd., Northwich, UK), to match the MC concentrate for volume and macronutrient content (Energy = 204 kcal, volume = 60 mL, carbohydrates = 49 g, protein = 2.2 g and fat = 0 g). The total anthocyanin content (used for colour purposes only) and total AOX capacity of the PLA were lower than the limits of detection, with trace amounts of phenolics (8.26 ± 0.04 mean gallic acid equiv/L). All drinks were prepared and all bottles were covered in tape prior to the study by a third party. Prior to study commencement, it was explained to participants that the aim of the study was to investigate the effect of a fruit juice on vascular function; therefore they were unaware which beverage was the experimental drink. Participants were instructed to follow a low phenolic diet for 48 h prior to each arm of the trial by avoiding fruits, vegetables, tea, coffee, alcohol, chocolate, cereals, wholemeal bread, grains and spices and were asked to refrain from strenuous exercise. Compliance with the dietary restrictions was assessed with a standardised, self-reported 2-day dietary record. All participants complied with the low phenolic diet and this was confirmed via visual inspection of the food diaries.

5.2.4 Cognitive tasks

All cognitive and mood measures were delivered using 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. This assessment system has previously been shown to be sensitive to nutritional interventions following both acute (Dodd et al., 2015) including acute supplementation with phenolics (Watson et al., 2015) and chronic supplementation (Stonehouse et al., 2013). At each of the aforementioned time points, a cognitive assessment test was completed. This assessment was a

collection of three tasks that lasted 9 minutes; this was performed twice, which equated to 18 minutes in total. The tests were performed twice in order to induce some level of cognitive fatigue. This was followed by a series of visual analogue scales to assess perceptions of fatigue and difficulty. The types of tests chosen have been previously used to detect changes in cognitive function following nutritional interventions (Scholey et al., 2010; Kennedy et al., 2010; Watson et al., 2015). In order to assess the relationship between specific brain regions and any changes in CBF, a selection of tasks that engender either higher or lower activation of the frontal cortex were employed. The "low activation" tasks comprised of a sustained attention test (digit vigilance). The "high activation" tasks (Rapid Visual Information Processing and Stroop tasks) entail a higher cognitive workload and have been shown to increase activity in the pre frontal cortex (Drummond et al., 1999; Lawrence et al., 2002). The battery of cognitive tasks is described in more detail below.

Digit Vigilance

The DV task is a measure of sustained attention and psychomotor speed (Coull et al., 1996). 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 target button on the response pad as quickly as possible every time the digit in the series matched the target digit. The task lasted three minutes in total. Task outcomes included accuracy (%) and reaction time for correct responses (ms)

Rapid Visual Information Processing (RVIP)

The RVIP task is a measure of sustained attention and working memory (Coull et al., 1996). This 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 target button on the response pad as quickly as possible. The task lasted three minutes in total. Task outcomes included number of target strings correctly detected (%) and average reaction time for correct detections (ms).

Stroop

The Stroop test is a measure of attention, inhibition and cognitive flexibility (Homack & Riccio, 2004). In this task, participants were presented with a colour name. The colour name presented was written in a coloured font, either the same "congruent" or a different "incongruent" font. Participants had to identify the colour of the font the word was written in, rather than the colour that the word was describing, via a response pad with coloured keys. Participants were presented with 90 stimuli in total taking ~3 minutes to complete. Task outcomes included number of correct responses (%) and the average response time for congruent and incongruent stimuli (ms).

Visual Analogue Scales

Participants were required to rate how "alert", "concentrated" and 'mentally fatigued' they felt and how 'difficult' they had found the tasks after each cognitive assessment repetition by indicating on a 100 mm line with the cursor ("not at all" at one end of the line and "extremely" at the other end) for alertness, fatigue and level of difficulty and ("very low" to "very high") for concentration. The VAS were scored as % along the line denoting more of the relevant adjective.

5.2.5 Blood pressure

Blood pressure was measured using a non-invasive digital automatic BP monitor (M10-IT Omron Healthcare, UK). The BP cuff was fitted by the same researcher at each of the six time points. The inter- and intra-trial %CV for this method was 4.2 and 1.3% respectively.

5.2.6 Cerebrovascular responses

5.2.6.1 Transcranial Doppler imaging

Cerebral blood flow velocity in the middle cerebral artery (CBFV) was determined using transcranial Doppler sonography (Doppler-Box, Compumedics DWL, Singen, Germany). A 2 MHz Doppler probe was positioned over the right middle cerebral artery using previously described search techniques (Aaslid et al., 1982), and secured with an adjustable headset (DiaMon, Compumedics DWL). The mean depth for Doppler signals was 62 ± 3 mm. All data were sampled at 200 Hz (PowerLab 16/30, ADInstruments Ltd, Oxfordshire, UK), and processed offline (LabChart version 5.4.2, ADInstruments Ltd).

5.2.6.2 Near Infrared Spectroscopy (NIRS)

The NIRS is a non-invasive brain imaging technique in which two nominal wavelengths of light, which are differentially absorbed by oxygenated (oxy-Hb) and deoxygenated haemoglobin (deoxy-Hb), respectively, are introduced through the skull via a laser emitter. They are then measured, following transit through the upper surface of the cortex, by an optode placed at a pre-set distance from the light source. NIRS has been used extensively as a technique for multiple-channel imaging of task-related brain activity over relevant areas of the head, including groups suffering from potential declines in CBF (Schecklmann et al., 2008). In the current study, cerebral oxygenation was assessed using near-infrared spectroscopy (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 bandage. The sensors alternately emit two wavelengths of near-infrared light (≈765 and 855 nm) with an emitter/optode separation distance of 4 cm. The NIRS data were acquired continuously and output every 5 s and recorded for later offline analysis. The NIRS data output was time stamped at the start of each task segment to assure that data corresponded to the relevant period of task performance. Relative concentration changes in oxy-Hb, deoxy-Hb and total-Hb were calculated. The experimental set up on study days is shown in Figure 17.

5.2.6 Statistical Analysis

Cognitive performance, BP and CBFV data were analysed by using a treatment × time point mixed model analysis of variance (ANOVA). Mauchly's Test of Sphericity was used to check homogeneity of variance for all ANOVA analyses; where necessary, violations of the assumption were corrected using the Greenhouse–Geisser adjustment. Significant main effects were followed up using LSD *post hoc* analysis. Planned comparisons were made between the MC and placebo condition for each variable at each of the time points [pre-supplement, 1, 2, 3 and 5 h]. The analysis of NIRS data was conducted with Minitab 15 for Windows (Minitab Inc, State College, PA). Prior to the primary analysis, a within subjects Analysis of Variance (ANOVA) was carried out with left/right optode included as a factor (hemisphere x treatment group) for each task. As there were

no treatment related interactions involving hemisphere the data from the 2 channels were averaged across hemispheres for the analysis and figures reported below. For each variable (oxy-Hb, deoxy-Hb and total Hb), data were converted to "change from baseline" (calculated from baseline pre-treatment period). Task length was fixed for the DV (180 s) and RVIP (180 s), but NIRS data from the Stroop test were truncated so that the same amount of data was analysed for all participants during each task period. Data from the 'resting/absorption' period (minutes 1-60) and the task performance were analysed separately for all time points [pre–supplement, 1, 2, 3 and 5 h]. Data from the 'resting/absorption' period was averaged across 6 equal 10-min epochs and analysed by two – way repeated measures analysis of variance (ANOVA) (epoch × treatment). Data from the task period data was averaged across 6 equal 3-min epochs. This data was analysed by a three-way repeated measures ANOVA (task (epoch) × treatment × time point).

In the absence of any directly relevant data, it was suggested that a sample size of twenty – four would be adequate to have greater than an 80% chance of detecting the medium effect sizes demonstrated in previous research assessing the effect of polyphenols on NIRS parameters (Wightman et al., 2014). The resultant sample size of 27 (for a within-subjects, crossover design) was in excess of the typical sample sizes for NIRS investigations.

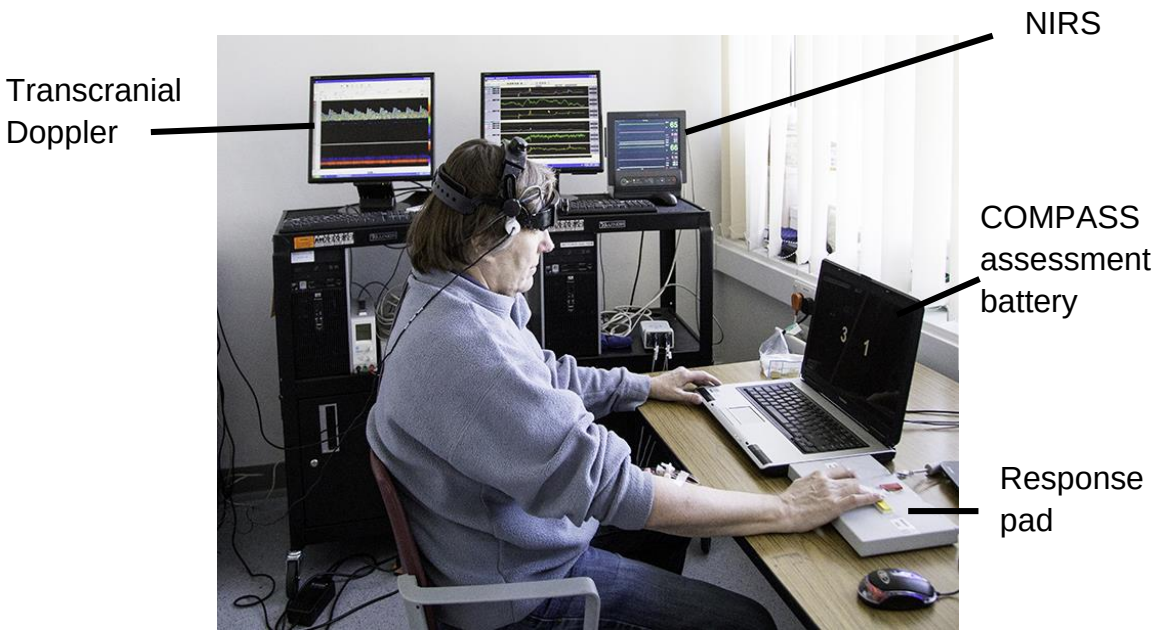

Figure 17 - The experimental set up on study days. Participants were fitted with a headpiece that continuously monitored cerebral blood flow velocity (TCD) and haemoglobin levels (NIRS) whilst performing several cognitive tests (COMPASS).

5.3 Results

Thirty male and female participants volunteered to take part in the study, but three participants voluntarily withdrew after the first study day (n=27). There were no adverse events reported in response to the intervention products. All participants complied with the low-polyphenolic diet according to the food diaries.

5.3.1 Cognitive performance and mood

No significant treatment-related differences were observed for any of the cognitive or mood measures (P > 0.05). The absolute values for task scores and mood ratings are given in Tables 13 and 14, respectively.

5.3.2 Blood pressure

Systolic blood pressure (SBP) exhibited a time (F = 15.856, P < 0.001), and treatment × time interaction effects (F = 4.825, P = 0.002). A *post-hoc* LSD test indicated that this difference occurred at 1, 2, 3 h post supplementation in the MC group, with peak reductions from baseline in postprandial SBP of 6 ± 2 mmHg at 1 h post MC consumption (Figure 18). There was no time, treatment or treatment × time interaction effects observed for diastolic blood pressure (DBP) (P > 0.05).

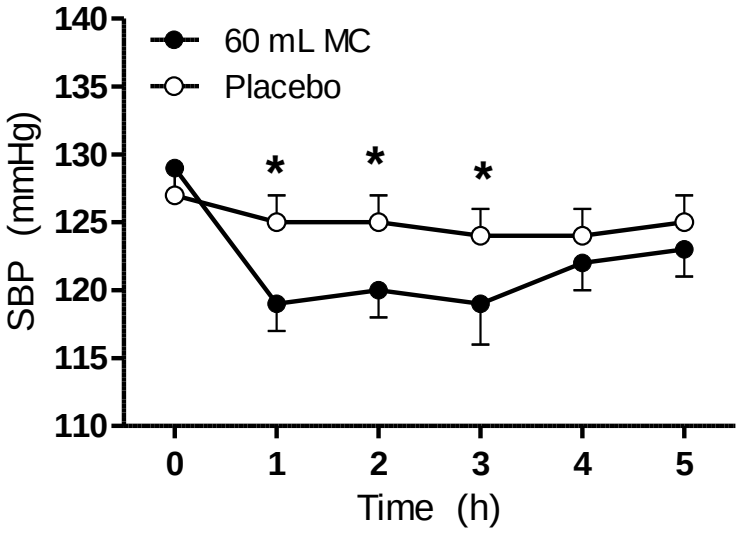

Figure 18 - Time course of systolic blood pressure (mean ± SEM) response after consumption of MC concentrate- and macronutrient – matched control (n=27). Significantly different from the placebo drink: * P < 0.05

Table 13 - Effects of MC concentrate and Pla on various aspects of cognitive performance in healthy middle aged adults.

		Task battery repetition										ANOVA		
		Baseline		1		2		3		5				
Measures	Treatment	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Effect	F	P
DV (%)	60 mL MC	94.2	1.1	94.3	1.4	93.6	1.4	92.3	1.7	92.3	2.0	T	0.087	0.771
	Pla	95.2	0.9	94.5	1.2	94.1	1.3	92.6	1.8	92.1	2.0	T × R	0.137	0.890
DV RT (ms)	60 mL MC	455.4	8.1	461.9	9.0	464.0	8.0	472.1	8.9	470.1	9.4	T	3.793	0.062
	Pla	455.5	8.2	461.0	8.6	465.8	9.1	454.6	11.1	444.0	16.2	T × R	2.109	0.135
RVIP (%)	60 mL MC	53.4	5.0	52.9	4.2	51.2	5.1	51.8	5.0	52.3	4.8	T	0.027	0.870
	Pla	51.7	4.3	55.1	4.7	51.3	4.9	53.5	4.5	52.2	4.7	T × R	0.391	0.759
RVIP RT(ms)	60 mL MC	491.6	32.2	517.1	12.1	522.4	11.0	528.0	11.0	504.4	11.8	T	0.269	0.608
	Pla	526.1	11.6	520.0	12.0	483.8	29.1	505.6	17.1	507.0	15.7	T × R	1.145	0.316
Stroop (%)	60 mL MC	98.7	0.2	98.8	0.3	98.7	0.2	98.8	0.2	98.7	0.3	T	0.960	0.414
	Pla	98.6	0.2	98.7	0.3	98.9	0.2	99.0	0.2	99.0	0.2	T × R	0.667	0.298
Stroop RT	60 mL MC	789.0	30.7	774.6	26.9	778.1	36.0	754.0	26.7	761.1	24.5	T	0.214	0.648
(ms)	Pla	814.9	37.5	764.0	29.2	764.0	29.2	763.4	29.8	805.3	68.2	T × R	0.677	0.487

All values are means ± SEM (n=27) T, treatment; R, repetition; DV, digit vigilance; RVIP, rapid visual information processing; RT, reaction time; Pla, Placebo.

Table 14 - Effects of MC concentrate and Pla on mood in healthy middle aged subjects.

		Task battery repetition												
		Baseline		1		2		3		5		ANOVA		
Measures	Treatment	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Effect	F	P
Alert	60 mL MC	35.67	4.55	35.39	3.55	40.31	4.11	42.91	4.41	45.22	4.44	T	0.415	0.525
	Pla	35.10	3.91	34.76	3.49	43.13	3.70	45.30	3.94	49.85	4.37	T × R	0.763	0.477
Concentration	60 mL MC	57.19	4.83	52.50	3.68	52.39	3.69	50.94	3.49	47.96	3.42	T	0.287	0.597
	Pla	51.75	3.61	57.02	3.19	48.80	3.44	49.15	3.50	46.93	3.52	T × R	1.417	0.250
Mental fatigue	60 mL MC	60.74	4.29	61.54	3.76	60.94	4.13	58.69	3.97	57.20	4.21	T	0.163	0.690
	Pla	61.65	3.95	63.44	3.32	55.76	3.82	54.30	4.09	56.85	4.08	T × R	1.281	0.288
Difficulty	60 mL MC	38.41	3.91	38.94	3.46	40.50	4.21	41.57	4.21	44.59	3.81	T	0.014	0.907
	Pla	40.73	3.69	36.65	3.41	39.78	3.08	38.96	3.43	44.96	3.50	T × R	0.631	0.579

All values are means ± SEM (n=27) T, treatment; R, repetition; Pla, Placebo.

5.3.3 Transcranial Doppler imaging

There was no time, treatment or treatment × time interaction effects observed for cerebral blood flow velocity (P > 0.05).

5.3.4 Near-IR spectroscopy parameters

5.3.4.1 Total haemoglobin (Total-Hb)

There was a significant interaction between treatment and post-treatment epoch on the initial ANOVA during the resting/absorption period (F = 5.234, P = 0.015). Reference to planned comparisons showed that there were significantly higher total-Hb concentrations during the last 3 10-min epochs of the resting/absorption period for MC concentrate. MC concentrate also resulted in higher total-Hb concentrations during each epoch of task performance 1 h post consumption (F = 16.452, P < 0.01). Thereafter, there were no significant differences in total-Hb (P > 0.05, Figure 19 A).

5.3.4.2 Oxygenated haemoglobin (oxy-Hb)

Similarly, there was a significant interaction between treatment and posttreatment epoch on the initial ANOVA during the resting/absorption period (F = 3.241, P = 0.029). Reference to planned comparisons showed that there were significantly higher oxy-Hb concentrations during the last 3 10-min epochs of the resting/absorption period for MC concentrate. MC concentrate also resulted in higher oxy-Hb concentrations during each epoch of task performance 1 h post consumption (F = 18.535, P = 0.019). Thereafter, there were no significant differences in total-Hb (P > 0.05, Figure 19).

5.3.4.3 Deoxygenated haemoglobin (deoxy – Hb)

There were no significant differences in terms of deoxy-Hb during either the resting/absorption or task performance periods (P > 0.05, Figure 20).

5.3.4.4 Task-related differences

There were no significant differences seen in the hemodynamic response to the DV, RVIP or Stroop tasks.

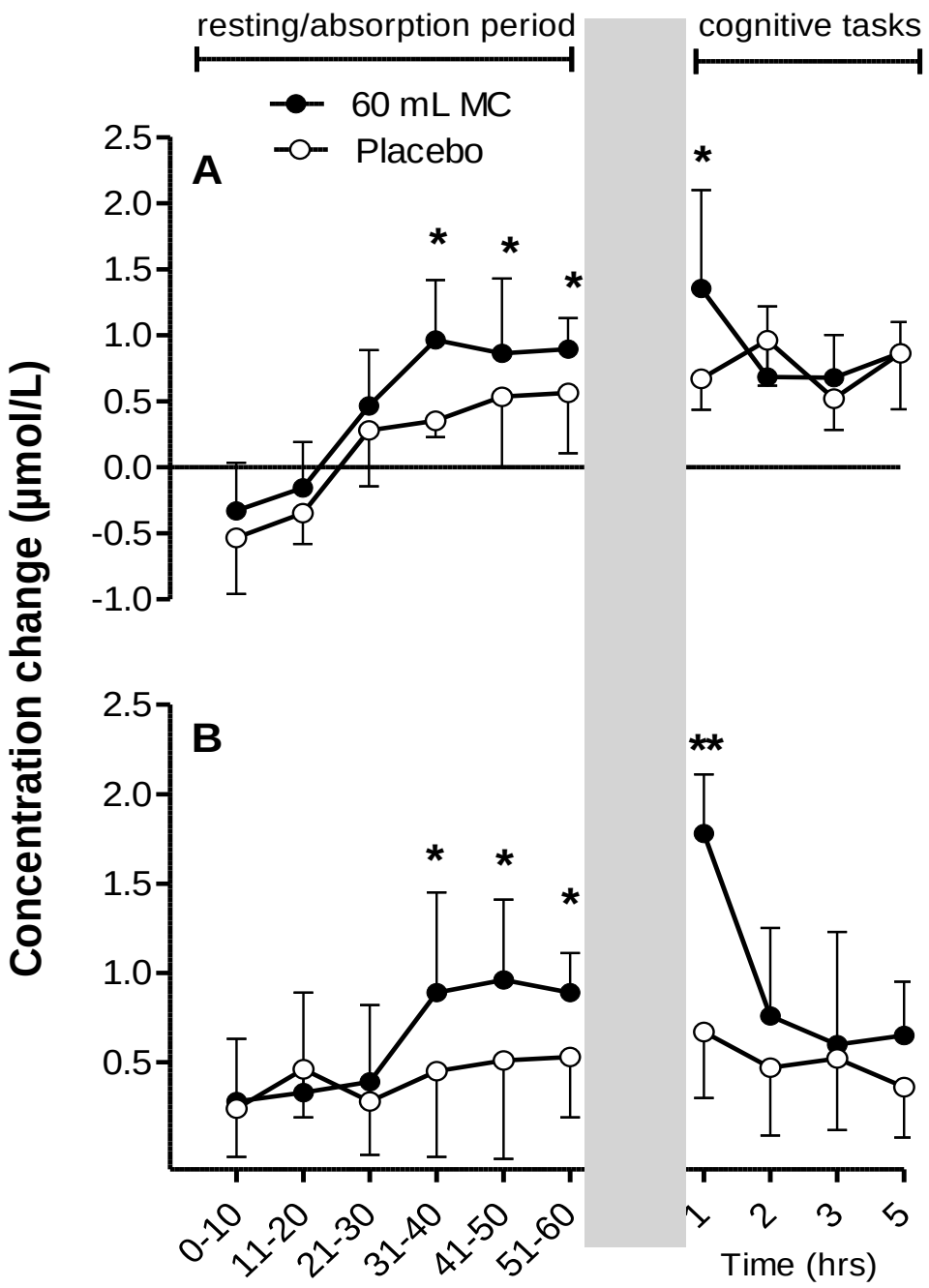

Figure 19 - A: Mean (± SEM) changes in concentrations of oxy-haemoglobin and B: total haemoglobin during a 60-min absorption period and subsequent cognitive task assessments 1, 2, 3 and 5 h post 60mL MC concentrate or placebo. Significantly different from the placebo: * $P < 0.05$, ** $P < 0.01$.

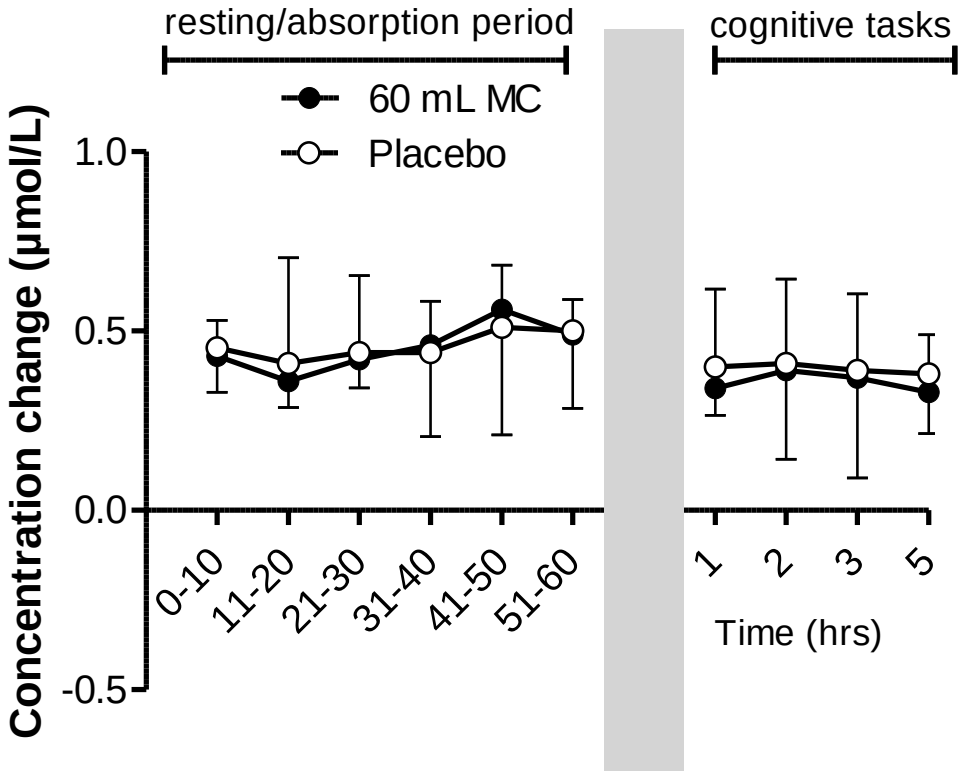

Figure 20 - Mean (± SEM) changes in concentrations of deoxy-haemoglobin during a 60-min absorption period and subsequent cognitive task assessments 1, 2, 3 and 5 h post 60mL MC concentrate or placebo.

5.4 Discussion

To the best of our knowledge, this study was the first to investigate the acute effects of Montmorency tart cherries consumption on cerebral blood flow variables and cognitive performance in a middle aged population. In support of our hypothesis, this study presents new information that in comparison to placebo, the consumption of a MC concentrate resulted in acute modulation of CBF parameters in the frontal cortex during task performance as indicated by the elevated concentration of total-Hb, with an identical pattern observed with oxy-Hb. This effect was evident for the cognitive assessment 1 h post MC consumption. These CBF observations were not associated with any significant modulation of cognitive performance or mood. There was also a significant reduction in SBP for up to three hours post MC consumption relative to the placebo.

Compromised cerebral blood flow has been suggested as a key contributor to cognitive function decline observed with advancing age and in a number of neurodegenerative diseases (Farkas et al., 2002). The results of the current study demonstrate that MC concentrate can modulate aspects of brain function, this was

evident 1 h post consumption. Total-Hb and oxy-Hb were increased toward the end of the 60-min resting/absorption period and during the cognitive assessment 1 h post consumption. However, there were no concomitant changes in deoxy-Hb across any of the time points. These results are consistent with previous studies using compounds and whole foods to demonstrate a positive effect on cognitive function and CBF. Kennedy and colleagues (2010) demonstrated an increase in total-Hb and oxy-Hb following single doses of orally administered resveratrol and more recently, Wightman et al., (2015) following beetroot juice ingestion. In both of these studies, total-Hb was increased during the first epoch of task performance. However, whilst total-Hb remained higher in the resveratrol study throughout the 40 minute cognitive assessment, it was decreased during the last 5 repetitions when participants were supplemented with beetroot juice. In the current study, no significant differences were seen following the first task period. The limitations associated with NIRS have consistently been highlighted (Murkin & Arango, 2009), and in the past few years, fMRI and other neuroimaging techniques have been used to assess the effect of a nutritional supplement on CBF (Isaacs, 2013; Bookheimer et al., 2013).

In terms of higher total-Hb concentrations, the modulation seen in the current study may be due to the vaso-relaxatory and antihypertensive properties of some of the phenolic acids (vanillic and protocatechuic acid) contained in the MC concentrate as demonstrated in Chapters 3 and 4 of this thesis. The time points (~1 hour post) at which these metabolites are seen in the plasma coincide with improvements in vascular function (Chapter 4), and modulation in CBF in the current study. Deoxy-Hb was not modulated in the current study. Furthermore, in the current study, task had no significant effect on cerebral modulation. It has previously been speculated that “high activation” tasks such as RVIP result in a higher increase in CBF than does performance in “low activation” tasks, for example digit vigilance. This can be largely attributed to the relative cognitive demands of the two tasks, with RVIP requiring the monitoring of rapidly changing digits along with a passive contribution from working memory. We speculate that these very early on effects on CBF in the current study might be associated with the sensory properties of the MC concentrate as previous studies have showed demonstrated that a number of sensory factors including differing taste and flavours are likely to modulate frontal cortex activity (Marciani et al., 2006; Smits et al., 2007). Marciani and colleagues previously demonstrated that several brain

areas were activated immediately after swallowing particularly when supplements had a strong (combined) taste or aroma. It could be argued that the MC concentrate was more sensory stimulating than the placebo, however this is speculative. Furthermore, a full analysis of sensory properties of MC and the placebo was outside the remit of the current study. In addition, NIRS only provides data on the relative concentration changes in each of the separate chromophores in response to tasks in the region of interest (the prefrontal cortex in the present thesis), and does not provide any information regarding the effects of the treatment on general cerebral blood flow.

Although the current study highlights an acute heightened NIRS response in brain regions responsible for task performance, there no was effect on cognitive performance. Supplementary oxygen (Moss et al., 1998) has been shown to positively influence cognitive performance in a healthy population. Therefore, it makes the expectation tenable that increases in CBF could be beneficial to acute cognitive performance via increasing the delivery of oxygenated blood metabolic substrate to, and efflux of metabolites from the brain, which is critical for brain function (Attwell et al., 2010). Importantly, despite some indication of improved blood flow, the current study showed no changes in cognitive task performance between experimental conditions. Nevertheless, these results do not stand alone; Caldwell et al., (2015) previously demonstrated that regardless of dose, cherry juice had no acute impact on cognitive function in young people, older people or dementia patients. They concluded that although cherry juice may have an acute impact on cardiovascular function, there was no change in cognitive performance 6 h post consumption. However, Caldwell and colleagues used sweet cherries as an intervention, it has been speculated that sweet cherries are not as rich in polyphenol compounds as tart/sour cherries (Kim et al., 2005).

Furthermore, cognitive assessments and blood flow measures were taken at baseline, 2 and 6 h post consumption, the current study attempted to explore the time points following consumption in more detail (hourly), however it is possible that any potential changes might still have been missed. Contrastingly, a chronic study by the same group (Kent et al., 2015) reported that the daily consumption of sweet cherries 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. Therefore, it is likely that regulation of blood flow and cognition are extremely complex, with multiple overlapping regulatory mechanisms paradigms and contributing structural components (Peterson et al., 2011), and therefore more likely to be influenced by chronic supplementation. This also accords with previous observations in similar trials, where Kelly et al., (2013) and Thompson et al., (2014) showed that after acute beetroot supplementation, there were no changes in cognitive performance for concentration, memory, attention or information processing ability. However, when older type 2 diabetics were supplemented with beetroot juice for 14 days, they experienced a significant improvement in simple reaction time compared to a control group (Gilchrist et al., 2014). These somewhat contrasting results may be partly explained by dose duration. It would seem that the cerebrovascular response required to elicit measurable changes in cognitive function can only be achieved with longer term dosing strategies that have the potential to induce sustained modifications to cerebrovascular function (Clifford et al., 2015).

However, contrary to this suggestion, two recent additions to the literature have demonstrated positive effects on cognitive function following acute blackcurrant (Watson et al., 2015) and wild blueberries (WBB) supplementation (Whyte et al., 2016). Acute blackcurrant supplementation was shown to improve RVIP accuracy and reaction time on the DV task in healthy people. Whilst, Whyte and colleagues demonstrated that acute cognitive benefits can be observed in 7-10-year old-children with an anthocyanin-rich blueberry intervention. The Page's test revealed the consistency and strength of this finding with WBB supplementation leading to significant overall improvements in cognition function, with the best change from baseline performance associated with 30g WBB treatment, intermediate performance with the 15g WBB treatment, and least effective performance with the vehicle treatment. Given that the protective cognitive effect of fruits is attributed to their high anthocyanin content, the anthocyanin dose in both of these studies was marginally higher than the current investigation (253 mg and 552 mg vs. 68 mg cyanidin-3-glucoside /L), this might go some way in explaining the inconsistent findings. It is also worthy to note, the cognitive tasks in the current study were selected on the basis of previous sensitivity to nutritional interventions (Scholey et al., 2010; Watson et al., 2015), however, these tasks may not be adequately sensitive to detect change in acute studies as perhaps this particular intervention

could affect different cognitive domains (i.e.. memory, recall). The most important consideration in setting up a suitable framework for measuring human cognitive function in polyphenol or flavonoid research is to determine methods that are sensitive to dietary changes and repeatable over time, simple to interpret and specific to cognitive domains (Macready et al., 2009).

Furthermore, all participants in the current study were healthy with no apparent issues pertaining to cerebral blood flow or cognitive ability. It is logical to question if that could mean that sufficient blood flow already exist for maximal cognitive performance and therefore, increasing blood flow beyond this threshold does not have any acute benefits on cognitive performance.

Additionally, there was no effect of the intervention on mood. This is somewhat surprising as mental fatigue has been previously shown to be receptive to cocoa flavanols in healthy adults (Scholey et al., 2010). However, Scholey and colleagues employed repeated 10-min cycles of a Cognitive Demand Battery (two serial subtraction tasks [Serial Threes and Serial Sevens] and RVIP), over the course of 1 h. Therefore, the cognitive assessment adopted in the current study might not have been as taxing on the brain as the aforementioned CDB with very limited rest time between repetitions.

There was a significant decrease in systolic blood pressure following MC supplementation when compared to placebo. These findings are in agreement with the previous chapter that reported a positive modulation of SBP in early hypertensive males following MC ingestion. This is not surprising, as participants in the current study had moderately elevated systolic blood pressure above the published ideal values at baseline - 128/82 mmHg. Kapil et al., (2010) noted that the magnitude of change in the BP response is directly related to baseline BP therefore, those who have a higher BP, will likely experience a greater change following an intervention. The current study is particularly noteworthy as data from prospective, observational studies have shown a reduction in mean SBP of 5-6 mmHg over a five year period was associated with 38% and 23% reduced risk of stroke and coronary heart disease, respectively (Collins et al., 1990). Here, we reported peak reductions in postprandial SBP of 6 ± 2 mmHg relative to the placebo. This finding, along with the modulation of CBF 1 h post MC consumption supports the growing body of evidence showing an inverse association between

the risk of chronic human diseases and the consumption of polyphenolic rich diet (King, 2000; Ginter & Simko, 2012).

The findings of the current study should be interpreted with a certain degree of caution because of the dietary restrictions imposed on participants. It is extremely unlikely that one would consume a diet that is free from polyphenol-rich foods and as a result, future work should attempt to demonstrate synergistic effects of MC supplementation within habitual dietary practices. Furthermore, a digital automatic BP monitor was used in the current study. The accuracy of this method has been called into question (Nelson et al., 2008). Future studies should consider using ambulatory blood pressure measurements, where readings are taking at regular intervals. Many studies have now confirmed that blood pressure measured over a 24-hour period is superior to clinic blood pressure in predicting future cardiovascular events (Ogedegbe, 2010). A timeframe of 5 h was utilized based on previous findings that phenolic compounds are quickly absorbed and/or excreted (Chapter 3) (Manach et al., 2005) and that any positive effects in vascular function are transient and return to baseline after four hours as evidenced in Chapter 4. However, it is possible that this timeframe may not long enough to capture the absorption of potentially other bioactive phenolic compounds provided by cherries in the colon.

In summary, the findings from this study suggest that MC concentrate can acutely modulate CBF in the prefrontal cortex characterized by increased concentrations of both total-Hb and oxy-Hb. Despite this evident modulation, these results do not translate to improvements in cognition or mood in the hours following consumption. Finally, this Chapter reaffirms previous findings that demonstrate a significant improvement in SBP following MC supplementation.

5.5 Perspectives

This Chapter addressed the third aim of this thesis to examine the effect of Montmorency tart cherry juice supplementation on cerebrovascular function and cognition. In participants supplemented with Montmorency tart cherry juice, total and oxy-Hb levels were elevated 1 h post consumption. Additionally, marked reductions in systolic blood pressure were observed following MC consumption, building on the findings from Chapter 4.

Whilst previous studies have assessed the impact of polyphenol supplementation on cognitive function and haemodynamics, prior to this study no attempt had been made to examine the haemodynamic response to tart cherries. The results from this Chapter add weight to the findings from Chapter 4, showing MC supplementation may be an efficacious strategy in modulating vascular function. Indices of cerebrovascular function (specifically total-Hb and oxy-Hb) were consistently higher in the MC supplemented group which may be of benefit to the aging population when trying to maintain sufficient blood flow to counteract the natural cognitive decline that occurs with age, although the lack of cognition or mood changes in this study, make this inference speculative. The findings from Chapter 4 suggest efficacy for MC in improving systolic blood pressure in those with early hypertension, and the results of the current Chapter provide further support those data.

Given the known vascular response following MC consumption provided by the current and preceding Chapters, and the interest surrounding polyphenol supplementation and its potential to improve exercise performance, Chapter 6 will directly assess the effects of acute tart cherry supplementation on plasma NO_2-concentration, blood pressure, $\dot{V}O_2$ kinetics, and exercise performance, compared with an energy-matched placebo.

6 Effects of Montmorency tart cherry consumption on plasma NO_2^- concentration, blood pressure, $\dot{V}O_2$ kinetics, and exercise performance.

6.1 Introduction

As previously highlighted, tart cherries are a food source rich in polyphenols (Wang et al., 1999; Seeram et al., 2001; Seynour et al., 2014; Bell et al., 2014) that include the flavonoids isorhamnetin, kaempferol, quercetin, catechin, epicatechin, procyanidins, and anthocyanins (Kim et al., 2005; Kirakosyan et al., 2009). Anthocyanins are a flavonoid group that has been associated with benefits for human health through anti-inflammatory effects (Zhu et al. 2013) and AOX activity (De la Cruz et al. 2013) (Section 2.2.3.1.4). These effects are of interest to counteract the production of reactive oxygen species during exhaustive exercise (Viña et al. 2000), which is the primary cause of exercise-induced disturbance in the oxidation–reduction status (i.e. redox balance) of skeletal muscle (Powers et al. 2004). In addition, the previous studies of this thesis demonstrated the positive effects of Montmorency tart cherry concentrate on various aspects of cardiovascular function, specifically enhanced cell migration, improvements in systolic blood pressure as well as elevated levels of total and oxy-Hb have been demonstrated. This may be mediated by the ability of polyphenols to increase nitric oxide by endothelial cells and also a reduced breakdown of nitric oxide by free radicals (Martin et al. 2002; Nagai et al. 2002) however this has not been investigated. Evidence shows that various polyphenols improve flow-mediated dilatation and endothelial function in humans by increasing endothelial nitric oxide synthesis (Nicholson et al., 2008; Ghosh & Scheepens, 2009). In sports where the rate of blood flow and maximum cardiac output are important determinants of cardiovascular performance, by acting on endothelial function, polyphenols could aid overall athletic performance

The cardio-protective properties of Montmorency tart cherries highlighted in this thesis, and the evidence that tart cherries can reduce oxidative stress and inflammatory markers (Wang et al., 1999; Howatson et al., 2010; Bell et al., 2014) may represent a potential ergogenic affect upon exercise performance. To date, there have only been two other studies that have investigated the effect of tart cherry supplementation on exercise performance. Clifford and colleagues (2014) first examined the effect of 200mg of CherryActive® capsules containing 216 mg of polyphenols on sub-maximal cycling and time trial performance. Results showed that no significant differences between trials were found for heart rate, respiratory exchange ratio, gross mechanical efficiency, oxygen consumption, blood lactate or final 20 km TT times ($P > 0.05$). In contrast, a more recent study

found that when participants were supplemented with powered Montmorency tart cherry capsules for 10-days, they averaged 13% faster half-marathon finish times (P= 0.001) and tended to have smaller deviations from predicted race pace (P = 0.091) compared to their placebo counterparts. The latter is in agreement with several studies reporting the significant beneficial AOX effects of natural polyphenols, in exercise-induced oxidative stress (Morillas-Ruiz et al., 2006; Panza et al., 2008). Previous studies have highlighted the performance-enhancing benefits of grape extract, chokeberries and blueberries (McAnulty et al., 2011; Lafay et al., 2009; Pilaczynska – Szczesniak et al., 2005). In one such study, Lafay et al (2009) supplemented handball players with a grape extract containing several polyphenols; the authors concluded that following grape extract supplementation, jump performance over 45-s significantly improved. The authors suggest that the enhancement in performance might be caused, at least in part, by the protective action of polyphenols during physical exercise. More recently, Cook et al. (2015) reported that following a seven-day intake of New Zealand blackcurrant extract, there was a significant improvement in cycling time-trial by 2.4% performance, coupled with an increase in fat oxidation. The authors speculated that this improvement may have been as a result of improved endothelial function and increased peripheral blood flow. Tart cherries have been shown to improve blood flow and blood pressure in earlier chapters; therefore if blood flow and cardiac output are important determinants of athletic performance, tart cherries may pose beneficial effects.

With the role of tart cherries in exercise recovery been widely discussed (Howatson et al., 2010; Bowtell et al., 2011 ; Bell et al., 2013; 2014; 2016), it is somewhat surprising that as of yet, there has been no attempt to investigate the effect of acute tart cherry ingestion on exercise performance. Therefore, this study addresses the specific aim of this thesis, which was to investigate the effects of acute tart cherry supplementation on plasma NO_2^- concentration ($[NO_2^-]$), a sensitive marker of NOS activity (Lauer et al., 2001), as well as blood pressure, $\dot{V}O_2$ kinetics, and exercise performance, compared with an energy-matched placebo. We hypothesized that, compared with placebo, MC supplementation would elevate plasma [NO_2-], reduce blood pressure, and improve $\dot{V}$O2 kinetics, cycling efficiency, and exercise performance.

6.2 Methods

6.2.1 Participants

Ten healthy male trained cyclists from a local cycling club volunteered to participate in the study (mean ± SD age; 28 ± 7 years, stature 1.83 ± 0.06 m, body mass 78.0 ± 8.5 kg and $\dot{V}O_{2peak}$ 59.0 ± 7.0 ml/kg/min). Exclusion criteria for the study were as follows: $\dot{V}O_{2peak}$ < 50 ml/kg/min (determined on visit 1), smokers, food allergy (as discussed with research team), history of gastrointestinal, renal or cardiovascular disease and current use of any food supplementations. All participants provided written consent prior to the commencement of the study. For 24 h prior to and for each of the testing days, participants were asked to avoid strenuous exercise, alcohol, caffeine, nutritional supplements and any anti-inflammatory drugs. Participants were also instructed to avoid polyphenol-rich foods and to record their dietary intake for 24 h prior to each testing day. Participants were asked to arrive at the laboratory in a rested and fully hydrated state, ≥10 h postprandial. All tests were performed at the same time of day. The study was conducted in accordance with the Helsinki Declaration and ratified by the University's Research Ethics Committee.

6.2.2 Study Design

Participants were required to report to the laboratory on five occasions over a 4-5 week period to complete the experimental testing (1 familiarization / $\dot{V}O_{2peak}$ visit and 4 experimental visits). On the first visit to the laboratory, participants completed a ramp incremental exercise test for determination of the gas exchange threshold (GET) and peak $\dot{V}O_2$ ($\dot{V}O_{2peak}$). Participants were also familiarized with the two exercise performance tests employed in the study on this visit to avoid any order effect on the performance results as a consequence of a potential "learning effect". Participants then returned to the laboratory on visits 2, 3, 4 and 5 to complete the experimental testing. During these tests, resting blood pressure, arterial stiffness, pulmonary $\dot{V}O_2$ kinetics, muscle oxygenation, and exercise performance was assessed, and venous blood samples were obtained. The MC concentrate and Placebo drinks were administered orally in a randomized order as part of a double – blind, crossover experimental design. Each supplementation day was separated by at least 3 days, but no longer than 7 days.

6.2.2.1 Incremental Test.

During the first laboratory visit, participants completed a ramp incremental cycle test on an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands). Initially, participants performed 3 min of baseline cycling at 0W: then the work rate was increased by 30 W/min until the limit of tolerance. The participants cycled at a self – selected pedal rate, which, along with saddle and handle bar heights and configuration, was recorded and reproduced in subsequent tests. Breath-by-breath pulmonary gas exchange data was collected continuously during the incremental tests and averaged over consecutive 10-s periods. The maximum $\dot{V}O_2$peak was taken as the highest 30-s mean value attained prior to the participant's volitional exhaustion in the test. The GET was determined as 1) the first disproportionate increase in CO_2 production ($\dot{V}CO_2$) from visual inspection of individual plots of $\dot{V}CO_2$ and $\dot{V}O_2$, and an increase in expired ventilation ($\dot{V}_E$)/ $\dot{V}O_2$ with no increase in ($\dot{V}_E$)/ $\dot{V}CO_2$. The work rate that's would require 90% of the GET (moderate – intensity exercise) and 70% Δ (GET + 70% of the difference between the work rate at the GET and $\dot{V}O_2$peak; severe intensity exercise) were calculated. The mean response time (MRT) for $\dot{V}O_2$ during ramp exercise was taken into account, therefore two – thirds of the ramp rate was deducted from the work rate at GET and peak (i.e.., 20W; Whipp et al., 1981).

Following the incremental test and a 45-minute rest, participants were familiarized with the exercise tests. Participants completed a severe – intensity step exercise test finishing with an all-out sprint followed, after a 30-minute passive recovery period, by a severe – intensity constant-work-rate step exercise test that was continued until the limit of tolerance.

6.2.2.2 Experimental tests.

On all subsequent visits, participants were required to rest in a seated position for 10 min in an isolated room. Thereafter, baseline blood pressure of the brachial artery was measured using an automated sphygmomanometer (M10-IT Omron Healthcare, UK). Additionally, pulse wave velocity and pulse wave analysis were determined by using Arterial Tonometry (SphygmoCor CPV system, ScanMed Medical, UK). Three measurements were taken, and the mean of the measurements was calculated. A venous blood sample was then collected into a lithium-heparin tube and centrifuged at 4,000 rpm at 4°C for 10 min, within 2 min of collection. Lithium-heparin plasma was subsequently extracted and immediately

frozen at -80°C for later analysis of [NO_2-] in duplicate via chemiluminescence (Wylie et al., 2013).

Participants were then provided with breakfast. Descriptive measures and a Physical Activity Level of 1.7 was used to calculate the participant's individual resting energy expenditure (Schofield Equation, 1985). This subsequently identified the amount of cereal (Rice Snaps, Tesco, Manchester, UK) and semi-skimmed milk (1g/kg/bm) each individual needed to consume to meet 10% of their daily energy requirements. This standardised fixed-energy breakfast meal consisted of a cereal: milk ratio of 30 g: 120 mL and delivered fat, protein and carbohydrate with a macronutrient composition of 14, 14 and 72%, respectively (Astbury et al., 2010). One-hour post breakfast consumption, participants received the intervention drink. Ninety minutes after ingestion of the supplement, vascular measures were reassessed and participants completed one of the two cycle tests described below, as published pharmacokinetic data have shown that this time frame should coincide with peak plasma concentrations of phenolic acids following MC supplementation according to both Chapters 3 and 4 of this thesis.

The exercise protocol consisted of three "step" exercise tests including two moderate – intensity step tests followed by one severe-intensity exercise bout. All participants performed a total of four bouts of moderate intensity exercise and two bouts of severe-intensity exercise for each experimental condition. This protocol replicated one used in a previously study by Bailey et al, (2015) when investigating the effect of L-Citrulline supplementation on $\dot{V}O_2$ kinetics and performance. Each transition began with 3 min of baseline cycling at 20W before an abrupt transition to the target work rate. Each moderate intensity bout lasted 6 min in duration. A passive recovery of 5 min separated the transitions. On two of the study visits (one occasion for each supplement), participants cycled for 6 minutes at a severe-intensity constant work rate (70% Δ), this was then followed immediately by a 60-s all-out sprint at maximum effort. The resistance on the pedals during this sprint was set using the linear mode of the Lode ergometer, so that each participant would attain the power output calculated to be 50% Δ when considering the participants preferred (linear factor = power/preferred cadence^2). Participants were provided with a 5-s countdown prior to the sprint. On the other two study visits (one occasion for each supplement), the severe-intensity constant-work-rate

bout was continued to the limit of tolerance, this limit was recorded as a fall in pedal rate >10rpm below the required pedal rate.

6.2.3 Treatments and dietary control

Participants consumed either 60 mL of MC concentrate or fruit-flavoured cordial in a double blind cross-over manner. This choice to use 60 mL was based on previous Chapters (Chapters 3 and 4) and work that showed a greater uptake of anthocyanin and phenolic acids *in vivo* post-consumption (Bell et al., 2014). The concentrate was diluted with 100 mL of water prior to consumption. The PLA supplement consisted of a commercially available, low fruit (<1%) squash (Kia Ora, Coca Cola Enterprises, Uxbridge, UK) cordial mixed with water, whey protein isolate (Arla Foods Ltd., Leeds, UK) and maltodextrin (MyProtein Ltd., Northwich, UK), to match the MC concentrate for volume and macronutrient content (Energy = 204 kcal, volume = 60 mL, carbohydrates = 49 g, protein = 2.2 g and fat = 0 g).

Prior to study commencement, it was explained to participants that the aim of the study was to investigate the effect of a fruit juice on vascular function. As a result, they were unaware which beverage was the experimental drink. Participants were instructed to follow a low phenolic diet for 24 h prior to each arm of the trial by avoiding fruits, vegetables, tea, coffee, alcohol, chocolate, cereals, wholemeal bread, grains and spices and were asked to refrain from strenuous exercise. Compliance with the dietary restrictions was assessed with a standardised, self-reported 1-day dietary record.

6.2.4 Measurements

During all tests, pulmonary gas exchange and ventilation were measured breath-by-breath. In order to do this, participants were required to wear a nose clip and breathe through a low-dead-space, low-resistance mouthpiece-and-impeller turbine assembly. The inspired and expired gas volume was continuously sampled at 100Hz; gas concentration signals were continuously sampled at 100 Hz using paramagnetic (O_2) and infrared (CO_2) analyzers (Oxycon, Care Fusion, Rolle, Switzerland). The gas analyzers were calibrated before each test using a calibrated 3-L syringe (Hans Rudolph Inc, Kansas City, Missouri, USA) and concentrations of known gases (16% O_2 and 5% CO_2). The breath-by-breath $\dot{V}O_2$ data from each test was linearly interpolated to provide second-by-second values,

and, for each individual, identical repetitions were time-aligned to the start of exercise and ensemble-averaged.

The oxygenation status of the vastus lateralis of the right leg was monitored using a commercially available using near-infrared spectroscopy (NIRS; INVOS 5100C, Somanetics, Troy, MI, USA). The system emits NIR light through fiber optic cables at two different wavelengths (765 nm and 855 nm) to the tissue and the light reflected from the tissue is detected by a photomultiplier tube. For each wavelength, the oximeter probe contains four light emitting fibers which are located at 2.0, 2.5, 3.0 and 3.5 cm from the detector, allowing ~1–2 cm measurement depth. The intensity of the transmitted light was continuously recorded at 1 Hz. Based on the absorption and scattering coefficients of light at each wavelength (Beer–Lambert Law), concentrations can be estimated for oxy(Hb + Mb), deoxy(Hb + Mb), and total(Hb + Mb). The leg was initially cleaned around the belly of the muscle, and the optodes were placed 20cm above the fibular head. The probes were secured to the skin surface and covered with an elasticized, tensor bandage to minimize the influence of extraneous light, and to avoid movement of the probe relative to the skin, while allowing unrestricted movement. The NIRS data were acquired continuously throughout the exercise protocol and output every 5 s and recorded for later offline analysis. The NIRS data output was time stamped at the start of each task segment to assure that data corresponded to the relevant period of task performance. Relative concentration changes in Oxy-Hb, Deoxy-Hb and Total-Hb were calculated.

PWV and PWA were determined by using Arterial Tonometry (SphygmoCor CPV system, ScanMed Medical, UK). The aortic pulse waveform and augmentation index were derived at the radial artery; PWV was determined between carotid and femoral sites. A pencil-type probe was used for all measurements and was held at the base of the neck over the carotid artery and at the inguinal crease over the right femoral artery. Recordings were taken when a reproducible signal was obtained with a high amplitude excursion. 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. Inter- and intra-trial %CV for this method was 3.3 and 3.1% respectively.

During the exercise trials, a blood sample was collected from a fingertip into a capillary tube at baseline, over the 20 s preceding the step transition in work rate,

the 20 s preceding the completion of 360 s of moderate- and severe – intensity cycling exercise, immediately following the all-out 60-s sprint and immediately after exhaustion during the severe-intensity constant-work-rate trial. These whole blood samples were analysed to determine blood lactate (Biosen C_Line, EKF Diagnostic, Barleben, Germany); intra-sample coefficient of variation for this instrument was< 1.8%.

6.2.5 Plasma [nitrate] and [nitrite] determination

All glassware, utensils, and surfaces were rinsed with deionized water to remove residual NO intermediates prior to [NO_2-] and [NO_3-] analysis. Plasma samples were deproteinized using zinc sulfate/sodium hydroxide precipitation prior to determination of [NO_3-]. Firstly, 500 µL of 0.18 N NaOH was added to 100 µL of sample followed by 5 min incubation at room temperature. Subsequently, samples were treated with 300 µL aqueous $ZnSO_4$ (5% w/v) and vortexed for 30 seconds before undergoing an additional 10 min incubation period at room temperature. Samples were then centrifuged at 4,000 rpm for 5 min, and the supernatant was removed for subsequent analysis. The [NO_3-] of the deproteinized plasma sample was determined by its reduction to NO in the presence of 0.8 % (w/v) VCl3 in 1M HCl within an air-tight purging vessel. Plasma samples were introduced to the vessel via 50 µL injections into the septum at the top of the vessel. The spectral emission of electronically excited nitrogen dioxide, derived from the reaction of NO with ozone, was detected by a thermoelectrically cooled, red-sensitive photomultiplier tube housed in a Sievers gas-phase chemiluminescence nitric oxide analyzer (Sievers NOA 280i. Analytix Ltd, Durham, UK). The [NO_3-] was determined by plotting signal (mV) area against a calibration plot of sodium nitrate standards. The [NO_2-] of the undiluted (non-deproteinized) plasma was determined by its reduction to NO in the presence of glacial acetic acid and aqueous NaI (4% w/v) from sodium nitrite standards. 100 µL injections were used for plasma [NO_2-] determination.

6.2.6 Statistical Analysis

Statistical analysis was performed using PASW Statistics 21.0 for Windows (SPSS, Inc., Chicago, IL.). All group characteristics were reported as means ± standard errors, unless otherwise stated. A two–way repeated measures ANOVA was employed to assess between – intervention differences in $\dot{V}O_2$, NIRS – derived [oxy-Hb], [deoxy-Hb], TOI, lactate and glucose. Mauchly's Test of Sphericity was

used to check homogeneity of variance for all ANOVA analyses; where necessary, violations of the assumption were corrected using the Greenhouse–Geisser adjustment. Significant main effects were followed up using LSD *post hoc* analysis. Blood pressure, arterial stiffness, NO_2^- and NO_3^- and exercise performance were analysed using a paired samples t-test. Statistical significance was accepted when $P < 0.05$.

6.3 Results

Eleven physically active males volunteered to take part in the study, but one participant voluntarily withdrew after the second study day (n=10). There were no adverse events reported in response to the intervention products. Subjects consumed all doses of the supplement for each experimental condition, and all participants complied with the low-polyphenolic experimental diet according to the food diaries. The $\dot{V}O_2$ peak attained in the ramp incremental test was 4.56 ± 0.3 l/min, which equated to a relative $\dot{V}O_2$ peak of 59 ± 7 $ml \cdot kg^{-1} \cdot min^{-1}$. The work rates that corresponded to 90% GET and 70%Δ were 121 ± 19 and 303 ± 28 W, respectively.

6.3.1 Pulmonary $\dot{V}O_2$ kinetics

The pulmonary gas exchange data for the moderate- and severe intensity cycle tests are reported in Table 15. There were no significant between-supplement differences for the baseline and end-exercise $\dot{V}O_2$ during the moderate-intensity step exercise tests ($P > 0.05$). Accordingly, the fundamental $\dot{V}O_2$ amplitude was not significantly different between the conditions (0.55 ± 0.09 and 0.60 ± 0.07 l/min with MC concentrate and Pla respectively, $P > 0.05$).

The baseline and end-exercise $\dot{V}O_2$ during severe-intensity exercise were not significantly impacted by the dietary interventions employed in this investigation ($P > 0.05$ for all comparisons). The $\dot{V}O_2$ at exhaustion was not significantly different between experimental conditions and was also not significantly different from the $\dot{V}O_{2peak}$ attained in the ramp incremental test ($P > 0.05$). No significant change from baseline to 120 s was observed (2.43 ± 0.10 and 2.37 ± 0.09 l/min with MC concentrate and Pla, respectively) or $\dot{V}O_2$ slow component (0.27 ± 0.02 and 0.35 ± 0.03 l/min with MC concentrate and Pla, respectively) were observed across the experimental conditions. There were no significant differences in $\dot{V}CO_2$ between the conditions during moderate- or severe-intensity cycle exercise ($P > 0.05$ for all comparisons).

Table 15 - Pulmonary gas exchange measures during moderate- and severe-intensity cycle exercise after MC and Pla supplementation.

	MC Concentrate	Placebo
	Moderate-intensity exercise	
$\dot{V}O_2$, l/min		
Baseline	1.67 ± 0.09	1.68 ± 0.11
End-exercise	2.22 ± 0.09	2.28 ± 0.10
Fundamental amplitude, l/min	0.55 ± 0.09	0.60 ± 0.07
	Severe – intensity exercise	
$\dot{V}O_2$, l/min		
Baseline	1.72 ± 0.04	1.74 ± 0.04
360 s	4.42 ± 0.12	4.36 ± 0.10
Exhaustion	4.50 ± 0.11	4.44 ± 0.09
Fundamental amplitude, l/min	2.43 ± 0.10	2.37 ± 0.09
Slow component amplitude l/min	0.27 ± 0.02	0.35 ± 0.03

All values are means ± SEM.

6.3.2 Exercise performance

The time to exhaustion during the severe-intensity constant-work-rate cycle trials (the exercise tolerance test) are shown in Figure 21, while the power profiles for the two experimental conditions during the 60-s all-out sprint that followed the 6-min bout of severe intensity exercise (the exercise performance test) are shown in Figure 22.

There were no significant differences in time to exhaustion during the exercise tolerance test between the experimental conditions (Figure 21). There were no significant differences in time to exhaustion during the exercise tolerance test between the experimental conditions (772 ± 34 vs 733.2 ± 34 s, $P > 0.05$).

A significant main effect for supplement was observed for the peak power attained and total work completed during the 60-s all-out sprint that concluded the exercise performance test ($P < 0.05$) in favour of the MC concentrate. Follow-up analyses demonstrated that, compared with Pla, MC concentrate supplementation increased the test peak power by 9.5% (363 ± 42 vs. 330 ± 26 W, $P = 0.033$; Figure 22) and the total work completed during the 60 s sprint by 10% between conditions (21 ± 3 vs. 19 ± 3 kJ, $P = 0.021$).

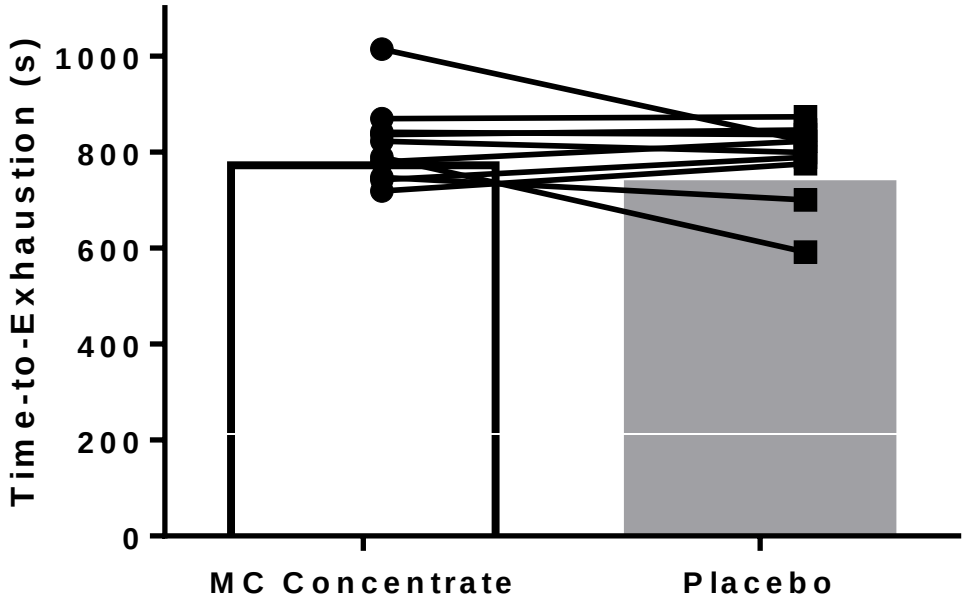

Figure 21 - Time to exhaustion during severe-intensity constant –work-rate cycle exercise after MC concentrate and placebo with individual responses to supplementation included.

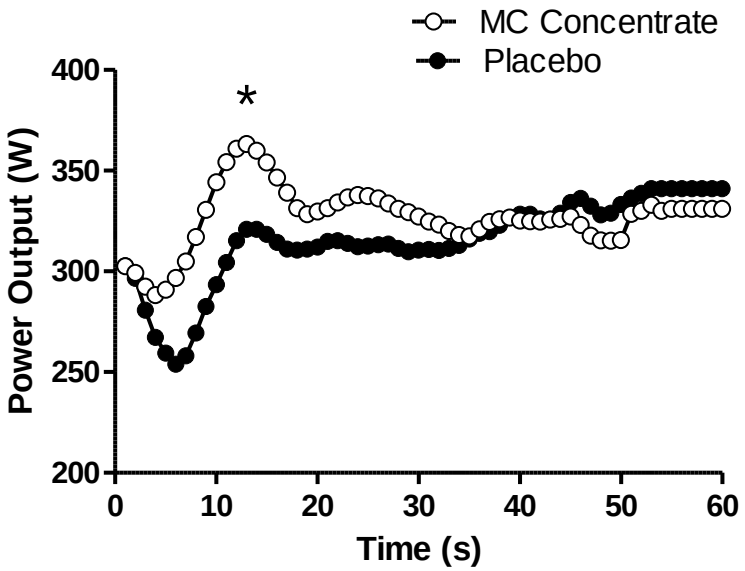

Figure 22 - Group mean power profiles during a 60-s all-out cycle sprint completed immediately after 6-min of severe-intensity cycle exercise following MC concentrate and Pla supplementation. Note significant increase in peak and mean power output during the 60s- all-out sprint after MC concentrate compared to Pla.

6.3.3 NIRS variables

The NIRS- derived muscle (deoxy-Hb, oxy-Hb and TOI) data during moderate- and severe-intensity cycle exercise with Pla and MC supplementation are reported in Table 16. There were no significant differences between the experimental conditions for any of the NIRS variables during the moderate or severe-intensity exercise ($P > 0.05$ for all comparisons).

Table 16 - Near – infrared spectroscopy measures during moderate- and severe intensity cycle exercise after MC and Pla supplementation.

	MC Concentrate	Placebo
	Moderate- intensity exercise	
Muscle [deoxy-Hb], AU		
Baseline	-1 ± 1	-2 ± 1
120 s	4 ± 1	5 ± 2
End-exercise	5 ± 1	5 ± 2
Muscle [oxy-Hb], AU		
Baseline	4 ± 1	4 ± 1
120 s	4 ± 1	4 ± 1
End-exercise	6 ± 1	5 ± 1
Tissue oxygenation index, %		
Baseline	67 ± 2	67 ± 2
120 s	67 ± 2	66 ± 2
End-exercise	66 ± 3	65 ± 1
	Severe-intensity exercise	
Muscle [deoxy-Hb], AU		
Baseline	-8 ± 1	-9 ± 1
120 s	15 ± 1	15 ± 1
End- exercise	15 ± 1	16 ± 1
Muscle [oxy-Hb], AU		
Baseline	10 ± 1	9 ± 1
120 s	-7 ± 1	-7 ± 1
End-exercise	-14 ± 2	-15 ± 1
Tissue oxygenation index, %		
Baseline	66 ± 3	68 ± 3
120 s	52 ± 4	54 ± 4
End- exercise	49 ± 3	48 ± 5

All values are means ± SEM. AU, arbitrary units.

6.3.4 Vascular measures

There was a significant main effect for supplement on SBP (P = 0.003), with follow-up analyses showing that SBP was lower 1.5 h post MC supplementation, with reductions of 5 ± 2 mmHg compared to the placebo trial. No other vascular variables (DBP, mean arterial pressure, PWV, AIx and AIx corrected for HR at 75

bpm) were altered after consumption of the MC concentrate compared to the placebo treatment. The absolute values for all variables are presented in Table 17.

Table 17 - Acute effects of tart Montmorency cherry juice and Pla on vascular function.

Variable	Baseline	1.5 h Post
SBP (mmHg)		
60 ml MC	118 ± 3	115 ± 2*
Pla	119 ± 3	120 ± 3
DBP (mmHg)		
60 ml MC	69 ± 2	68 ± 3
Pla	67 ± 3	68 ± 2
PWV (m/s)		
60 ml MC	6.0 ± 0.3	5.9 ± 0.4
Pla	6.0 ± 0.3	6.0 ± 0.3
MAP (mmHg)		
60 ml MC	85 ± 2	84 ±2
Pla	83 ±2	85 ± 3
AIx (%)		
60 ml MC	9.4 ± 2.0	9.1 ± 1.7
Pla	8.3 ± 2.0	7.5 ± 1.6
AIx @ 75bpm (%)		
60 ml MC	9.2 ± 2.3	9.9 ± 2.2
Pla	9.8 ± 1.8	10.3 ± 2.3

All values are means ± SEM (n=10). * Significant difference between Placebo and cherry concentrate treatment (2-factor repeated measures ANOVA) $P < 0.05$: SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; PWV, pulse wave velocity; AIx, augmentation index; MC, Montmorency cherry concentrate; Pla, Placebo.

6.3.5 Plasma [NO_2^-] [NO_3^-]

The plasma concentrations [NO_2^-] [NO_3^-] for the MC and Pla conditions are reported in Table 18. There were no significant changes reported for NO_2^- or NO_3^- in the MC supplemented trial when compared to the Placebo ($P > 0.05$).

Table 18 - Plasma [NO_2^-] and [NO_3^-] at baseline and 1.5 h following MC concentrate/Pla supplementation.

	MC Concentrate		Pla	
	Pre	**Post**	**Pre**	**Post**
Plasma [NO_2^-] nM	65 ± 9	68 ± 8	63 ± 7	63 ± 9
Plasma [NO_3^-] nM	19 ± 2	18 ± 2	20 ± 2	18 ± 2

All values are means ± SEM (n=8). MC, Montmorency Cherry; Pla, Placebo.

6.3.6 Lactate and glucose

There was no treatment or treatment × time interaction effect observed in blood [lactate], however there was a significant time effect identified during both the exercise performance and tolerance test ($F = 168.179$, $P < 0.001$). Glucose demonstrated a significant treatment × time interaction effect in the exercise performance and tolerance test ($F = 3.050$, $P = 0.029$). Glucose levels were significantly different between MC and placebo at time points 2 and 3 (20 s preceding the step transition in work rate, the 20 s preceding the completion of 360 s of moderate intensity exercise) during the exercise performance test and at time point 2 (20 s preceding the step transition in work rate) during the tolerance test. No other differences were reported. Absolute values are presented in Table 19.

Table 19 - Acute effects of tart Montmorency cherry juice and Pla on lactate and glucose following exercise performance and tolerance test.

	Time points				
	1	**2**	**3**	**4**	**5**
Lactate (mmol/L)	*Exercise performance test*				
60 ml MC	1.7 ± 0.2†	2.3 ± 0.2†	2.6 ± 0.2†	9.5 ± 0.8†	10.6 ± 0.6†
Pla	1.7 ± 0.2†	2.2 ± 0.2†	2.9 ± 0.4†	10.0 ± 0.8†	11.6 ± 0.8†
	Exercise tolerance test				
60 ml MC	2.0 ± 0.1†	2.2 ± 0.2†	2.6 ± 0.2†	9.8 ± 0.7†	12.5 ± 0.8†
Pla	2.1 ± 0.2†	2.4 ± 0.2†	2.7 ± 0.2†	9.9 ± 0.5†	11.2 ± 0.7†
Glucose (mmol/L)	*Exercise performance test*				
60 ml MC	3.3 ± 0.3	3.8 ± 0.3*	4.0 ± 0.3*	3.5 ± 0.3	3.6 ± 0.2
PLA	3.2 ± 0.3	3.2 ± 0.2	3.4 ± 0.3	3.8 ± 0.2	3.8 ± 0.2
	Exercise tolerance test				
60 ml MC	3.6 ± 0.2	3.9 ± 0.2*	3.7 ± 0.2	4.0 ± 0.2	4.3 ± 0.2
PLA	3.4 ± 0.3	3.2 ± 0.2	3.6 ± 0.2	3.7 ± 0.2	3.8 ± 0.3

All values are means ± SEM (n=10). † Significant difference between time points from baseline, * Significant difference between placebo and cherry concentrate treatment, $P < 0.05$.

6.4 Discussion

To the best of knowledge, this study was the first to investigate the effects of acute tart cherry supplementation on plasma NO_2- concentrations, blood pressure, $\dot{V}O_2$ kinetics, and exercise performance, compared with an energy-matched placebo. The principal novel findings from this study are that acute MC supplementation enhanced exercise performance in the absence of changes in $\dot{V}O_2$ amplitudes, NO_2- concentrations or muscle oxygenation. Additionally, SBP was significantly decreased 1.5 h post consumption in the MC trial, relative to the Pla.

Peak power output and total work completed over a 60-s sprint was increased by 6 and 10% respectively, during an exercise performance test following MC supplementation relative to the Pla. While tart cherry supplementation has been shown to improve exercise recovery (Howatson et al., 2010; Bowtell et al., 2011; Bell et al., 2014), decrease markers of inflammation and oxidative stress (Wang et al., 1999; Bell et al., 2014; Howatson et al., 2010), studies investigating the effects

of tart cherries on exercise performance are limited and equivocal. One recent addition to the literature investigated the effects of powdered Montmorency tart cherry supplementation on acute endurance exercise performance in aerobically trained individuals (Levers et al., 2016). Results of this study revealed that short-term supplementation of Montmorency powdered tart cherries not only attenuated markers of muscle damage, but also increased performance in aerobically trained individuals evidenced by a quicker overall race pace compared to the Pla group.

Similar findings have been reported in other studies with whole polyphenol compounds (Nieman et al., 2010) and in studies where polyphenolic content of a fruit – derived supplement is similar to tart cherries (Kang et al., 2012). The current study cannot determine any physiological mechanisms behind the improved performance, but the significant improvement in sprint performance accompanied by an insignificant small trend toward lower blood lactate levels following MC ingestion might suggest that MC supplementation may have contributed to less production of or enhanced removal of lactate during intense exercise, although this is speculative. Although, *in vitro* and *in vivo* animal studies suggest that polyphenols could really play a role in improving exercise performance (Davies et al., 2010); on the other hand, data in favour of polyphenol supplementation in humans are sparse and future research is warranted to fully understand if polyphenol intake enhances exercise performance. It is work commenting that MC supplementation could have prolonged the duration for which the participants were in the optimal cellular redox state for force production such that when they were require to produce an all-out sprint, they produced a higher peak power and completed more work. Studies on intact muscle have shown that the cellular redox balance may have an important influence on contractile function. That is, optimal contractile function may depend on the redox state of some, or all the participating cellular elements. In accordance with this, a study by Reid et al. (1993) demonstrated in rat diaphragm bundles that brief exposures to H_2O_2 increased twitch forces and prolonged the time to peak tension and the half-relaxation time. However, the current study did not measure any redox markers and this should be explored in future work.

Nitric oxide is a key regulator of vascular integrity. This multifaceted physiological signalling molecule can be synthesized endogenously through the nitrate-nitrite (NO_2-) – NO pathway (Lundberg & Weitzberg, 2009). Furthermore, it can be

generated through NOS and plasma [NO_2-] has been reported to reflect NOS activity as well as the capacity for O_2-independent NO generation (Jones et al., 2014). Dietary nitrate supplementation has previously been shown to increase NO biomarkers, prevent platelet aggregation (Webb et al, 2008), modulate cellular respiration (Cooper & Giulivi, 2007), reduce blood pressure (Hobbs et al., 2013) and improve exercise efficiency in healthy adults (Bailey et al., 2009; 2010). No significant difference in NO_2- concentration was reported between the MC and Pla trial in the current study. This is somewhat in agreement with the findings from Chapter 3, where no time, treatment, or time × treatment interaction effect for plasma nitrate or nitrite was observed following 60mL MC supplementation using an ELISA kit. The lack of a change in plasma NO_2- concentrations in this study extends previous findings by using a sensitive method to detect plasma [NO_2-] in the nM range and this better reflects NOS activity than plasma [NO_3-]. Well trained cyclists were recruited in the current study as research has demonstrated a clear moderate improvement of performance following polyphenol supplementation (Somerville et al., 2017); an improvement that would prove advantageous in sporting situations where very little separates opponents. However, it is likely that eNOS is optimally functioning in this cohort and therefore no changes were observed.

Furthermore, a study (Nabrzyski & Gajewska, 1994) that investigated the content of nitrates and nitrites in fruits, vegetables and other foodstuffs reported relatively small concentrations in cherries (from 0.0 to 36.0 mg NO_3/kg product). Previous studies have examined the impact of processing on cherry anthocyanins and polyphenolics (Ferretti et al., 2010; Chaovanalikit & Wrolstad, 2004) and have reported that pre-harvest factors and temperature, light intensity, fruit crops maturity may affect the content and stability of polyphenols and the nutritional value in cherries. In addition, post-harvest factors such as transport and storage can also influence polyphenol composition of food crops. As a result, it makes the argument tenable that the little amount contained in the whole cherry as previously alluded to, could be further diminished or removed during processing. Therefore, it would appear that the health benefits associated with tart cherry consumption as discussed previously are not likely to be attributed to NO, at least systemically. A second, and perhaps more likely method by which MC supplementation improves exercise performance, as outlined in Chapter 2, is based on the high polyphenol content of cherries and the subsequent uptake of downstream metabolites. This

mechanism as previously described to in Chapter 2 and 3 has been suggested to be at least partly responsible for acute improvements in indexes of cardiovascular function.

It was hypothesized that because of the AOX properties and pro-circulatory effects of the polyphenols contained in tart cherries; this would lead to an overall improvement in aerobic metabolism during highly intense exercise. However, in contrast with a recent addition to the literature concerning dietary polyphenol supplementation and metabolic rate (Jo et al., 2016), and our experimental hypothesis, acute MC supplementation did not significantly alter $\dot{V}O_2$ during moderate- or severe intensity cycle ergometry exercise. Unsurprisingly, as capacity of aerobic metabolism during highly intense exercise depends on efficient blood supply to the contracting muscle (Mortensen et al., 2008), there were no significant differences in any of the NIRS variables between conditions. Conversely, it has previously been shown that blackcurrant intake inhibited the decrease in total-Hb and oxy-Hb that occurred in a model of experimentally induced disturbances to the local circulation (i.e.. typing activity) (Matsumoto et al., 2004). The authors speculated that this effect was largely caused by the high anthocyanin content of blackcurrants. Although tart cherries have also previously been shown to have a high anthocyanin content [specifically cyanidin and peonidin (Ou et al., 2012)], these findings were not replicated in the current study. Overall, there is insufficient and inconsistent evidence to conclude whether or not polyphenol supplementation can alter muscle oxygenation and improve oxidative metabolism, as a consequence of differences in experimental design including the type of supplementation, subjects and protocol (Malaguti, 2013).

A primary outcome of enhanced NO synthesis is a reduction in blood pressure owing to NO-induced smooth muscle relaxation (Gruetter et al., 1979). The current study reported a significant reduction in SBP 1.5 h post MC ingestion relative to placebo, however this augmented modulation occurred in the absence of changes in NO biomarkers. These results do not stand alone; a recent study demonstrated that when participants were supplemented with L-Citrulline or L-arginine, plasma NO_2- was not increased, however significantly reduced blood pressure was observed (Bailey et al., 2015; Vanhatalo et al., 2013). Previous Chapters have reported significant improvements in SBP following MC consumption in males with early hypertension [Chapter 4] and middle aged adults

[Chapter 5]. Chapter 4 also highlighted than an increase in downstream phenolic acids, protcatechuic and vanillic acid, have been reported after acute MC consumption, which suggests that circulating phenolic metabolites derived from Montmorency tart juice are, at least in part, responsible for acute improvements in SBP. As mentioned earlier, further work is required to elucidate if a similar mechanism is responsible for the improvement in exercise performance in the current study.

In conclusion, the findings of the current study demonstrate that acute supplementation with tart cherry juice has the ability to lower SBP and improve exercise performance with no changes in oxidative metabolism or muscle oxygenation. Further research is required to determine whether the effects reported would be amplified using a chronic supplementation strategy and to further confirm the link between cellular protective action, and performance enhancement effects caused by the consumption of tart cherries in elite and occasional athletes.

6.5 Perspectives

This Chapter aimed to investigate the effect Montmorency tart cherry juice supplementation would have on exercise performance in well-trained individuals. Results demonstrated that participants supplemented with Montmorency tart cherry juice performed better in a 60 s all-out sprint following moderate- and severe intensity exercise. This was characterized by higher peak power output attained and total work competed over the course of the 60 s. Additionally, SBP was reduced 1.5 h post MC supplementation. Despite these changes, there were no differences in $\dot{V}O_2$ kinetics, NO_2- plasma concentrations or muscle oxygenation between the two conditions.

The findings from this Chapter extend the application of MC supplementation to wider group of individuals, specifically to athletic populations in a bid to improve exercise performance. The concept of marginal gains has revolutionised many sports particularly if a sprint finish could potentially be the difference between winning and losing. After completing exercise that was deemed both mechanically and metabolically stressful, when participants were supplemented with MC, they performed better over a 60-s sprint. Importantly, regardless of the mechanism, these improvements in performance are of most interest to the athlete, applied coach or sports scientist. The moderate- and severe intensity exercise task used in

this Chapter presented as a good model to assess $\dot{V}O_2$ kinetics and cycling economy with both the presence and the absence of a $\dot{V}O_2$ slow component.

7 General Discussion

7.1 Experimental Chapter Synopsis

The first experimental Chapter in this thesis (Chapter 3) was designed to (1) examine the time course of selected phenolic compounds following ingestion of a Montmorency cherry (MC) concentrate, and (2) identify the effects of the selected phenolic compounds on vascular smooth muscle cell (VSMC) behaviour *in vitro*. Previous work has suggested that the health-related benefits of tart cherries arise from their high anthocyanin content (Bell *et al.,* 2015); however, given the poor bioavailability of whole anthocyanins it would appear that the biological effectiveness of Montmorency cherries might be due to the downstream metabolites of anthocyanins and the polyphenol interactions, which accomplish complementary effects. Such synergies occur when combinations of bioactive substances exert effects at target sites that are greater than the sum of individual components (Lila & Raskin, 2004). Ou and colleagues (2012) previously established that the anthocyanins (in order of decreasing prevalence) in processed tart cherry products were cyanidin-3-glucosylrutinoside, cyanidin-3-rutinoside and peonidin-3-rutinoside. As a result, Chapter 3 aimed to identify the primary downstream metabolites of the anthocyanins, cyanidin and peonidin, in the plasma post ingestion. HPLC/LCMS identified two dihydroxybenzoic acids, protocatechuic and vanillic acid (PCA and VA) in plasma following MC concentrate consumption. Both compounds were most abundant 1-2 h post-initial ingestion with traces detectable at 8 h post-ingestion. In addition, VSMC migration increased when the cells were treated in concert with both metabolites, demonstrating that these metabolites, at a similar level to that seen in plasma, can alter VSMC function. There was no effect on cell proliferation observed.

The second investigation (Chapter 4) aimed to identify the effects of Montmorency tart cherry consumption on vascular function in males with early hypertension. Cherry extracts have been shown, in cell and animal models, to exert a range of cardio-protective effects that include increasing nitric oxide production and AOX status, reducing lipid oxidation and inhibiting inflammatory pathways (Wang et al., 1999; Seeram et al., 2001). However, the potential cardio-protective effects of cherries have not been investigated in humans to the same degree. Montmorency tart cherries are high in numerous polyphenols, such as anthocyanins, and as previously discussed these compounds have poor bioavailability. However as demonstrated in Chapter 3, it is likely that they contribute to the overall appearance of downstream metabolites in the plasma following MC ingestion and

that the resulting metabolites can alter vascular function *in vitro*. As such, it makes the expectation tenable they would positively modulate vascular function *in vivo*. Results of Chapter 4 demonstrated that MC supplementation significantly reduced SBP for up to 3 hours post-prandial, with peak reductions of 7 ± 3 mm Hg at 2 h after MC consumption relative to the placebo. Furthermore, findings suggested that circulating phenolic metabolites (PCA and VA) derived from MC juice are at least partly responsible for acute improvements in SBP in men with early hypertension. This was the first study to demonstrate the efficacy for MC supplementation as an adjuvant in the management of hypertension.

The third experimental study (Chapter 5) of this thesis was designed to build on the findings from Chapter 4 and further explore the health applications of tart cherries. Conceptually, in light of the vaso-modualtory effects of polyphenol-rich foods, and the findings of Chapter 3 and 4, it was not unrealistic to speculate that Montmorency cherries might also be capable of conferring a positive cognitive effect by improving cerebral blood flow. The aim of this study was to examine the effects of Montmorency tart cherry consumption on cerebral blood flow and cognition in middle aged adults. The results of this study demonstrated that in comparison to a placebo, the consumption of a MC concentrate resulted in acute modulation of CBF parameters in the frontal cortex during cognitive task performance as indicated by the elevated concentrations of total-Hb, with an identical pattern observed with oxy-Hb. This effect was evident during the last 3 10-min epochs of the 60-min resting/absorption period and for the cognitive assessment 1 h post MC consumption. However, these CBF observations were not associated with any significant modulation of cognitive performance or mood. Similar to the results observed in Chapter 4, there was a significant reduction in SBP for up to three hours post MC consumption relative to the placebo trial. The results from this Chapter add weight to the findings from Chapter 4, suggesting that MC supplementation may be an efficacious strategy in modulating other aspects of (cerebro-) vascular function.

Given the abundance of previous literature supporting the use of MC supplementation as a recovery aid (Howatson et al., 2010; Bowtell et al., 2011; Bell et al., 2014) and previous studies that have reported improved exercise capacity and efficacy in association with improved vascular function (Oh et al., 2010), the final experimental Chapter of this thesis (Chapter 6) directly assessed

the effects of acute Montmorency tart cherry supplementation on plasma NO_2^- concentrations, blood pressure, $\dot{V}O_2$ kinetics, and exercise performance, compared with an energy-matched placebo. Results demonstrated that acute MC supplementation increased peak power by 6% (363 ± 42 vs. 341 ± 16 W) and the total work completed during the 60 s sprint by 10% between conditions (21 ± 3 vs. 19 ± 3 kJ). This improvement in performance occurred in the absence of changes in $\dot{V}O_2$ kinetics, NO_2^- concentrations or muscle oxygenation. Similar to the previous experimental chapters, SBP was significantly decreased 1.5 h post consumption in the MC trial, relative to the placebo. The findings from this chapter extend the application of MC supplementation to wider group of individuals, specifically those individuals with impaired physical mobility due to cardiovascular and cardiorespiratory impairment and also to athletic populations in an attempt to improve exercise performance

7.2 Main findings

The overall aim of this thesis was to investigate the bioavailability of compounds in Montmorency tart cherry juice and their potential application to modulate aspects of health relating to vascular function. A number of relevant issues have been previously discussed throughout each of the experimental chapters, however the following sections discusses the main findings of this thesis in the context of existing literature, the limitations of the work and potential future areas of investigation.

7.2.1 Bioavailability

The initial aim of the thesis was to assess the polyphenol uptake following human consumption of Montmorency tart cherries. Data from the investigational chapters of this thesis have identified the presence of both PCA (2.75 and 2.76 µg) and VA (0.29 and 0.30 µg) in the plasma post MC supplementation. To date, the majority of work involving MC supplementation focuses on the absorption of primary anthocyanins. Given that the bioavailability of these compounds is poor (Patras et al., 2010), exploring the presence and absorption time of their primary downstream metabolites may be more appropriate when trying to determine the compounds eliciting positive health benefits. These two dihydroxybenzoic acids are the main degradation metabolites of cyanidin and peonidin, the most abundant anthocyanins present in MC concentrate (Wang et al., 1997; Kirakosyan et al.,

2000). Chapter 3 demonstrated that physiological concentrations of these phenolic acids are most bioavailable 1-2 h post MC consumption; this finding was further supported in Chapter 4 of this thesis. Both of these phenolic acids have been found to possess several health-promoting properties including chemo-preventative, antibacterial, AOX, antidiabetic, anticancer, antiulcer, antiaging, anti-inflammatory and anti-atherosclerotic effects (Kamat et al., 2000; Kakker & Bais, 2014). Chapter 3 used a 30 and 60 mL dose of MC as previous studies investigating the pharmacokinetics and biological effects of MC used an identical dosing strategy (Bell et al., 2014) or 45 and 90 whole cherries (Seymour et al., 2014). Doses higher than this may infer some gastrointestinal discomfort (Bell et al., 2014)

7.2.2 Vascular function *in vitro*

Within Chapter 3, this thesis also examined the effect of PCA and VA on VSMC behaviour. VSMC are responsible for the provision of vascular tone in normal, healthy blood vessels, and their behaviour is critical in the development of atherosclerotic plaques (Ross, 1993). Chapter 3 demonstrated that cell migration increased when the VSMC were exposed to both VA and PCA, demonstrating that these metabolites, at a similar level to that identified in the plasma following MC supplementation, can alter cell function. Importantly, migration of de-differentiated VSMC is required for vessel remodelling which occurs from exercise and vascular injury. The VSMC migration in advanced atherosclerotic plaques is often considered to be protective as it increases stability, protecting against plaque rupture and ensuing vascular trauma such as myocardial infarction or stroke (Louis & Zahradka, 2010). By increasing VSMC migration, the metabolites may potentially be beneficial for blood vessel remodelling, although this would require further investigation. Cell proliferation was not altered over the course of this experiment. Noticeably, the time frames and period of investigation in this chapter were different for the *in vivo* and *in vitro* work i.e. 8 h vs 24 h. This was the case as it takes much longer for cells to react to a particular stimulus, and thus the time frame was extended accordingly.

7.2.3 Vascular function *in vivo*

The secondary aim of the thesis was to assess the impact of MC supplementation on vascular function in humans. In the literature, there is very little evidence in humans to support the modulation of vascular function following cherry supplementation; in fact the most recent literature has reported no changes (Lynn et al., 2014). The current thesis advanced on the study by Lynn and colleagues by investigating a population with a higher CV risk and incorporating more robust methods for vascular assessment. Throughout this thesis, there was an observable impact of MC supplementation on systolic blood pressure. Data from the investigational Chapters of this thesis give some indication of an augmentation of vascular function in various populations following acute supplementation with Montmorency tart cherry concentrate. It must be noted, that there appeared to be a clear pattern of modulation of blood pressure across all chapters. In Chapter 4, systolic blood pressure was reduced by 7 ± 3 mm Hg in males with early hypertension following 60 mL MC supplementation. Similarly, in Chapter 5, the MC supplement resulted in peak reductions in SBP of 6 ± 2 mmHg relative to the placebo in middle aged adults. Additionally in Chapter 6, SBP was significantly reduced 1.5 h post MC supplementation in male cyclists. In both Chapter 4 and 5, SBP had returned to near baseline 5 h post supplementation, however this was not explored in Chapter 6. The studies in this thesis are particularly noteworthy because data from prospective observational studies have shown a reduction in mean SBP of 5–6 mm Hg over a 5-y period was associated with 38% and 23% reduced risk of stroke and coronary artery disease, respectively (Collins et al., 1990).

An earlier study from Lynn and colleagues (2014) did not detect any changes in BP or any other vascular measures following tart cherry consumption, however, this was in normotensive subjects (~111/70 mm Hg). Both Chapter 4 and 5 of this thesis found significant reductions in SBP following MC supplementation in individuals whose baseline BP was 137 / 82 mm Hg (Chapter 4) and 128 / 82 mm Hg (Chapter 5), therefore it makes the argument tenable that the magnitude of change in the BP response is directly related to baseline BP (Kapil et al., 2010). In support of this finding, there is a plethora of data suggesting improvements in blood pressure following the consumption of other polyphenol-rich fruits such as cocoa, beetroot, and grape extract (Grassi et al., 2005; Hobbs et al., 2013; Draijer

et al., 2015). And although it has previously been reported and demonstrated in Chapter 4, that concurrent improvements in all measures of vascular function are not always observed (Hobbs et al., 2013), modulations of central haemodynamics were observed after supplementation of MC juice in Chapter 5.

A continuous, oxygen-rich supply of blood is vital for general health. Poor circulation of blood and reduced oxygenation is associated with a number of disorders including cardiovascular disease (Zeiher et al., 2003). Of importance here, blood flow and oxygenation are especially vital for neurocognitive performance; with research correlating reduced CBF and poorer cognitive function in humans (Celsis et al., 1997). Conversely, improvements to aspects of cognitive function can be seen with supplementary inspiration of pure oxygen in deprived participants (Weiskopf et al., 2002) and in young, healthy participants (Moss et al., 1998). Changes in concentrations of either oxy-Hb and deoxy-Hb are related to changes in cerebral metabolic rates (Tamura et al., 1997), and the concentration of deoxy-Hb and to a lesser extent oxy-Hb, is strongly correlated with the fMRI blood-oxygenation-leveldependent (BOLD) signal (Huppert et al., 2006).

fMRI is the most comparable neuroimaging tool to NIRS with both measuring the CBF response to preceding neural activation; the 'neurovascular coupling'. Neural activation instigates an increase in CBF which is primarily seen as increased concentrations of total and oxy-Hb. This CBF response is greater than the metabolic rate of oxygen extraction/utilization (deoxy-Hb) and, as such, the concentration of deoxy-Hb can be observed to decrease during cognitive performance (Hasegawa et al., 2002). This relative fall in deoxy-Hb is the product ofthe local haemoglobin becoming more oxygenated and results in a slight increase in the magnetic signal; as haemoglobin is diamagnetic when oxygenated and paramagnetic when deoxygenated (Buxton et al., 2004). As stated above, this BOLD signal is strongly correlated to that measured by NIRS; indeed the two techniques have been converged successfully in the past, and a relative rise in total and oxy-Hb with a concomitant reduction in deoxy-Hb is the typical hemodynamic response to cognitive workload as assessed by NIRS (Tamura et al., 1997).

When compared to the placebo, MC consumption resulted in significant modulations in prefrontal cortex haemoglobin concentrations during the absorption period and cognitive task performance. Increases in oxy-Hb of 0.76 µmol/L and

total Hb of 1.43 µmol/L were seen during the cognitive task period 1 h after consumption of MC juice relative to the placebo. No effect of task type was evident. These increases coincide with the appearance of phenolic acid concentrations highlighted in earlier chapters. This is the first study to investigate the acute effects of Montmorency tart cherries on cerebral blood flow variables and cognitive function in a middle aged population. The findings from this Chapter are in agreement with previous studies using polyphenol compounds and whole foods to demonstrate a positive effect on cerebral blood flow (Kennedy et al., 2010; Wightman et al., 2015). Reductions in cerebral blood flow have been associated with natural aging (Leenders et al., 1990) and neurological diseases (Dede et al. 2007). Results from this thesis outline the ability of tart cherries to increase cerebral blood flow in a middle aged population.

However, it must be noted that NIRS only provides data on the relative concentration changes in each of the separate chromophores (oxy-Hb, deoxy-Hb, total-Hb) in response to tasks in the region of interest (the prefrontal cortex in the present thesis), and does not provide any information regarding the effects of the treatment on general cerebral blood flow. In addition, a disadvantage of the NIRS imaging technique is that it has low spatial resolution in comparison to other methods; however it does have the benefit of providing high temporal resolution (Obrig & Villringer, 2003). Therefore, although the results presented in Chapters 5 are promising, the paradigm could be improved in future interventions.

In Chapter 4 and 5 of this thesis, the greatest improvements in SBP occurred in association with peak plasma PCA and VA as highlighted in earlier Chapters, indicating that both of these metabolites could be partly responsible for the effects observed, particularly given that these hydroxybenzoic acids were shown in the Chapter 3 to modulate vascular smooth muscle cell behaviour *in vitro.* Furthermore, we speculate that these very early on effects on CBF in the current study might be associated with the sensory properties of the MC concentrate as previous studies have showed demonstrated that a number of sensory factors including differing taste and flavours are likely to modulate frontal cortex activity (Marciani et al., 2006; Smits et al., 2007). Marciani and colleagues previously demonstrated that several brain areas were activated immediately after swallowing particularly when supplements had a strong (combined) taste or aroma. It could be argued that the MC concentrate was more sensory stimulating than the

placebo; however this is speculative as full analysis of sensory properties of MC and the placebo was outside the remit of the current study.

To conclude the vascular function findings from this thesis; initial findings would suggest that MC supplementation can reduce SBP. The pattern of evidence from the subsequent studies in this thesis supports this modulation of systole. This finding, along with the modulation of CBF post MC consumption supports the growing body of evidence showing an inverse association between the risk of chronic human diseases and the consumption of polyphenolic rich diets (Pandey & Rizvi, 2009; Lima et al., 2014).

7.2.4 Cognitive function

Although Chapter 5 highlights an acute heightened NIRS response in brain regions responsible for task performance (increases in oxy-Hb of 0.76 µmol/L and total Hb of 1.43 µmol/L respectively), this modulation of shallow pre-frontal cortical haemodynamics was not associated with any improvement or decrement to measured behavioural parameters. Supplementary oxygen has been shown to positively influence cognitive performance (Moss et al., 1998). However, to the best of the author's knowledge, there are no studies that show that this pattern of CBF modulation following polyphenol supplementation is associated with cognitive improvements in middle aged adults. Yet, the results of this study do not stand alone. Kennedy et al., (2010) showed that resveratrol supplementation increased pre-frontal cerebral blood flow in a similar pattern to Chapter 5, with no change in cogntion. In addition, other berry studies (Krikorial et al., 2012) have shown that although polyphenol supplementation results in changes in haemodynamics, which are particularly evident during cognitive demand, this does not necessarily manifest into improved cognitive performance.

Conceivably, all participants in the current study were healthy with no apparent issues pertaining to cerebral blood flow or cognitive ability. It is logical to question if that could mean that sufficient blood flow already exist for maximal cognitive performance and therefore, increasing blood flow beyond this threshold does not have any acute benefits on cognitive performance. Therefore it is more likely that regulation of blood flow and cognition are extremely complex, with multiple overlapping regulatory mechanisms paradigms and contributing structural components and are not easily affected by nutritional interventions.

7.2.5 Exercise performance

A secondary aim of this thesis was to assess the effect of MC supplementation on exercise performance. While tart cherry supplementation has been shown to improve exercise recovery (Howatson et al., 2010; Bowtell et al., 2011; Bell et al., 2014), decrease markers of inflammation and oxidative stress (Wang et al., 1999; Bell et al., 2014; Howatson et al., 2010), studies investigating the effects of tart cherries on exercise performance are limited and equivocal. Chapter 6 of this thesis demonstrates that MC supplementation could improve exercise performance in trained male cyclists.

Peak power output and total work completed over a 60-s sprint was increased by 6 and 10% respectively, during an exercise performance test following MC supplementation relative to the Pla. This study is in agreement with a recent addition to the literature where the effects of powdered Montmorency tart cherry supplementation on acute endurance exercise performance in aerobically trained individuals was investigated (Levers et al., 2016). Results of this study revealed that short-term supplementation of Montmorency powdered tart cherries not only attenuated markers of muscle damage, but also increased performance in aerobically trained individuals evidenced by a quicker overall race pace compared to the Pla group. Similar findings have been reported in other studies with whole polyphenol compounds (Nieman et al., 2010) and in studies where polyphenolic content of a fruit – derived supplement is similar to tart cherries (Kang et al., 2012).

Although physiological mechanisms for this observation are unclear, previous studies have shown that L-citrulline and beetroot supplementation resulted in improvements in skeletal muscle oxygenation and performance during endurance exercise in healthy subjects (Bailey et al., 2015; Suzuki et al., 2015). These improvements are paralleled by increases in nitric oxide production, decreases in blood pressure and flow mediated vasodilation. However, improvements in exercise performance in Chapter 6 occurred in the absence of changes in $\dot{V}O_2$ kinetics, NO_2- plasma concentrations or muscle oxygenation, however there was an overall reduction in SBP post MC supplementation. Both Chapters 3, 4 and 5 highlighted than an increase in PCA and VA might, at least in part, be responsible for acute improvements in vascular function. In agreement with this theory, previous studies have reported that improvements in performance may be due to the ability of polyphenol flavonoids to exert a vasodilatory effect which may

promote blood flow to the working muscle (Pietta, 2000; Oh et al., 2010) However, these possibilities need to be verified in the future.

7.3 Clinical applications

Although the focus of this thesis was to investigate the efficacy of MC supplementation on various aspects of health and exercise performance in healthy participants, these studies have highlighted the potential clinical application of Montmorency tart cherries. Section 2.6 of the literature review discussed some of the previous work where MC supplementation was used in a more clinical setting i.e.. gout and sleep disturbance. Furthermore, it was highlighted in both section 2.5 and 2.6 that supplementation with MC significantly decreased markers of inflammation and oxidative stress and this in itself may have implications for the management of clinical pathologies associated with high levels of inflammation and oxidative stress and suggests that MC consumption may have the potential to reduce cardiovascular or chronic diseases in humans. In support of this, the results from all of the studies in this thesis demonstrate the potential cardio-protective benefits of Montmorency tart cherries. In Chapter 3, cell migration of vascular smooth muscle cell was increased post MC supplementation. Migration of de-differentiated VSMC is required for vessel remodelling which occurs from exercise but also following vascular injury which may be of clinical relevance.

Additionally, in Chapter 4, 5 and 6, it was reported that MC supplementation significantly lowered systolic blood pressure in (1) males with early hypertension (2) middle aged adults (3) trained cyclists, suggesting application to those with diagnosed hypertension, a major risk factor of several cardiac diseases. Further to this, in Chapter 5 the consumption of a MC concentrate resulted in acute modulation of CBF parameters in the frontal cortex during task performance as indicated by the elevated concentrations of total-Hb, with an identical pattern observed with oxy-Hb. Compromised cerebral blood flow has been suggested as a key contributor to cognitive function decline. Therefore, the application of tart cherries in groups who experience natural decrements in CBF and oxygenation would be worthwhile i.e.. cohorts experiencing natural age-associated reductions in CBF and/or cognitive function and. neurodegenerative or stroke patients. Naturally, cardiovascular variables are inextricably linked to cerebral blood flow and metabolism in the brain, and therefore, they covary with the incidence of age-

related cognitive decline, dementia, and mood disorders. Notably, polyphenol-related increases in vasodilation, cerebral blood flow, and NO synthesis are also implicated in hippocampal angiogenesis and neurogenesis, processes that are implicated in learning, memory, and neuroprotection. Lastly, the findings from Chapter 6 extend the application of MC supplementation to wider group of individuals, specifically those individuals with impaired physical mobility due to cardiovascular and cardiorespiratory impairment.

7.4 Limitations of findings

A number of limitations exist in the interpretation of the findings from this thesis which have been discussed in each chapter. The controls put in place allow for targeted analyses, however in most instances, this is at the expense of a degree of ecological validity. The following over-arching limitations are potential issues and criticisms of the work.

The strict dietary restrictions imposed on participants throughout the thesis are a particular limitation and as a result, findings should be interpreted with a certain degree of caution. In order to examine the true efficacy of tart cherry supplementation on various aspects of cardiovascular function and exercise performance, it was important that participants were restricted from consuming any other foods rich in polyphenols that might affect results. This approach allowed for greater confidence when interpreting the influence of tart cherries on various health and exercise parameters. However, it is extremely unlikely that one would consume a diet that is free from polyphenol-rich foods and as a result, future work should attempt to demonstrate synergistic effects of MC supplementation within habitual dietary practices. In Chapters 3, 4 and 5, dietary restrictions were imposed 48 h prior to the visit; however this was shortened to just 24 h in the final experimental chapter. The reason for this was twofold, the population in Chapter 6, well trained cyclists had five visits to the laboratory (twice as many as other studies), in addition they also found the diet far more difficult to maintain. Secondly, and more importantly, it became apparent in Chapter 3 and 4 that the compounds of interest were either absorbed or excreted 5 h post consumption. In order to take into account enterohepatic metabolism which is not limited to a few hours, a 24 h restriction was imposed.

An acknowledged limitation of the current study is that the analysis was not exhaustive and so not every polyphenol was analysed; instead, the focus was on the degradation products of two of the main anthocyanins reported in the MC juice (Ou et al., 2012; Kirakosyan et al., 2009) that could exert a positive effect on vascular function. In addition, we did not investigate compounds with poor bioavailability due to their instability, large molecular weight or quick excretion. Conceivably, these compounds might also contribute to any potential physiological effects exerted by MC and cannot be excluded. Furthermore, enterohepatic metabolism could predict that the absorption of polyphenols and their metabolites are not limited to just a few hours after intake (Seymour et al., 2014; de Ferrars et al., 2014). As a result, the timeframe of all the studies (≤ 8 hours) might not have been long enough to fully capture the entire absorption profile of the selected phenolic acids.

As previously described in section 2.3.2, there are several factors that affect the polyphenol content of foods. The batch-to-batch variation in MC supplement is a further limitation of this work. Unfortunately due to the resource available, it was not possible to analyse each batch for individual polyphenol or polyphenol content. However in an attempt to counteract this, total anthocyanins, total phenolic content and Trolox equivalent AOX capacity was identified for all studies.

7.5 Future research directions

The aims of this thesis have been addressed in the 4 experimental Chapters, and more importantly have contributed to the existing literature. The data from this thesis has provided an insight into the application of Montmorency tart cherry supplementation in both health and exercise settings. Consequently, a number of potential avenues for future research have been identified.

Chapters 3 and 4 of this thesis have previously established the time course at which some polyphenolic compounds are most bioavailable (peak absorption) following acute doses of tart cherry juice. Following chronic consumption of MC, it makes the expectation tenable that continued or elevated bioavailability could increase a number of health parameters, particularly in relation to markers of vascular reactivity (Jin et al., 2011) and consequently a more pronounced effect on blood flow and vasodilation following a longer period of consumption.

If continuing with samples from young, healthy participants, future studies may want to bear in mind the difficulty in observing vascular improvements and cognitive enhancement in this population. These participants are likely to have good cognition (Ronnlund et al., 2005); as a result any treatment-related improvements would undoubtedly be small. More recently, there has been emphasis on the use of functional foods and cherries to improve the prognosis in clinical populations such as those with cardiovascular disease and neurological disorders.

Foods such as Montmorency cherries contain hundreds of polyphenolic compounds that give rise to a myriad of different metabolites present in biological samples in a wide range of concentrations. Until quite recently, this complexity has meant that it was virtually impossible to identify how supplements influence metabolism and how these changes can be related to health effects. However, through the developments in technology this is changing and there have been numerous reports describing the use of 'omics' technology to investigate aspects of human nutrition (Primrose et al., 2011). As a result, we can comprehensively analyse the whole metabolome (all metabolites synthesized by an organism) (Fiehn, 2002) and focus on the measurements of metabolite concentrations and secretions in cells and tissues. This will allow for the identification of small molecules that make the difference between the effects of different diets and, in so doing, deepen our knowledge of human health and the interacting and regulatory roles of nutrition.

Furthermore, it appears different compounds get metabolised and absorbed at different time points in the colon. The enterohepatic circulation of colonic metabolites requires further investigation and hepatic metabolism of colonic metabolites of plant phenolic compounds should be addressed in the future. Additionally, one of the major gaps in the literature at present, is the lack of knowledge concerning hepatic conversion of small colon-derived phenolic acids, which may be missed in analyses of human body fluids or cell lines due to their hydrophilic nature. Finally, the influence of consuming a mixed diet i.e.. "real" complex meals, on bio-accessibility absorption is poorly understood. Future work should attempt to investigate the potential synergistic effects of MC supplementation within habitual dietary practices.

In conclusion, the series of investigations in this thesis have provided the first evidence that a commercially available Montmorency tart cherry concentrate is rich in polyphenols and has potential to modulate aspects of vascular function and improve exercise performance. These findings are of importance as they highlight a natural, alternative solution to pharmacological interventions in managing blood pressure and enhancing performance in diverse populations. As Wersching (2011) points out, the direct interplay between nutrients in whole Montmorency tart cherries may be more important than the unique nutrients on their own in the reduction of risk in cardiovascular disease and performance improvement. With that said, it is critically important that future work focus on the bioavailability and kinetics of absorption with the goal to find the combinatorial mix that acts synergistically upon the various targets, to then as a whole reduce the economic burden of cardiovascular disease and to promote healthy aging.

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9 Appendices

8.1 Appendix 1: Example informed consent document

INFORMED CONSENT FORM

Project title:

Participant ID:

Principal Investigator: Karen Keane

Investigator contact details: karen.keane@northumbria.ac.uk

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 FOR REMOVAL AND STORAGE OF TISSUE

Project Title:

Participant ID:

Principal Investigator: Karen Keane

Investigator contact details: karen.keane@northumbria.ac.uk

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

Tissue/Bodily material	**Purpose**	**Removal Method**

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.....……..

8.2 Appendix 2: Example Health Questionnaire Document (Ch 3, 5 and 6)

Health Questionnaire

STRICTLY CONFIDENTIAL

Participant Code:

Height:

Weight:

Please answer these questions truthfully and completely. The sole purpose of this questionnaire is to ensure that you are fit and healthy to follow the proposed research programme.

1. How would you describe your present level of activity?

Vigorous activity:

Less than 1x per month	Once a month
Once a week	Two/three times a week
Four/five times a week	More than five times a week

2. Are you currently taking any form of medication? If yes, please give brief details.

Yes No

3. Have you suffered from a bacterial or viral infection in the last two weeks?

Yes No

4. Have you had cause to suspend physical activity in the last two weeks for any reason?

Yes No

5. If you currently suffer from or have previously suffered from any of the following conditions you will be unable to take part in the study. Please inform the researcher (without specifics) if as a result you will now be unable to take part in the study.
   - heart complaint/condition
   - asthma
   - diabetes (Type 1 or 2)
   - high blood pressure
   - blood borne disease or infection

6. Is there any reason why you should not embark on the proposed research programme?

You are not required to provide specifics of any condition that precludes you from taking part in the study. However, if you are not sure if any such condition will affect your ability to participate, please feel free to discuss this with the research team, although you are not obliged to do so.

Signature:

Date:

Sports Scientist:

8.3 Appendix 3: Example Health Questionnaire Document (Ch 4)

Health Questionnaire

STRICTLY CONFIDENTIAL

Participant Code:

Height:

Weight:

Please answer these questions truthfully and completely. The sole purpose of this questionnaire is to ensure that you are fit and healthy to follow the proposed research programme.

7. How would you describe your present level of activity?

Vigorous activity:

Less than 1x per month	Once a month
Once a week	Two/three times a week
Four/five times a week	More than five times a week

8. Are you currently taking any form of medication? If yes, please give brief details.

Yes No

9. Have you suffered from a bacterial or viral infection in the last two weeks?

Yes No

10. Have you had cause to suspend physical activity in the last two weeks for any reason?

Yes No

11. If you currently suffer from or have previously suffered from any of the following conditions you will be unable to take part in the study. Please inform the researcher (without specifics) if as a result you will now be unable to take part in the study.
    - heart complaint/condition
    - asthma
    - diabetes (Type 1 or 2)
    - blood borne disease or infection

12. Is there any reason why you should not embark on the proposed research programme?

You are not required to provide specifics of any condition that precludes you from taking part in the study. However, if you are not sure if any such condition will affect your ability to participate, please feel free to discuss this with the research team, although you are not obliged to do so.

Signature:

Date:

Sports Scientist:

8.4 Appendix 4: Example Diet Record and Instructions Document

CONFIDENTIAL

Participant Code: _____

FOOD RECORD DIARY

Please record everything you eat and drink for **1/2 days (depending on study) prior to your trial and throughout the trial (96 hours).** You will then be asked to consume identical amounts of the same food and drink prior to and during the second trial. **Please avoid consuming the foods listed at the back of this document throughout the 1/2 days prior to and during the trial periods. This is vitally important to the results of the study so please adhere to this strictly.** Instructions and examples are given inside.

Information about your diet will be treated in confidence.

If you have problem, please contact: Karen (Karen.keane@northumbria.ac.uk)

Department of Sport Exercise and Rehabilitation

Faculty of Health and Life Sciences

Northumbria University

Newcastle Upon Tyne

NE1 8ST

INSTRUCTION FOR USING THE FOOD DIARY

- Everything that you eat and drink over the course of the day should be recorded.
- Do not forget to record second helpings and between meal snacks.
- Eating Out – Most people eat foods away from home each day, please do not forget to record these. Take your diary with you where ever it is possible. If this is too inconvenient just record the type of food eaten.
- Names and descriptions of foods should be as detailed as possible, including the brand name and any other information available.

e.g. Cheese – is insufficient information.

Cheese, cheddar (Shape reduced fat) – is sufficient information.

Start a new page in your diary for each day, and record each item on a separate line. Record the time of day in the first column of each line.

e.g. 10:30 am McVities Digestive Biscuits (2) 50g

The space provided at the foot of each page for general comments is for you to give any further information about your diet and your training/activity for that day.

e.g. Steady run, morning 1 hour.

Missed lunch due to stomach pains

DAY 1 (96 hours pre-trial 1)

Date: / /

Please use a separate line for each item eaten; write in weight of Plate; leave a line between different 'Plate' entries.

A	B		C	D	E	F	Office Use
Time	Food eaten		Brand name of each item (except fresh food)	Full description of each item including: -whether fresh, frozen, dried, canned -cooked: boiled, grilled, fried, roasted. -what type of fat food fried in	Weight Served	Weight of Leftovers	Actual Weight
am/pm	home	away			(gms)	(gms)	(gms)

GENERAL COMMENTS and ACTIVITY UNDERTAKEN:

DAY 2 (72 hours pre-trial 1)

Date: / /

Please use a separate line for each item eaten; write in weight of Plate; leave a line between different 'Plate' entries.

A	B		C	D	E	F	**Office Use**
Time	Food eaten		Brand name of each item (except fresh food)	Full description of each item including: -whether fresh, frozen, dried, canned -cooked: boiled, grilled, fried, roasted. -what type of fat food fried in	Weight Served	Weight of Leftovers	Actual Weight
am/pm	home	away			(gms)	(gms)	(gms)

GENERAL COMMENTS and ACTIVITY UNDERTAKEN:

Dietary Restrictions

Please see below for foods that you can/cannot eat during the period 1/2 days prior to the experimental trial. It is vitally important that you strictly follow these restrictions in order to make sure any changes we see in your blood samples are directly as a result of the supplements you are provided with. The foods on the 'should NOT' eat list are those are high in polyphenols (AOXs), if you come across foods not listed there but you know are high in AOXs, please aV̈Oid these also.

Foods that should NOT be eaten during the diet

- Tea, coffee, drinking chocolate, alcohol (especially red wine and apple cider), fruit juice
- Fruits and Vegetables
- Chocolate and chocolate products
- Cereals / wholemeal bread / grains
- Spices (such as curry) and herbs

Foods that you may eat

- White bread
- Butter, vegetable oil (aV̈Oid olive oil)
- Pasta, rice
- Meat, eggs, fish
- Peeled potatoes (mash, crisps, French fries)
- Mushrooms
- Digestive biscuits
- Milk and milk products (Plain yogurts, cheese)

EXAMPLES OF DIET FOR THE DAYS PRIOR THE STUDY

Breakfast:

- Toast (white bread) with butter (**NO** jam)
- Glass of milk or Plain yogurt (or Greek yogurt but without Honey)
- Scrambled eggs
- Bacon and sausages (**NO** tomato sauce)
- Croissant, pastries (**NO** jam or chocolate coated)
- Waffles, pancakes
- Rice Krispies

Lunch:

- Sandwich (white bread, white bagels, white pitta bread, or panini) with any of the following fillings (**NO** lettuce, or tomatoes):
  - Chicken
  - Cheese
  - Bacon, Ham, Corned beef
  - Tuna
  - Prawns
  - Butter
  - Mayonnaise
- Sausage roll, pork pies
- Pack of crisps (Ready salted)
- Burger and chips

Dinner:

- Fish and chips with salt and vinegar (**NO** tomato sauce)
- Chicken with mushrooms and mash potatoes or chips (**NO** vegetable or peas)
- Chicken and fried rice / fried noodles with eggs, and oyster sauce (**NO** peas and **NO** soya sauce)
- Macaroni and cheese (cream cheese sauce)
- Tuna, salmon, cod, with rice or pasta (**NO** tomato-based or vegetable-based sauce)
- Steak, pork chops
- Spaghetti carbonara (cream cheese sauce, bacon)

Snacks:

- Digestive biscuits (**NO** chocolate coated)
- Short breads
- Custard rice pudding
- Crisps (Ready salted)
- Ice cream (vanilla flaV̇Our)
- Vanilla flaV̇Our milk-shake
- Plain scones with butter, clotted cream

You can drink water at any time.

WHAT YOU MUST NOT EAT OR DRINK

- Coffee
- Tea
- Alcohol (especially red/white wine, apple cider, beer)
- Fruits (especially apples, pears, apricots, prunes, plums, cherries, peaches, blueberries, grapes)
- Vegetables (especially tomatoes, spinach, asparagus, cabbage, carrots, peppers, onions, broccoli, cauliflower)
- Fruits juices (especially Ribena), soft drinks (coca cola, irn bru…)
- Chocolate / chocolate products (biscuits, cakes, chocolate bars) / drinking chocolate
- Wholemeal bread, granary bread, brown bread
- Wholegrain cereals (such as Weetabix, bran flakes, Corn flakes, oat cakes, porridge, Muesli)
- Wholemeal food (such as wholemeal pasta, wholemeal rice…)
- Beans (such as baked beans, green beans), lentils, peas
- Curry dishes, curry spice, chilli, paprika
- Herbs (such as basil, parsley, coriander, chives…)
- Tomato sauce (such as Ketchup, ready-made pasta sauce in jar…)

We are aware that this may be difficult for you to follow such a diet, as it may be really different from your usual diet. The reason we ask you to change your normal diet is that the foods listed above contain similar components that we want to be testing in the fruit juice. Therefore, if you do eat the foods above, this may interfere with the study.

We recommend that you eat plenty of pasta or rice the night before the any of the trial days, to avoid being too hungry in the morning of the study and provide a good energy source.

Thank you for your co-operation.

8.5 Appendix 5: Time course of systolic blood pressure in Chapter 4

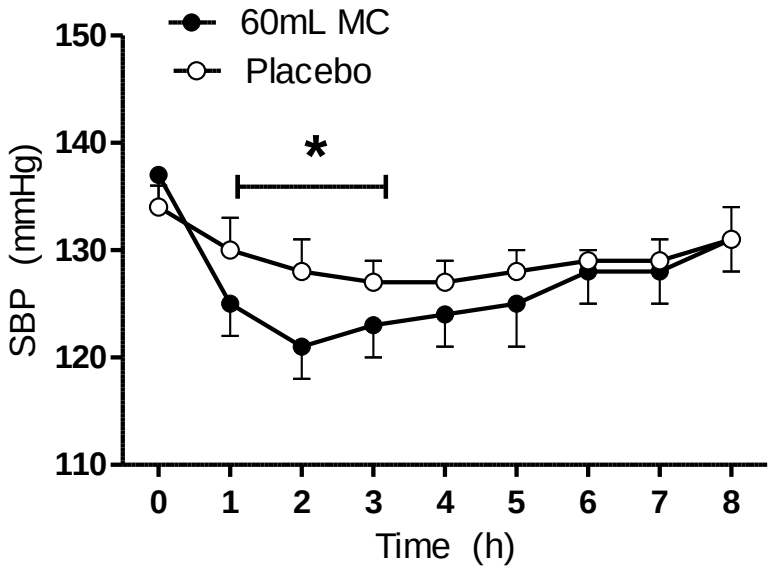

Figure 23 - Time course of systolic blood pressure (mean ± SEM) response after consumption of MC concentrate- and macronutrient – matched control (n=15). Significantly different from the placebo drink: * $P < 0.05$

8.6 Appendix 6: Baseline NIRS data collected in Chapter 5

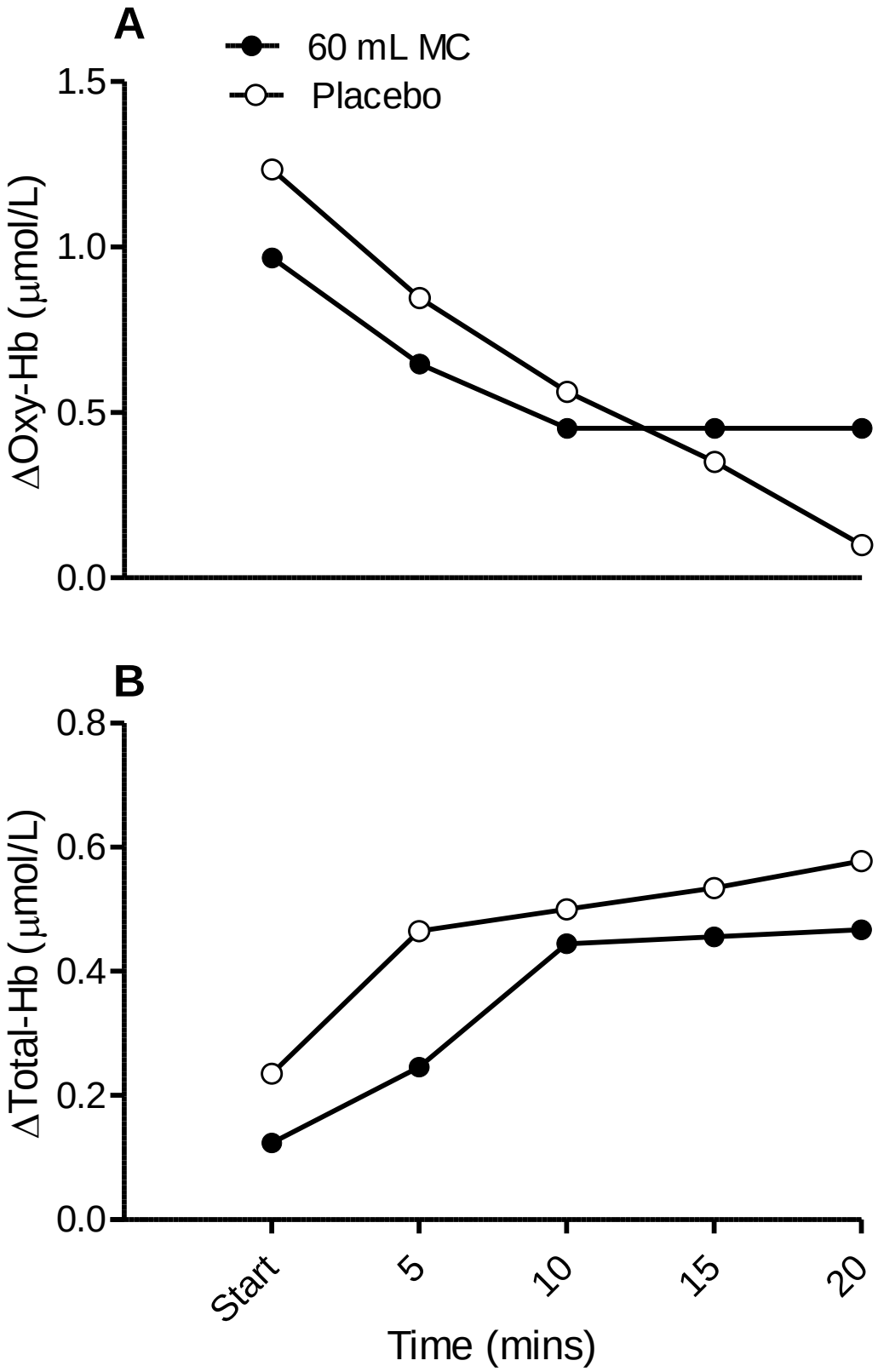

Figure 24 Baseline data for oxy-Hb and total-Hb, by treatment visit. Mean values over the course of the baseline assessment for oxy-Hb are 0.59 µmol/L (MC) and 0.62 µmol/L (Placebo). Mean values for total-Hb are 0.35 µmol/L (MC) and 0.46 µmol/L (Placebo).