Prevention Is Better Than Cure:

Cardio-Metabolic Responses to Montmorency Tart Cherry Supplementation With and Without Exercise

Terun Desai

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Abstract

Globally, cardio-metabolic diseases and their associated complications are the leading cause of mortality and morbidity, and also the single largest contributor to health care expenditure (World Health Organisation, 2017). Hitherto, much effort has been placed on attempting to cure rather than prevent these diseases; hence rates are projected to increase over the next 25 years (Cho *et al.*, 2018). Consequently, a change in approach towards prevention would be more effective at minimising the burden of these diseases. Hence, this thesis focussed on implementing lifestyle interventions, Montmorency tart cherries (MTC) (*Prunus cerasus* L.) and FATMAX exercise, to improve biomarkers associated with Metabolic Syndrome (MetS), the precursor to developing cardiovascular disease and type II diabetes (Wilson *et al.*, 2005).

Tart cherries are a dietary source rich in many polyphenolic compounds, particularly anthocyanins, which have been increasingly investigated for their ability to optimise health over the past 20 years (Wang, 1998; Seeram *et al.*, 2001; Bell *et al.*, 2014a; Kelley, Adkins and Laugero, 2018). *In vitro* (Wang, 1998; Seeram *et al.*, 2001; Keane *et al.*, 2016a) and animal (Seymour *et al.*, 2008, 2009) models have provided strong evidence for the anti-oxidative, anti-inflammatory, anti-diabetic and cardio-protective properties of tart cherries. Inconsistent findings from human trials limit the conclusions that can be drawn, however positive results relating to improved cardio-metabolic function (Ataie-Jafari *et al.*, 2008; Martin *et al.*, 2010; Keane *et al.*, 2016b; Keane *et al.*, 2016c; Chai *et al.*, 2018) and recovery from exercise (Howatson *et al.*, 2010; Bell *et al.*, 2014d) have been reported. These cardio-metabolic responses were observed in various human populations, but never in a MetS cohort, and mechanisms of observed effects have not been delineated. Consequently, the overarching aim of this thesis was to establish whether MTC supplementation with and without FATMAX exercise could improve biomarkers associated with cardio-metabolic health; and delineate potential mechanisms of action.

The results from this thesis have corroborated previous literature but also identified novel and clinically relevant findings. The first study **(Chapter 4)**, examined health responses to a combination of MTC juice supplementation with exercise for the first time. However, no significant improvements on cardio-metabolic biomarkers with MTC consumption were observed in healthy humans. Additionally, based on hormesis, **chapter 4** also suggested short-term MTC supplementation may be most effective. The next investigation **(Chapter 5)** was the first to assess cardio-metabolic responses to MTC consumption, in humans with MetS. Results indicated acute supplementation of MTC capsules and juice reduced insulin concentrations; demonstrating a health benefit of MTC capsules for the first time in any human population. However, responses between MTC juice and capsules were not different. Corroborating previous research, MTC juice reduced systolic blood pressure acutely, by a clinically relevant margin. Similarly, and arguably the most clinically important finding of this thesis was reported in **chapter 6**, as 7 days MTC juice consumption significantly reduced 24-hour ambulatory blood pressure in a population of individuals with MetS. Hence, these findings indicate MTC juice may be used as an anti-hypertensive intervention. Moreover, **chapter 6** revealed for the first time an MTC intervention improved fasting glucose, total cholesterol, TC:HDL ratio and lowered resting RER in any human population, after 6 days of MTC juice supplementation compared to a control group. The last experimental study attempted to elucidate the mechanistic pathways for observed responses with MTC **(Chapter 7)**. Findings demonstrated dilutions of MTC concentrate extended lifespan in *Caenorhabditis elegans*; and identified MTC may act as a calorie restriction mimetic via the PPAR signalling pathway.

Together these observations promote the integration of MTC, as a safe, pragmatic, naturally-occurring dietary intervention, into habitual consumption for the prevention and amelioration of cardio-metabolic dysfunction. Substantiation of these results from future, well-designed clinical trials is necessitated to support the implementation of MTC in practice.

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Acknowledgements

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I've come to the end of my PhD rollercoaster ride but have shared it with a small, tight-knit, bonkers yet brilliant group of PhD students; each of which are at varying points along that ride. As the first to finish of those to have passed through C100, I'm happy to report that the end will come…one day! I will miss the many antics that we have shared from working late-nights and having to be kicked out by the campus security guards to road trips and playing table tennis in the lab. I'm lucky to now be friends with a group of people who are always clowning-around and up for a laugh, because without that we would all have gone crazy.

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Now, there is a great sense of déjà vu writing this part of the acknowledgements section. It only seems like yesterday that I was acknowledging Dr. Lindsay Bottoms as my undergraduate supervisor, yet incredibly almost a decade has passed since our paths crossed. Hence, I regard you more as a friend than a supervisor. This thesis is a far cry from thermoregulation, cooling and table tennis; but that is testament to how well we work together. Who knows what we will research in the future…

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Lastly, contrary to popular belief with PhD students, I can wholeheartedly say that I have thoroughly enjoyed my PhD experience from start to finish and those mentioned above have made that possible; Thank You!

Contributions

The following table outlines significant contributions from other individuals in the studies included within this thesis.

I would like to sincerely thank Dr. Samantha Hughes and colleagues at HAN BioCentre, HAN University of Applied Sciences, Nijmegen, The Netherlands for their contribution to some of the work conducted in this thesis.

Section	Contributors	Description
Chapter 7	• Terun Desai (TD) and others at University of Hertfordshire • Samantha Hughes (SH) and others at HAN University of Applied Sciences	Chapter 7 details an investigation into: Using *Caenorhabditis elegans* to reveal the molecular basis of the health benefits from Montmorency tart cherry (*Prunus cerasus* L.). This work was conducted in collaboration with colleagues at HAN BioCentre, HAN University of Applied Sciences, Nijmegen, The Netherlands. Conceptualisation; TD (60%) and SH (40%). Resources; TD (5%) and SH (95%). Methodology; TD (10%) and SH (90%). Formal Analysis; TD (15%) and SH (85%). Writing; TD (35%) and SH (65%).

Publications

Peer Reviewed Publications

Desai, T., Roberts, M. and Bottoms, L. (2019). Effects of Montmorency tart cherry supplementation on cardio-metabolic markers in metabolic syndrome participants: A pilot study. *Journal of Functional Foods, 57,* pp. 286-298. DOI:10.1016/j.jff.2019.04.005

Desai, T., Bottoms, L. and Roberts, M. (2018). The effects of Montmorency tart cherry juice supplementation and FATMAX exercise on fat oxidation rates and cardio-metabolic markers in healthy humans. *European Journal of Applied Physiology, 118* (12), pp. 2523-2539. DOI:10.1007/s00421-018-3978-9

Conference Communications

Desai, T., Bottoms, L. and Roberts, M. Acute cardio-metabolic responses to different forms of Montmorency tart cherry supplementation in humans with Metabolic Syndrome: a pilot study. International Conference on Polyphenols. Madison, Wisconsin, USA, 16-20 July 2018.

Desai, T., Bottoms, L. and Roberts, M. The effects of Montmorency tart cherry juice supplementation on fat oxidation during FATMAX exercise and cardio-metabolic markers at rest. European College of Sports Science Conference. MetropolisRuhr, Essen, Germany, 5-8 July 2017.

Desai, T., Bottoms, L. and Roberts, M. The effects of Montmorency tart cherry juice supplementation and FATMAX exercise on cardio-metabolic markers in healthy humans. World Congress on Polyphenol Applications. University of Vienna, Vienna, Austria, 20-21 June 2017.

Desai, T., Bottoms, L. and Roberts, M. The effects of tart cherry juice supplementation on fat oxidation, body composition and cardio-metabolic markers with cycling exercise. BASES Annual Student Conference. Bangor University, Bangor, UK, 23 March 2016.

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Abbreviations

ABPM	Ambulatory Blood Pressure Monitor
ACE	Angiotensin-I-converting Enzyme
AIx	Augmentation Index
AIx at HR75	Augmentation Index at Heart Rate 75 bpm
AMPK	Adenosine Monophosphate Kinase
ANOVA	Analysis of Variance
AP	Augmentation Pressure
ATP	Adenosine Triphosphate
AU	Arbitrary Units
AUC	Area Under Curve
BMI	Body Mass Index
BP	Blood Pressure
CHD	Coronary Heart Disease
CHO	Carbohydrate
CI	Confidence Interval
CO	Cardiac Output
CONSORT	Consolidated Standards of Reporting Trials
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
CR	Calorie Restriction
CRM	Calorie Restriction Mimetic
CRP	C-reactive Protein
CV	Coefficient of Variation
CVD	Cardiovascular Disease
DBP	Diastolic Blood Pressure
DNA	Deoxyribonucleic Acid
DOMS	Delayed Onset Muscle Soreness
dsRNA	Double-Stranded Ribonucleic Acid

EE	Energy Expenditure
EGCG	Epigallocatechin-3-gallate
EIMD	Exercise Induced Muscle Damage
ELISA	Enzyme Linked Immunosorbent Assay
eNOS	Endothelial Nitric Oxide Synthase
EN-RAGE	Extracellular Newly Identified Receptor for Advanced Glycation End-Products Binding Protein
FATMAX	Intensity of MFO conveyed as $\%\dot{V}O_2max$
FATMIN	Regions of Low or Slight Fat Oxidation Rates
FFA	Free Fatty Acid
FFM	Fat Free Mass
FMD	Flow-mediated Dilatation
FOXO1	Forkhead Box Protein O1
GAE	Gallic Acid Equivalents
GLUT	Glucose Transporter
GPx	Glutathione Peroxidase
GTE	Green Tea Extract
HbA_{1c}	Glycated Haemoglobin
HDL	High-density Lipoprotein
HOMA/HOMA2	Homeostatic Model Assessment
HOMA2-%S	Homeostatic Model Assessment for Insulin Sensitivity
HOMA2-IR	Homeostatic Model Assessment for Insulin Resistance
HOMA2-β	Homeostatic Model Assessment for pancreatic β-cell function
HPMC	Hydroxypropyl Methylcellulose
HR	Heart Rate
HR_{max}	Maximal Heart Rate
HRP	Horseradish Peroxidase
hsCRP	High-Sensitivity C-reactive Protein
ICC	Intraclass Correlation Coefficient
IGF-1	Insulin-like Growth Factor-1

IL	Interleukin
ILS	Insulin-like Signalling
LDL	Low-density Lipoprotein
LoA	Limits of Agreement
LOX	Lipoxygenase
MAP	Mean Arterial Pressure
MAPK	Mitogen-activated Protein Kinase
MetS	Metabolic Syndrome
MFO	Maximal Fat Oxidation Rate
MTC	Montmorency Tart Cherry/Cherries
MTCC	Montmorency Tart Cherry Capsules
MTCJ	Montmorency Tart Cherry Juice
MTCJ60	Montmorency Tart Cherry Juice (60 mL Concentrate)
NF-κB	Nuclear Factor kappa-light-chain-enhancer of Activated B Cells
NGM	Nematode Growth Media
NHANES	National Health and Nutrition Examination Survey
NO	Nitric Oxide
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
NZBE	New Zealand Blackcurrant Extract
NZBP	New Zealand Blackcurrant Powder
ORAC	Oxygen Radical Absorbance Capacity
ox-LDL	Oxidised LDL
PAI-1	Plasminogen Activator Inhibitor-1
PCA	Protocatechuic Acid
PGC-1α	Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-Alpha
PLA	Placebo
PP	Pulse Pressure
PPAR	Peroxisome Proliferator-Activated Receptor
PPARα	Peroxisome Proliferator-Activated Receptor Alpha

PPARγ	Peroxisome Proliferator-Activated Receptor Gamma
PVC	Plasma Volume Change
PWA	Pulse Wave Analysis
PWV	Pulse Wave Velocity
RCT	Randomised Control Trial
RER	Respiratory Exchange Ratio
RM-ANOVA	Repeated-Measures Analysis of Variance
RMR	Resting Metabolic Rate
RNAi	RNA Interference
ROS	Reactive Oxygen Species
RPE	Rating of Perceived Exertion
RQ	Respiratory Quotient
SBP	Systolic Blood Pressure
SD	Standard Deviation
SEVR	Subendocardial Viability Ratio
SGLT-1	Sodium-dependent Glucose Cotransporter-1
SIRT-1	Sirtuin-1
SM	Skeletal Muscle
SOD	Superoxide Dismutase
SPSS	Statistical Package for the Social Sciences
SREBP-1c	Sterol Regulatory Element-Binding Protein-1
SV	Stroke Volume
sVCAM-1	Soluble Vascular Cell Adhesion Molecule-1
T2D	Type II Diabetes Mellitus
TAS	Total Anti-oxidant Status
TC	Total Cholesterol
TC:HDL	Total Cholesterol to HDL Ratio
TG	Triglycerides
TMB	Tetramethylbenzidine
TNF-α	Tumour Necrosis Factor Alpha

TPR	Total Peripheral Resistance
URTI	Upper Respiratory Tract Infection
VA	Vanillic Acid
$\dot{V}CO_2$	Volume of Carbon Dioxide Production
VLDL	Very Low-density Lipoprotein
$\dot{V}O_2$	Volume of Oxygen Uptake
$\dot{V}O_{2max}$	Volume of Maximal Uptake
$\dot{V}O_{2peak}$	Peak Volume of Oxygen Uptake
VSM	Vascular Smooth Muscle
W	Watts
W_{max}	Maximal Wattage
XO	Xanthine Oxidase

Chapter 1

Introduction

Chapter 1. Introduction

Prevention is better than cure, especially when there is no cure! Founded on this ideology, the research conducted as part of this thesis set out to use lifestyle interventions to prevent the development of cardio-metabolic diseases.

Cardio-metabolic disease encompasses cardiovascular diseases (CVD), type II diabetes mellitus (T2D) and chronic kidney disease which are associated with modifiable risk factors including excess adiposity, hypertension, hypercholesterolaemia and hyperglycaemia (Danaei *et al.*, 2014). Cardio-metabolic disease has become a major, global public health concern (Kraushaar and Krämer, 2009) as its constituent diseases are the leading cause of mortality and morbidity worldwide, and is also the single largest contributor to health care expenditure (World Health Organisation, 2017). Globally in 2015, there was an estimated 422.7 million prevalent cases of CVD and 17.92 million deaths (Roth *et al.*, 2017), representing 31% of all deaths (World Health Organisation, 2017). Moreover, global prevalence of diabetes amongst individuals aged 18-99 years was 8.4% (451 million) as of 2017 and is expected to increase to 9.9% (693 million) by 2045; suggesting a concomitant increase in CVD related deaths and placing evermore pressure on healthcare systems (Cho *et al.*, 2018). These figures starkly highlight the magnitude and urgent need to reverse this pandemic.

Metabolic Syndrome (MetS) encompasses a cluster of different cardio-metabolic criteria (abdominal obesity, pre-hyperglycaemia, pre-hypertension and atherogenic dyslipidaemia), and has become of increased interest in clinical and academic settings (O'Neill and O'Driscoll, 2015). MetS is considered to be the precursor to CVD and T2D and confers a 2- and 5-fold greater risk of developing these diseases, respectively (Alberti *et al.*, 2009). Approximately, a quarter of the global population are thought to have MetS, equating to 1.75 billion people (Saklayen, 2018). This thesis targeted individuals with MetS, yet to be diagnosed with a cardio-metabolic disease, to provoke a meaningful contribution towards lessening the burden of cardio-metabolic dysfunction on a global and individual scale. This was primarily achieved

through focussing on reducing incidence rates of cardio-metabolic disease and therefore curbing the physical and financial resources spent on treating disease.

Cardio-metabolic disease is commonly caused by an unhealthy lifestyle characterised by high levels of sedentary behaviour and disproportionately higher caloric intake relative to expenditure (Kaur, 2014). Strikingly, 80% of diagnosed cardio-metabolic diseases are preventable through improving lifestyle (Piepoli *et al.*, 2017). Since CVD and T2D are closely interrelated, a multifactorial approach to prevent complications is recommended through diet, exercise, aggressive blood pressure control and treatment of dyslipidaemia (Einarson *et al.*, 2018).

The efficacy of exercise and improving habitual dietary practice on the risk of developing cardio-metabolic disease is remarkable, such that medical associations recommend these as primary tools for the prevention of CVD and T2D (Sigal *et al.*, 2004; Rosenzweig *et al.*, 2008). Yamaoka and Tango (2012) reported either dietary intervention or a combination of dietary and exercise interventions resolved MetS in twice as many people compared to a control group. Subsequently, this provides rationale for the use of dietary and exercise interventions, in the present thesis, as a two-pronged approach to elicit cardio-metabolic health improvements.

Modest daily improvements in health from food consumed throughout a lifetime is more noteworthy and safer than ‘higher risk’ pharmacological drugs consumed in the short-term to treat specific conditions (Chiva-Blanch and Visioli, 2012). Diets rich in fruits, vegetables, legumes and whole grains provide protection from cardio-metabolic dysregulation typical of MetS (Pitsavos *et al.*, 2006; Kastorini *et al.*, 2011). These foodstuffs contain polyphenols, which are ubiquitous, naturally-occurring phytochemicals derived from plant-based foods (Manach *et al.*, 2004). Polyphenols have been heavily researched over the past 25 years to exploit their myriad of health-promoting properties, including anti-oxidant, anti-inflammatory, anti-hypertensive, anti-hyperglycaemic and lipid-lowering (Manach *et al.*, 2004); thus may

defend against CVD and diabetes development (Scalbert *et al.*, 2002; Stevenson and Hurst, 2007; Bahadoran, Mirmiran and Azizi, 2013). Polyphenols are divided into many different subclasses, of which anthocyanins are one (Manach *et al.*, 2004). Recently, anthocyanins have gained plentiful attention for their health-promoting properties, particularly due to their potent anti-inflammatory and anti-oxidant properties (He and Giusti, 2010). There is growing evidence that anthocyanins and anthocyanin-rich foods confer significant improvements to cardio-metabolic health (Pojer *et al.*, 2013; Wallace, Slavin and Frankenfeld, 2016; Fairlie-Jones *et al.*, 2017; Yang *et al.*, 2017).

A food source which has received substantial attention in clinical and exercise science fields over the past 10 years, are tart cherries (*Prunus cerasus* L.). Tart cherries have been shown to possess a high phytochemical content, including phenolic acids, catechins, proanthocyanidins, melatonin, quercetin, kaempferol and particularly anthocyanins (Seymour *et al.*, 2009; Ou *et al.*, 2012). These phytochemicals are thought to contribute to the potent anti-oxidative and anti-inflammatory properties of tart cherries, which exceed that of common non-steroidal anti-inflammatory drugs such as ibuprofen (Wang *et al.*, 1999). Human studies have shown tart cherries to effectively combat oxidative stress (Traustadottir *et al.*, 2009; Bell *et al.*, 2014c; Bell *et al.*, 2016), inflammation (Howatson *et al.*, 2010; Martin *et al.*, 2011; Bell *et al.*, 2014bc) and promote post-exercise recovery (Howatson *et al.*, 2010; Bowtell *et al.*, 2011; Bell *et al.*, 2015; Levers *et al.*, 2016). The anti-inflammatory and anti-oxidative properties of tart cherries conveniently target the underlying chronic low-grade inflammation and pro-oxidant status that is central to MetS pathophysiology. Indeed, tart cherries were demonstrated to improve body composition and cardio-metabolic markers in rodents (Seymour *et al.*, 2008, 2009) and humans (Ataie-Jafari *et al.*, 2008; Martin *et al.*, 2010; Bell *et al.*, 2014b; Keane *et al.*, 2016; Chai *et al.*, 2018), suggesting this fruit may ameliorate symptoms associated with MetS. However, human trials with tart cherry interventions in individuals with MetS have yet to be conducted.

Despite prolonged interest in polyphenols, researchers have been unable to delineate the mechanisms of their health-promoting effects (Watson, Preedy and Zibadi, 2013). Improved cardio-metabolic health makes the expectation tenable that lifespan may also be extended (Heilbronn and Ravussin, 2003). Hence, this thesis attempted to delineate the mechanisms of action of observed effects with Montmorency tart cherries (MTC) and determine whether they act as a calorie restriction mimetic to extend lifespan in a *Caenorhabditis elegans* (*C. elegans*) model.

Lastly, some of the issues with current interventions include the lack of individualisation of interventions and limited knowledge of the cause and effect relationship between interventions and their overall impact on disease (Kraushaar and Krämer, 2009). Currently, reversal of the cardio-metabolic disease epidemic will not be achieved without more effective preventative interventions (Kraushaar and Krämer, 2009). Thus, identification of more efficacious interventions is required; this programme of research aimed to contribute to resolving this issue by examining MTC and individualised FATMAX exercise (the intensity of maximal fat oxidation conveyed as $\%\dot{V}O_2max$) as potential interventions with simple and feasible application to daily life. Presently, no research has been conducted in participants with MetS nor has MTC supplementation been combined with exercise in any population. Therefore, this thesis addressed these gaps and aimed to present data evaluating the use of these interventions as preventative measures against cardio-metabolic diseases, in the view to conduct future large-scale clinical trials.

Chapter 2

Literature Review

This literature review is partitioned into 3 parts. **Part 2.1** will review literature pertaining to functional foods and their health-promoting properties. Particular focus will be attributed to polyphenols, mainly anthocyanins as they are the most abundant polyphenol in tart cherries, and their metabolism in humans before reviewing literature on their effects on health. This section will end with a comprehensive review of research examining metabolism of cherry products and their effects in both exercise science and clinical nutrition domains. **Part 2.2** will review the epidemiology, pathophysiology and interventions used to combat MetS. As both areas encompass a wide range of sub-topics in relation to health, a detailed analysis of each is beyond the remit of this review. Subsequently, focus will be placed on cardio-metabolic health. **Part 2.3** will review literature relating to the use of the model organism, *Caenorhabditis elegans*, for human health, particularly cardio-metabolic. The literature explores potential mechanisms through which polyphenolic dietary interventions may extend lifespan and improve healthspan, thus propose mechanistic pathways for the action of MTC.

Part 2.1. Polyphenols and Health

2.1.1. Functional Foods and Nutraceuticals

"Let food be thy medicine and medicine be thy food". This belief was first embraced 2500 years ago by Hippocrates (Hasler, 2002). Since the development of modern drugs, this concept has been marginalised until relatively recently; and the role of diet in disease prevention and amelioration has now become tremendously topical (Hasler, 2002; Bultosa, 2015). 'Functional foods' is a term that was marketed by the food industry to describe the health benefits of food and therefore a universal definition has not been formulated (Hasler, Bloch and Thomson, 2004). However, the British Nutrition Foundation (2016) define functional foods as foodstuffs that deliver enhanced benefits over and above their basic nutritional value. Herein lies the benefit of functional foods, since all foods are 'functional', the ability of certain foodstuffs with added physiological and psychological benefits that prevent or ameliorate chronic disease

renders them extremely desirable (Hasler, 2002; Hasler, Bloch and Thomson, 2004; Bultosa, 2015). The health promoting effects of functional foods pertain mainly to growth and development, cardio-metabolic and gastrointestinal physiology, defence against oxidative stress, improved cognition and physical performance and fitness (Tur and Bibiloni, 2016).

Functional foods are whole foods occurring in their natural form or fortified/enhanced with bioactive components such as fish oils, vitamins and minerals (Hasler, Bloch and Thomson, 2004). In contrast, nutraceuticals refer to non-conventional foods with health promoting properties but occurring as an isolated or purified product, commonly consumed in capsule, tablet or liquid form (Hasler, Bloch and Thomson, 2004). The advantage of functional foods over nutraceuticals lie in the greater safety of consuming conventional whole foods, since nutraceuticals provide bioactive agents in dosages that far exceed those attainable from conventional whole foods (Hasler, 2002).

Research has reported positive results on the effect of both functional foods and nutraceuticals against chronic diseases including CVD (Sikand, Kris-Etherton and Boulos, 2015), diabetes mellitus (Sikand, Kris-Etherton and Boulos, 2015), cancer (Aghajanpour *et al.*, 2017), respiratory (Hwang and Ho, 2018), gastrointestinal (Salminen *et al.*, 1998; Cencic and Chingwaru, 2010) and cognitive diseases (Lang *et al.*, 2013).

Examples of functional foods which have demonstrated health benefits include beetroot (Clifford *et al.*, 2015), green tea (Cabrera, Artacho and Giménez, 2006), chocolate (Ackar *et al.*, 2013), red wine (grapes) (Yoo, Saliba and Prenzler, 2010), berries (Paredes-López *et al.*, 2010; Vendrame *et al.*, 2016) and cherries (Bell *et al.*, 2014a; Kelley, Adkins and Laugero, 2018). A commonality between these foods is their abundance of polyphenols. Polyphenols are a group of phytochemical compounds ubiquitously found in various plant-based dietary sources and the most abundant anti-oxidants in the human diet (Scalbert *et al.*, 2002). In the UK, average consumption of polyphenols (1521 $mg.day^{-1}$) is surprisingly higher than other

European regions such as the Mediterranean (1011 mg.day^{-1}) (Zamora-Ros *et al.*, 2016). Polyphenols are divided into many classes including hydroxybenzoic acids, hydroxycinnamic acids, flavonoids, stilbenes, and lignans, of which flavonoids have sub-classes including anthocyanidins, proanthocyanidins, flavonols, flavones, flavanols, flavanones and isoflavones (Manach *et al.*, 2005) (Figure 2.1). Although not considered a polyphenol, phenolic acids are increasingly being recognised for their health promoting attributes. Phenolic acids are secondary metabolites of parent polyphenols after they undergo metabolism (Scalbert *et al.*, 2002). They are significantly more bioavailable thus responsible for a greater contribution of the anti-oxidant potential than parent polyphenols (Manach *et al.*, 2005).

Polyphenols have traditionally been targeted for their powerful anti-oxidative properties, however the mechanism through which they exert their health effects extends beyond just their free radical scavenging ability (Chiva-Blanch and Visioli, 2012). Alternative mechanisms that polyphenols may operate via include activation of endogenous anti-oxidant and phase II detoxification enzymes, modulation of gut microbiota and activation of molecular and cellular signalling cascades regulating inflammation and cardio-metabolic function (Chiva-Blanch and Visioli, 2012).

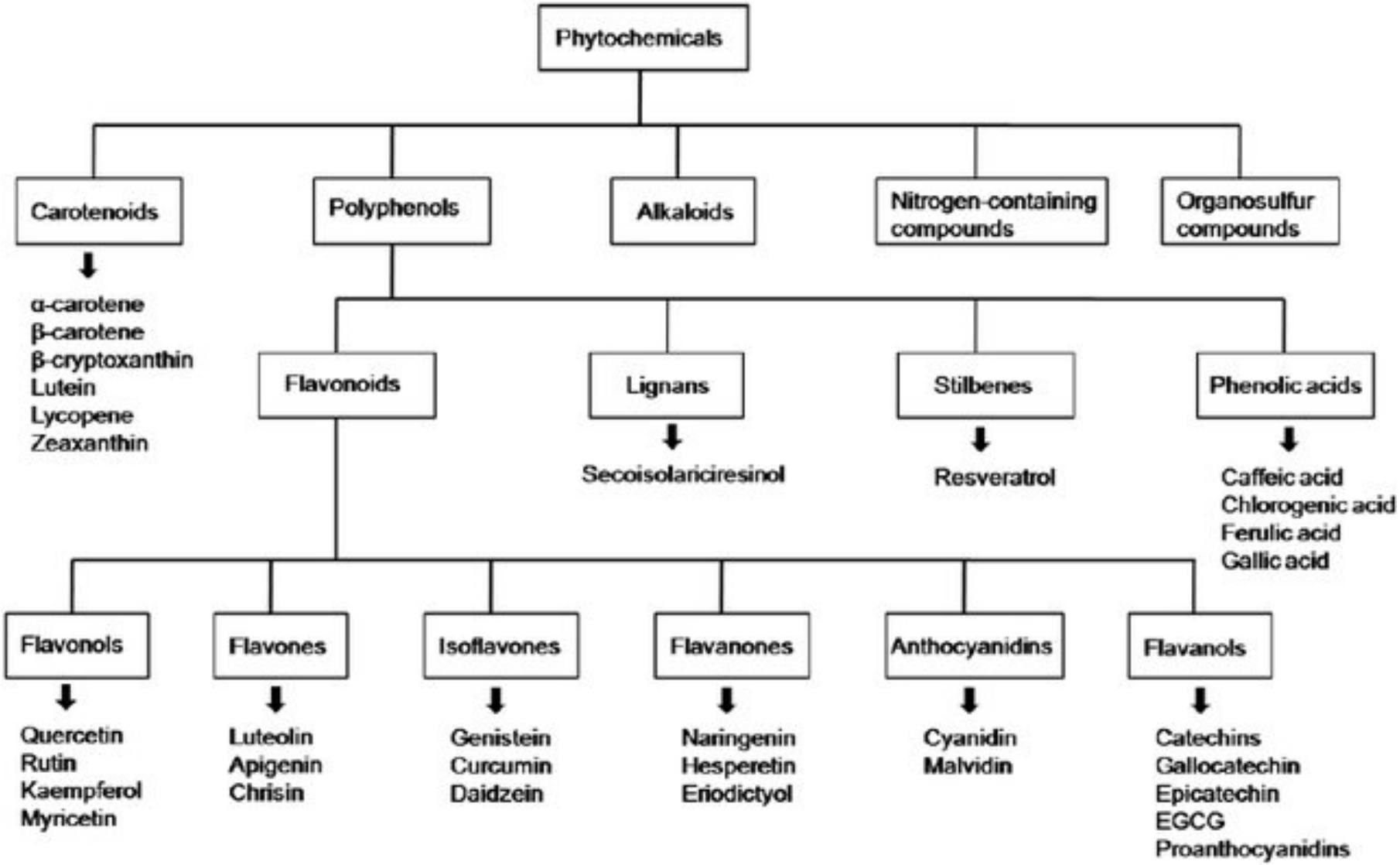


Figure 2.1. Classification of phytochemicals and the main natural compounds in each group (Ramos, Lima and Pereira-Wilson, 2011).

2.1.2. Polyphenol Structure

The chemical structure of phenolics is characterised by attachment of one or more hydroxyl groups to at least one aromatic ring (Zhong, 2011). Over 8000 phenolic compounds have been discovered, with many occurring naturally in plants (Strack, 2007). These compounds are concentrated in the skin and flesh of fruits and vegetables and are occasionally found in other plant parenchyma such as bark, leaves, seeds and roots (Zhong, 2011).

Flavonoids are a large group of C_{15} polyphenolic compounds arranged as two aromatic-rings (A and B) linked by a three-carbon bridge (C_6-C_3-C_6) that forms an oxygenated heterocycle (C ring) (Zhong, 2011). Flavonoid sub-classes are thus determined by the variation of the type of heterocycle (C ring) (Figure 2.2).

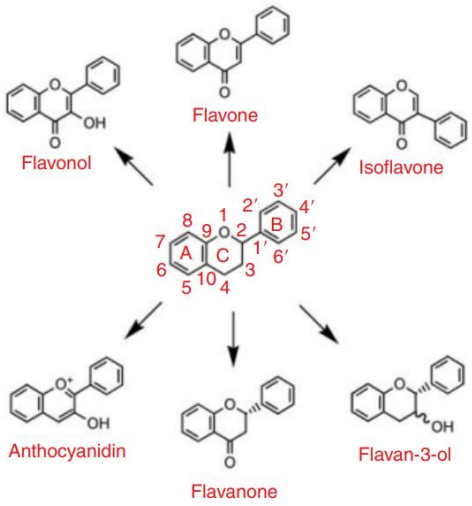
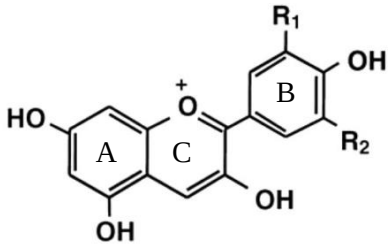


Figure 2.2. Generic chemical structure of major flavonoids.

Structural differences in relation to anthocyanidins, are responsible for determining variants of anthocyanidins (cyanidin, delphinidin, petunidin, peonidin, pelargonidin and malvidin) and consequently anthocyanins (Pojer *et al.*, 2013) (Figure 2.3). Sugar moieties attached to anthocyanidins include glucose, galactose, rhamnose, arabinose and xylose (Mazza and Miniati, 2018). Amongst aglycones, hydroxyl groups attached to the B-ring display blue/purple

pigments and while attachment of methoxyl groups present red pigmentation (He and Giusti, 2010).



R_1	R_2	Anthocyanidin
H	H	Pelargonidin
OH	H	Cyanidin
OCH_3	H	Peonidin
OH	OH	Delphinidin
OCH_3	OH	Petunidin
OCH_3	OCH_3	Malvidin

Figure 2.3. Generic anthocyanidin chemical structure.

2.1.3. Polyphenol Absorption, Distribution, Metabolism and Excretion

Polyphenol absorption is affected by molecular weight and the degree of glycosylation and esterification with heavier molecules (Scalbert *et al.*, 2002). Flavonoids, apart from catechins and proanthocyanidins, in whole foods are present in glycosylated forms, which the body is unable to absorb (Scalbert *et al.*, 2002). Thus, metabolism of flavonoids is initiated in the oral cavity by salivary enzymes and β-glucosidase derived from oral epithelial cells (Fernandes *et al.*, 2014). Furthermore, oral microbiota has been shown to enact phase II metabolism on anthocyanins, indicating the oral cavity as an important region of polyphenol metabolism (Larsen *et al.*, 2011).

Intestinal enzymes, lactase phlorizin hydrolase and β-glucosidase, facilitate the hydrolysis process in the gut to produce a polyphenolic aglycone and a glycoside (sugar) moiety; considered phase I metabolism (D'Archivio *et al.*, 2010) (Figure 2.4). However, anthocyanins are an exception in that the glycosylated moieties attached to the anthocyanidin can be absorbed and detected in systemic circulation (D'Archivio *et al.*, 2010). Absorption of anthocyanins has

been shown to occur from the stomach, small intestine and colon via active transporters, sodium-glucose cotransporter-1 (SGLT-1) and glucose transporter 2 (GLUT-2) (Faria *et al.*, 2009). In contrast to other flavonoids, anthocyanin absorption from the stomach via bilitranslocase is common and likely responsible for their rapid appearance in systemic circulation, between 6-20 minutes post-consumption (Pojer *et al.*, 2013). The acidic conditions in the stomach facilitates anthocyanin stability and thus augments bioavailability, however during intestinal transit, stability decreases since pH increases (McGhie and Walton, 2007).

After deglycosylation, the aglycones and glycoside moieties are absorbed across the gut lumen into epithelial cells via passive diffusion or epithelial transporters (Cassidy and Minihane, 2017). Once absorbed, metabolites undergo some phase II metabolism (conjugation reactions including, glucuronidation, methylation, sulphation and to a lesser extent acetylation) in the gut epithelium and are then transported to the liver via the portal vein where further phase II metabolism may occur; after which metabolites enter into systemic circulation for distribution to other tissues (Marín *et al.*, 2015) (Figure 2.4). Phenolics not absorbed in the stomach or small intestine, travel downstream to the colon where they undergo hydrolysis or deconjugation by colonic microbiota to further promote absorption (Marín *et al.*, 2015).

Efflux of aglycones, phenolic acids and conjugated metabolites from the body occurs either through the kidneys in urine, or biliary excretion from the liver into the intestine (Cassidy and Minihane, 2017). Intestinal enzymes hydrolyse metabolites while microbial enzymes (β-glucosidase, β-glucuronidase, α-rhamnosidase) deconjugate metabolites into deconjugated compounds and aglycones in the colon (Duda-Chodak *et al.*, 2015) (Figure 2.4). Deconjugated compounds and aglycones undergo demethylation to phenolic acids and aldehydes by colonic microbiota, then secondary reabsorption in an enterohepatic cycle to further promote absorption (Corona *et al.*, 2013). Any unabsorbed metabolites after colonic microbiota metabolism are excreted in faeces (Marín *et al.*, 2015).

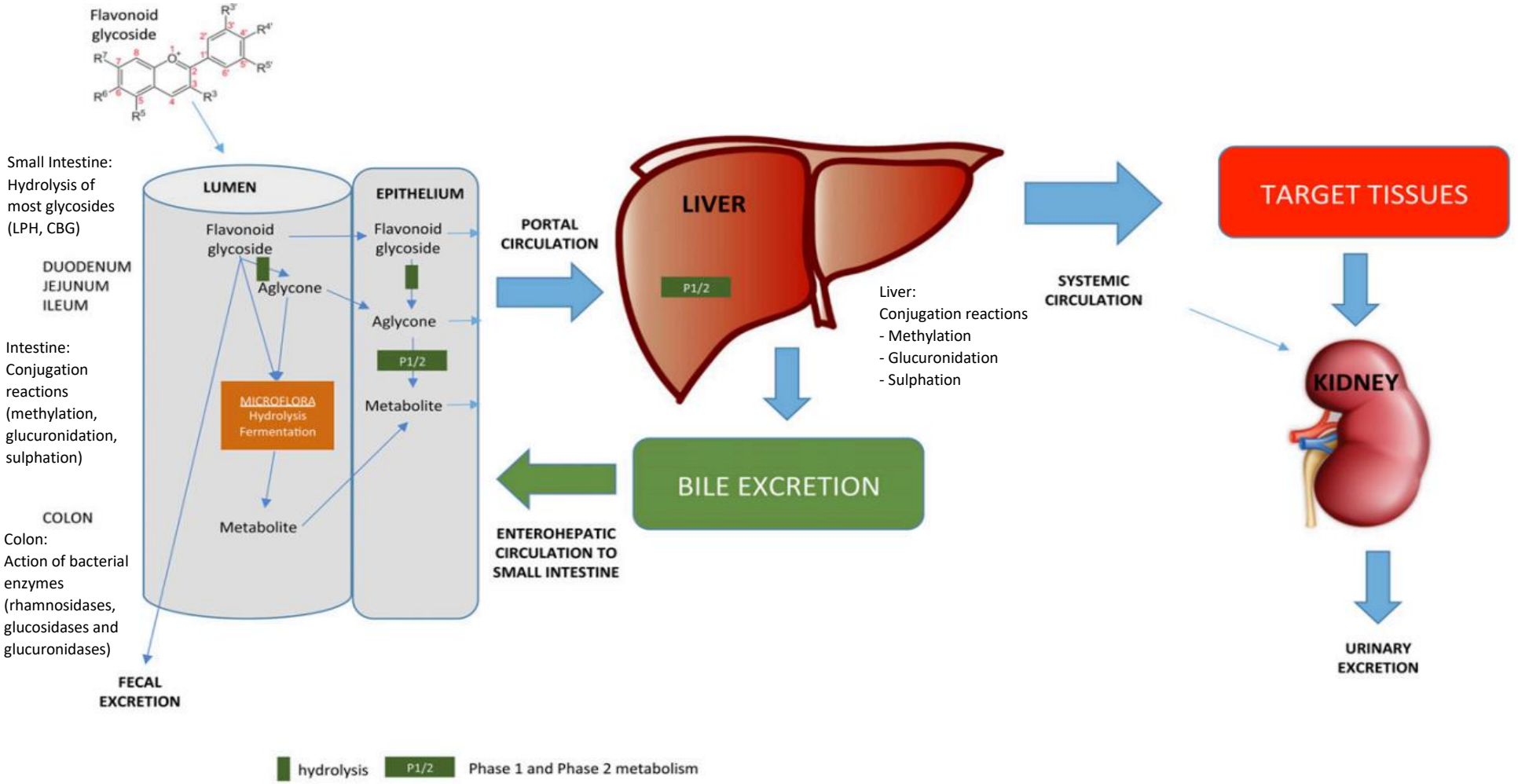


Figure 2.4. General absorption and metabolism routes of parent flavonoid compounds, their secondary metabolites and phase II conjugates (Adapted from Cassidy and Minihane, 2017). CBG (cytosolic β-glucosidase); LPH (lactase phlorizin hydrolase).

2.1.4. Anthocyanin Bioavailability

Porrini and Riso (2008) defined bioavailability as the portion of an ingested compound or nutrient, relative to the total amount consumed, that reaches the site where it can exert its biological action.

As mentioned previously, glycosylated anthocyanins are able to be absorbed into systemic circulation intact and pass into urine as such without undergoing hydrolysis or phase II conjugation, unlike other flavonoids (D'Archivio *et al.,* 2010). However, similar to other flavonoids, glycosylated and acylated forms of anthocyanins are less bioavailable and thus undergo hydrolysis into their aglycone (anthocyanidin) and the sugar moiety (Wallace, 2011). This process increases the bioefficacy of dietary anthocyanins but at the expense of stability, hence previous literature consistently reporting low bioavailability of anthocyanins (~0.4%) (Wallace, 2011). More recently however, Kay *et al.* (2017) found anthocyanins may be more bioavailable than first thought when considering their secondary metabolites and phenolic catabolites. These are suggested to be the main bioactive components responsible for the health-promoting properties of anthocyanins (Espín, González-Sarrías and Tomás-Barberán, 2017; Kay *et al.*, 2017). Czank *et al.* (2013) found similar anthocyanin recovery rates (12.3%) to flavan-3-ols and flavones (2.5-18.5%). Specifically, when a major anthocyanin of tart cherries, 500 mg cyanidin-3-glucoside, was provided to humans, 0.18% was recovered from blood, 5.37% from urine, 6.91% from breath and 32.13% from faeces, with the remaining 56.1% unaccounted for (Czank *et al.,* 2013). This is likely due to methodological procedures which examined anthocyanin bioavailability being setup to detect disappearance of the main flavylium cation only; and not other smaller phenolic compounds after biotransformation, which are more abundant *in vivo* (McGhie and Walton, 2007). Consequently, this is a major reason for the poor understanding of anthocyanin bioavailability (McGhie and Walton, 2007). After phase I metabolism of 500 mg cyanidin-3-glucoside, secondary metabolites,

protocatechuic acid (PCA) and phloroglucinaldehyde, were detected in serum 6 hours post-bolus at concentrations of 0.72 μmol.L^{-1} (Czank *et al.,* 2013); indicating rapid metabolic transformation. However, PCA-derived metabolites were observed in serum up to 48 hours after cyanidin-3-glucoside consumption, prolonging bioavailability (Czank *et al.,* 2013).

In human pharmacokinetic studies, anthocyanin plasma concentrations peaked between 1.4-592 nmol.L^{-1} and occurred at 0.5-4 hours post-consumption of dosages ranging from 68-1300 mg (Kay, 2006). Peak concentrations (~100 nmol.L^{-1} after dosages ≤500 mg anthocyanins) of secondary metabolites occurred between 0.5-3 hours in systemic circulation and remained in urine up to 24 hours after consumption (Czank *et al.,* 2013). Approximately 32% of the compounds found in serum and urine were parent anthocyanins with secondary metabolites accounting for the remaining 68% (Kay, Mazza and Holub, 2005).

Phytochemical bioavailability is affected by a variety of factors as shown in Table 2.1. Anthocyanin bioavailability is particularly low due to poor intestinal absorption, high metabolism rates and rapid elimination (Manach *et al.,* 2004). Bioavailability of polyphenols in humans is notoriously poor (Porrini and Riso, 2008) as highlighted by the literature above. Therefore, it has been posited that augmenting bioavailability of a polyphenol would enhance its effectiveness on health (Bohn, 2014).

Table 2.1. Factors affecting bioavailability of polyphenols in humans. Adapted from D'Archivio *et al.* (2010).

Pre-Harvest Factors	Cultivation procedures; harvesting timings; climate conditions (temperature, light, water, altitude, UV radiation), physical (soil); use of fertilisers and pesticides; attack by pathogens and pests.
Post-Harvest Factors	Thermal treatments; homogenisation; lyophilisation; methods of cooking; storage and shipping conditions.
Polyphenol Related Factors	Chemical structure and size; concentration in food; dosage administered.
Food Related Factors	Food matrix; co-ingestion of positive (e.g. dietary fat) or negative (e.g. dietary fibre, protein) effectors of absorption; *in vivo* structural interaction with other compounds (binding with proteins e.g. albumin) or with polyphenols possessing similar mechanism of absorption.
Host Physiological Factors	Host characteristics (genetics, illness history, sex, age, training status, habitual polyphenol intake); enteric and intestinal pH concentration; enzyme activity; intestinal transit time; colonic microbiota.

2.1.4.1. Effect of Food Matrix on Anthocyanin Bioavailability

Consideration of the vehicle by which anthocyanins are delivered into the body must be made when assessing their bioavailability. Anthocyanins can be provided in a variety of matrices/forms including whole fruit, dried fruit, juice concentrate, juice drink (extract with water) and capsules. Greater absorption of anthocyanins occurs in supplements provided in liquid form, however at the expense of bioavailability (McGhie and Walton, 2007). This is due to the exposure of anthocyanins to external factors that affect their stability such as pH, temperature, light, oxygen, enzymes, oral microbiota and sugars (Robert and Fredes, 2015). Capsules are a medium by which the influence of these factors may be mitigated due to protection of the anthocyanins by the exterior capsule shell (Robert and Fredes, 2015).

Oidtmann *et al.* (2012) compared encapsulated (pectin or whey protein isolate shells) and non-encapsulated bilberry anthocyanin concentrations after *in vitro* incubation with gastric and fed-

state intestinal fluids. During and after incubation, total anthocyanin concentrations were 23% higher with the encapsulated bilberry extract compared to non-encapsulated, suggesting encapsulation protected anthocyanins from enteric degradation. An obvious limitation is that *in vitro* incubation of anthocyanins with gastric and intestinal fluids does not mimic the complexity of *in vivo* anthocyanin metabolism in humans. The role of salivary amylase and colonic microbiota as part of the metabolism of anthocyanins (McGhie and Walton, 2007) has been overlooked in this study, limiting the application of the findings.

Kamonpatana *et al.* (2012) found oral microbiota significantly degraded cyanidin glycosides from chokeberry extracts by 50%, one hour after incubation with human saliva. This finding suggests that a glycoside of the main anthocyanidin found in tart cherries is considerably affected by salivary degradation, indicating that consideration of the method of delivering tart cherry anthocyanins to ensure maximal bioavailability is necessary. Capsules, which bypass the salivary degradation of anthocyanins, may therefore be a suitable option of achieving this. However, the bioavailability of anthocyanin-rich cherry capsules has not been assessed nor have their health effects.

2.1.5. Effects of Anthocyanins on Cardio-Metabolic Health

Anthocyanins are predominantly responsible for the dark red, blue, black and purple pigments found in various fruits and vegetables (Wu *et al.*, 2006). Anthocyanins are abundant in a variety of common dietary sources (e.g. berries, cherries, red/black grapes, pomegranates, purple corn, purple cabbage) which are available all year round, particularly in well-developed regions (He and Giusti, 2010). Thus, of all the polyphenols, anthocyanins are thought to be consumed in the greatest amounts from food sources present in habitual diet (He and Giusti, 2010). Considering this greater intake, it has been postulated that anthocyanins may exert a greater effect on health compared to other polyphenols (Landete, 2013). However, the abundance of

anthocyanins in the diet does not necessarily correlate with high bioavailability in systemic circulation or target tissues (D'Archivio *et al.,* 2010).

The average estimated anthocyanin intake from habitual diet in the United States is 12.5 $mg.day^{-1}$ (Wu *et al.,* 2006), whereas in Europe consumption differs significantly from 19.8 (Netherlands) to 64.9 $mg.day^{-1}$ (Italy) for men, and for women, from 18.4 (Spain) to 44.1 $mg.day^{-1}$ (Italy) (Zamora-Ros *et al.,* 2011). Incidentally, UK average anthocyanin consumption for men (22 $mg.day^{-1}$) and women (24 $mg.day^{-1}$) tends to fall near the lower range amongst European countries (Zamora-Ros *et al.,* 2011).

Many reviews and meta-analyses have reported largely beneficial cardio-metabolic health responses from anthocyanin consumption (Wallace, 2011; Reis *et al.*, 2016; Wallace, Slavin and Frankenfeld, 2016; Yang *et al.*, 2017; Daneshzad *et al.*, 2018; Kimble *et al.*, 2018). Epidemiological evidence suggested anthocyanin consumption reduced risk of coronary heart disease and CVD mortality by 9% and 8%, respectively (Kimble *et al.,* 2018). Dietary anthocyanin consumption was associated with a 15% T2D risk reduction, and berry consumption with an 18% risk reduction (Tan *et al.*, 2016). Recently an epidemiological study monitoring 124,086 individuals for up to 24 years, provided encouraging evidence that anthocyanins may aid weight management (Bertoia *et al.,* 2016).

Randomised control trials (RCT) investigating the impact of anthocyanins on cardio-metabolic biomarkers in humans, have reported some equivocal findings (Wallace, Slavin and Frankenfeld, 2016; Yang *et al.*, 2017; Daneshzad *et al.*, 2018). Overall, anthocyanins improved biomarkers that were not optimal at baseline (Wallace, 2011; Wallace, Slavin and Frankenfeld, 2016; Yang *et al.*, 2017; Daneshzad *et al.*, 2018). However, the RCTs included in these reviews displayed much variation in the supplements administered (purified/whole food/extract), form (matrix), supplementation length, anthocyanin dose administered, composition of the

anthocyanin-rich extracts, dietary control measures, control comparators, baseline status of the biomarkers, and differences in the reporting of outcome measures. Subsequently, it is difficult to reach a consensus on optimal anthocyanin supplementation strategies to improve particular aspects of cardio-metabolic health. Hence, further well-designed trials are required to explore the optimal dosage, duration, matrix and anthocyanin formula for improving cardio-metabolic health through specific manipulation of individual parameters in various populations.

2.1.5.1. Acute and Short-term Anthocyanin Feeding Studies on Cardio-Metabolic Health

The low feasibility and high economic cost of consuming anthocyanins for extended periods has led to interest in assessing the benefits of acute (≤24 hours) and short-term (≤7 days) supplementation. Short-term supplementation of anthocyanin-rich dietary interventions (mean 244 $mg.day^{-1}$, range from 6.8–724 $mg.day^{-1}$) has led to improved insulin sensitivity (Willems *et al.*, 2017; Solverson *et al.*, 2018), lowered brachial systolic blood pressure (SBP) (Keane *et al.*, 2016b; Stote *et al.*, 2017), central SBP (Jennings *et al.*, 2012), increased 24-hour fat oxidation rate (Solverson *et al.*, 2018) and flow-mediated dilatation (FMD) (Rodriguez-Mateos *et al.*, 2013, 2016). Anthocyanins were able to modulate arterial stiffness, measured by pulse wave velocity (PWV) and analysis (PWA) (Fairlie-Jones *et al.*, 2017), and improve cardiac haemodynamic parameters including stroke volume (SV), total peripheral resistance (TPR), cardiac output (CO) (Willems *et al.*, 2015) and mean arterial pressure (MAP) (Jennings *et al.*, 2012; Cook *et al.*, 2017a).

The main advantage of acute anthocyanin supplementation is maximising its bioavailability and coinciding parent anthocyanin and metabolite pharmacokinetics with observed health effects. However, a limitation of these studies is the lack of follow-up after discontinuing supplementation. Therefore, the biological significance of these anthocyanin-rich interventions

in relation to long-term cardio-metabolic health remains unknown. Further research would be required to provide greater biological significance, ecological validity and clinical benefit.

2.1.5.2. Mechanisms of Anthocyanins on Health

2.1.5.2.1. Anti-oxidant Theorem and Hormesis

Oxidative stress has been considered an important characteristic of ageing and chronic disease due to the free radical theory of ageing (Harman, 1956). The theory posits that organisms age because cells accumulate oxidative damage induced by free radicals (Sohal and Weindruch, 1996). Free radicals can inflict damage in various ways: destructing deoxyribonucleic acid (DNA) and cellular structures, activating signalling pathways that increase reactive oxygen species (ROS) production, altering gene and enzyme function and disrupting normal repair processes (Poulsen *et al.*, 2000). Age-related chronic diseases including CVD, T2D and MetS (*section 2.2.4*) have oxidative stress ascribed as a core component of their underlying pathophysiology (Liguori *et al.*, 2018); based on many studies reporting a low anti-oxidant status/pro-oxidant balance being associated with ageing (Sohal and Weindruch, 1996). This led to the 'anti-oxidant theorem/hypothesis' that age-related chronic diseases may be prevented with provision of anti-oxidants to return the oxidant balance to homeostasis (Stanner *et al.*, 2004).

The concept of hormesis, emerged from toxicology, and describes the biphasic dose-response where a chemical can induce biologically opposite effects at different doses (Calabrese *et al.*, 2007). Commonly, at low doses the chemical has stimulatory/beneficial effects and at high doses inhibitory/toxic effects (Calabrese *et al.*, 2007), giving rise to an inverted-U curve (Figure 2.5). In relation to oxidative stress, evoking a slight stress response from low concentrations of ROS prompts a stronger, more potent anti-oxidative response against subsequent deleterious bouts of oxidative stress and thus protects against free radical damage (Masoro, 2000).

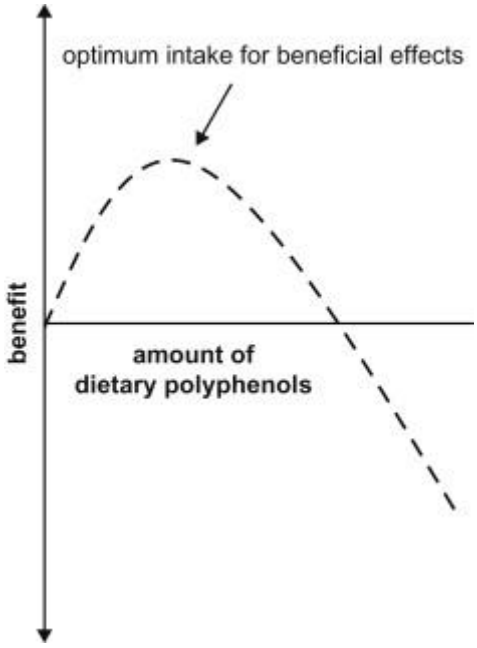


Figure 2.5. Hormetic curve indicating optimum dose of polyphenols for beneficial effects (De Roos, Arbones-Mainar and Gutiérrez, 2010).

Dietary phytochemicals operate via hormetic mechanisms and are therefore termed 'hormetins' (Rattan, 2008). The action of polyphenols and specifically anthocyanins against chronic disease is thought to operate via direct and indirect anti-oxidant mechanisms (Stevenson and Hurst, 2007). Direct anti-oxidant action against oxidative stress was initially purported to be the mechanism of action of anthocyanins, as *in vitro* studies have demonstrated their ability to quench free radical damage, particularly lipid peroxidation (Stevenson and Hurst, 2007). However, *in vivo* studies have suggested anthocyanins are unlikely to significantly contribute to the overall total anti-oxidant capacity given the relatively low bioavailability and plasma concentrations in which they appear (Landete, 2013). Anthocyanins in plasma only reach nM and low μM concentrations, thus it has been reported that anthocyanins would only contribute a transient 2-4% increase in plasma total anti-oxidant capacity (Stevenson and Hurst, 2007). In further discouragement of the direct anti-oxidant mechanism, anthocyanins in plasma typically occur in their phase II metabolite form, which have significantly less anti-oxidant potential

than their parent compounds (Liu, 2003). Consequently, this would explain the lack of effect in studies supplementing single anti-oxidant nutrients to improve chronic diseases (Liu, 2003).

An indirect anti-oxidant mechanism may therefore be in play. The concept of hormesis inducing slight oxidative stress and upregulating endogenous anti-oxidant enzyme production has been proposed as an indirect mechanism (Stevenson and Hurst, 2007). Traustadottir *et al.* (2009) proposed three mechanisms by which tart cherries act against oxidative stress. These include scavenging free radicals directly, formation of cyanidin–DNA complexes resistive to oxidative damage and activation of protective xenobiotic responses via the upregulation of endogenous anti-oxidants and Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) activation (Traustadottir *et al.*, 2009). Nrf2 also transactivates anti-oxidant response elements, under oxidative stress conditions encountered in cardio-metabolic diseases (Reis *et al.*, 2016). Therefore, Nrf2 is a key regulator of endogenous anti-oxidant enzymes including but not limited to glutathione-S-transferase, glutathione peroxidase and haem oxygenase-1 (Sykiotis *et al.*, 2011). Šarić *et al.* (2009) found upregulation of the endogenous enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPx) in mice after consumption of tart cherry juice. In addition to the anti-oxidant activity, Nrf2 also contributes to anti-inflammatory processes through crosstalk with the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signalling pathway (Ahmed *et al.*, 2017). Low-grade, chronic inflammation is inherent in vascular and metabolic pathologies; thus the ability of anthocyanins to influence the NF-κB signalling pathway and reduce the inflammatory response plays an important role in improving vascular and metabolic function (Wallace, 2011).

Overall, hormetic pathways that polyphenols, including anthocyanins, function through include: Nrf2/anti-oxidant response element pathway, NF-κB pathway and sirtuin-FOXO pathway (Son, Camandola and Mattson, 2008). This provides evidence that polyphenols including anthocyanins exert their health-promoting effects via a myriad of hormetic signalling

pathways. Importantly, the anti-oxidant dose required to elicit the positive effects are small, so as not to induce the toxic effects of anti-oxidants and thus comply with the hormetic curve.

2.1.5.2.2. Modulation of Gut Microbiota

Lastly, modulation of gut microbiota is increasingly recognised as a potential mechanism of the cardio-metabolic protective effects of polyphenols (Cardona *et al.*, 2013; Espín, González-Sarrías and Tomás-Barberán, 2017), including anthocyanins (Faria *et al.*, 2014; Igwe *et al.*, 2018). A systematic review by Igwe *et al.* (2018), examining the effects of anthocyanins on gut microbiota showed upregulation of beneficial microbiota including *Bifidobacterium* and *Lactobacillus* and simultaneous reductions of pathogenic microbiota including *Clostridium*.

2.1.6. Cherries

Cherries belong to the Rosaceae family and the genus *Prunus*; they are commonly found in temperate climates in northern regions (McCune *et al.*, 2011). There are over a hundred cultivars of cherries, but the main types are sweet (*Prunus avium* L.) and tart (or sour) (*Prunus cerasus* L.) (McCune *et al.*, 2011). The sweet cherry is commonly grown from the Bing cultivar and the tart cherry from the Montmorency cultivar (Kelley, Adkins and Laugero, 2018).

2.1.6.1. Phytochemical Content of Cherries

Tart, sweet and Cornelian (*Cornus mas* L.) cherries have been assessed for their health-promoting abilities (McCune *et al.*, 2011; Kelley, Adkins and Laugero, 2018). Cherries possess a high variety of bioactive compounds including vitamin C, fibre, carotenoids, hydroxycinnamates, melatonin, anthocyanins, flavonols and flavan-3-ols such as catechin, epicatechin, quercetin-3-glucoside, quercetin-3-rutinoside and kaempferol (Ferretti *et al.*, 2010). Of these, anthocyanins were found to be the most abundant (Ferretti *et al.*, 2010). The anthocyanin content of Montmorency tart cherries (MTC) compares favourably to other anthocyanin-rich dietary sources (Table 2.2).

Table 2.2. Anthocyanin content of various whole foods and beverages [adapted from Clifford (2000)]. Units for whole foods in $mg.kg^{-1}$ and for juices/wine in $mg.L^{-1}$.

Food Source	Anthocyanin Content ($mg.L^{-1}$ or $mg.kg^{-1}$)
Montmorency Tart Cherry (juice)	9117
Blackberry	1150
Blueberry	825-4200
Blackcurrant	1300-4000
Cabbage (purple)	250
Chokeberry	5060-10000
Elderberry	2000-10000
Grape (red)	300-7500
Blood Orange (juice)	2000
Raspberry (black)	1700-4277
Raspberry (red, whole fruit)	100-600
Raspberry (red, juice)	4-1101
Strawberry	150-350
Sweet Cherry	20-4500
Wine (red)	240-350

In whole tart cherries the parent anthocyanidin, cyanidin, accounts for 93% of anthocyanins in the Montmorency cultivar (Kirakosyan *et al.*, 2009). In MTC concentrate the most abundant anthocyanins include, cyanidin-3-glucosylrutinoside, cyanidin-3-rutinoside and peonidin-3-rutinoside (Bell *et al.*, 2014b).

MTC possess less anthocyanins than other tart cherry varieties but contain more total phenolics (Kirakosyan *et al.*, 2009; McCune *et al.*, 2011). It has been postulated that this greater quantity of total phenolics may increase bioavailability and thus improve MTC bioefficacy *in vivo* (Seymour *et al.*, 2008). Kirakosyan *et al.* (2009) assessed the quantity of total anthocyanins and phenolics in dried (with and without added sugar), frozen, concentrate and individually quick-frozen powdered Montmorency cherries. Frozen whole tart cherries contained the most total anthocyanins and phenolics, followed by individually quick-frozen powder, concentrate, dried without sugar and dried with sugar (Kirakosyan *et al.*, 2009); suggesting increased acidity provides more phenolics and anthocyanins.

In another study, analysis of MTC in dried, frozen and diluted juice (30 mL, equivalent to 90-110 whole tart cherries, and 60 mL concentrate) forms showed the greatest concentrations of total anthocyanins, phenolics, anti-oxidant capacity (Trolox), PCA, vanillic acid (VA) and chlorogenic acid were in juice form, with values linearly increasing in a dose-dependent manner (Keane *et al.*, 2016a). Total anthocyanin and (phenolic) content of 30 mL concentrate, 60 mL concentrate, dried and frozen MTC were 312.7 $mg.L^{-1}$ cyanidin-3-glucoside equivalents (713.7 $GAE.L^{-1}$), 624.7 $mg.L^{-1}$ (1427.3 $GAE.L^{-1}$), 0.08 $mg.g^{-1}$ (0.05 $GAE.g^{-1}$) and 0.3 $mg.g^{-1}$ (0.06 $GAE.g^{-1}$), respectively (Keane *et al.*, 2016a). Moreover, tart cherry concentrate was shown to have the highest oxygen radical absorbance capacity (ORAC) capacity of any other tart cherry product (frozen, canned, dried) and other anthocyanin-rich foods (plums, grapes, strawberries and blueberries) at common habitual intake portion sizes (Ou *et al.*, 2012). Therefore, MTC concentrate may be the most suitable form of supplementation to induce beneficial health responses.

2.1.6.2. Cherry Pharmacokinetics

Plasma concentrations of the anthocyanins cyanidin-3-glucosylrutinoside and cyanidin-3-rutinoside, were found to be greatest in healthy human participants after consuming whole tart cherries (Seymour *et al.*, 2014). After consuming 45 (total anthocyanins 12.96 mg) whole cherries, concentrations peaked at 2 hours post-ingestion, cyanidin-3-rutinoside also peaked 2 hours after consuming 90 (total anthocyanins 25.83 mg) whole cherries, however cyanidin-3-glucosylrutinoside peaked at 4 hours with 90 cherries (Seymour *et al.*, 2014). Consumption of 45 whole cherries was not significantly different compared to 90 cherries in terms of plasma anthocyanin concentrations over a 12-hour time period, although 90 cherries tended to be more bioavailable (Seymour *et al.*, 2014). The authors also noticed a delayed yet statistically significant elevation in plasma anti-oxidant capacity between 8-12 hours post-ingestion of 45 and 90 cherries compared to baseline. Time of appearance of metabolites in urine peaked 6-8

hours post-ingestion with 90 cherries. These findings align with the general pharmacokinetics of anthocyanins in humans (Kay, Mazza and Holub, 2005).

Keane *et al.* (2016a) studied plasma concentrations of secondary metabolites after human consumption of Montmorency tart cherry juice (MTCJ) made with 30 or 60 mL concentrate in a randomised, counterbalanced design. Plasma concentrations of PCA, VA and chlorogenic acid in twelve, healthy, males were measured at baseline, 1, 2, 3, 5- and 8-hours post-consumption. PCA was significantly elevated from baseline one-hour post-consumption in both conditions (Figure 2.6), however peak concentrations and total AUC between conditions were not significantly different. A significant time effect was observed for VA after 60 mL consumption (Figure 2.6). A similar response was observed with 30 mL as demonstrated by non-significant differences between AUC responses, however peak concentration occurred 2-hours post-consumption, suggesting the lower total anthocyanin and phenolic content may have been a limiting step for the slower production of VA after metabolism by gut microbiota. The pharmacokinetics of these secondary metabolites are in accordance with Seymour *et al.* (2014). Two limitations of Keane's *et al.* (2016a) study include the lack of a standardised washout between conditions (minimum 10 days). This may have affected baseline values of the second trial, particularly given the high inter-individual variance of anthocyanin metabolism (Czank *et al.,* 2013). Secondly, blood sampling time points limited complete examination of metabolites as enterohepatic anthocyanin metabolism occurs up to 48 hours post-consumption (Czank *et al.,* 2013).

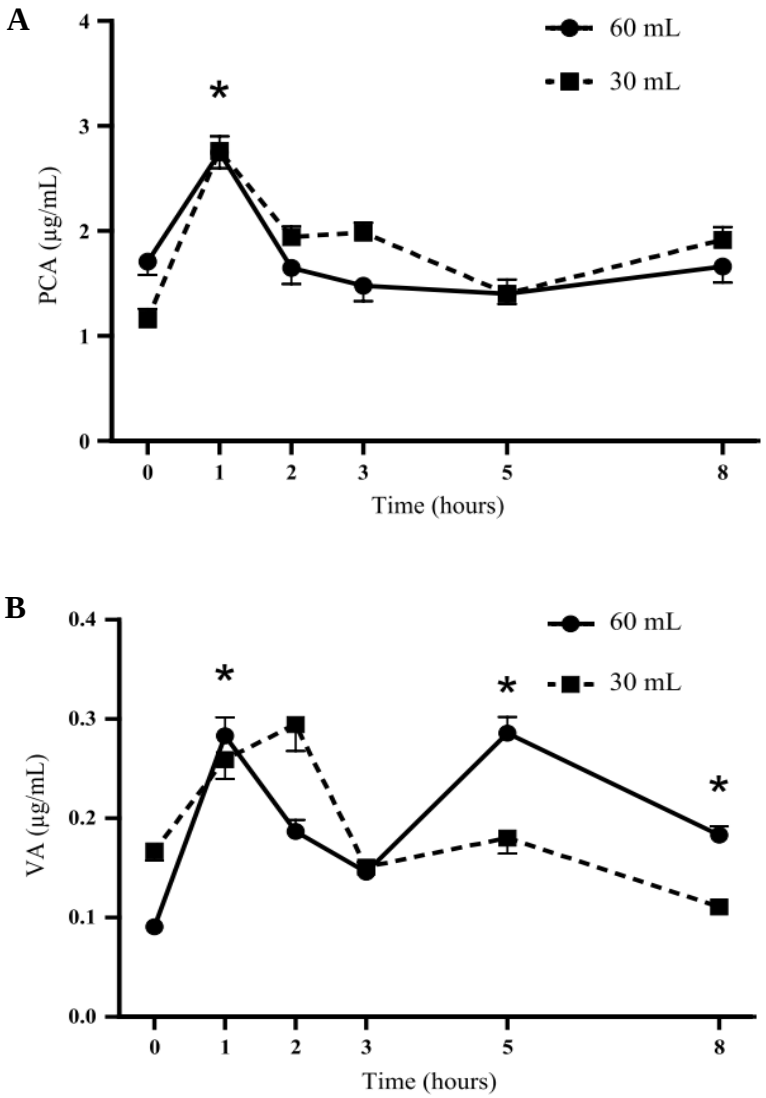


Figure 2.6. **(A)** PCA responses to 30 and 60 mL Montmorency cherry concentrate (MC). Absolute baseline values were 1.16 ± 0.326 and 1.70 ± 0.435 µg/mL for 30 and 60 mL, respectively. Asterisk indicates a significant time effect ($P < 0.05$) (30 and 60 mL dose); data presented as mean ± SEM. **(B)** VA responses to 30 and 60 mL Montmorency cherry concentrate (MC). Absolute baseline values were 0.158 ± 0.031 and 0.093 ± 0.024 µg/mL for 30 and 60 mL, respectively. Asterisk indicates a significant time effect ($P < 0.05$) (60 mL dose only); data presented as mean ±SEM (Keane *et al.*, 2016a).

2.1.6.3. Tissue Bioavailability of Cherries

Understanding the effectiveness of tart cherry anthocyanins requires awareness of the tissue bioavailability. Kirakosyan *et al.* (2015) identified tart cherry anthocyanins in several tissues in healthy rats using liquid chromatography-mass spectrometry, after supplementation of 1% or 10% wet weight tart cherry powder. The anthocyanins found by Bell *et al.* (2014b) in MTC concentrate, were differentially distributed in various tissues within the rats (Kirakosyan *et al.*,

2015). Bladder and kidney tissue contained the greatest amounts of total anthocyanins, with moderate amounts in cardiac, brain and liver tissues and none found in retroperitoneal fat. Currently, it is unknown in which tissues tart cherry anthocyanins would be most bioavailable in humans. Further research is required in assessing tissue bioavailability of anthocyanins, phenolics and secondary metabolites after consumption of MTC in healthy and diseased human populations.

2.1.6.4. Cherries and Exercise

The effects of cherries within the exercise science field have predominantly focused on treatment of exercise-induced muscle damage (EIMD) and associated complications, including exercise-induced oxidative stress, inflammation and delayed onset muscle soreness (DOMS). The ability of athletes to recover and return to play after strenuous activity is important for performance (Howatson and van Someren, 2008). Cherry supplementation, especially the Montmorency cultivar, has been shown to alleviate DOMS, upper respiratory tract infection (URTI), inflammation, oxidative stress and recover muscle function quicker after strenuous exercise (Bell *et al.*, 2014a; Connolly, 2015) (Table 2.3). However, the mechanisms of action by which tart cherries exert their beneficial effects to improve functional and performance parameters are unknown (Bell *et al.*, 2014a).

Table 2.3. Summary of studies examining the effects of tart cherry supplementation on physiological and functional parameters after exercise.

Study	Study Design, Sample Size and Cohort	Cherry Supplementation Strategy and Dietary Control	Study Details	Significant Results with Cherry Supplementation compared to Placebo	Conclusion
Connolly, McHugh and Padilla-Zakour, (2006)	Randomised, Crossover, Placebo Controlled 16 healthy, active, males	355 mL twice daily for 8 days – Juice No dietary control	Eccentric elbow flexion contractions (2 x 20 maximum contractions) was performed on the fourth day of supplementation	↓ Strength Loss ↓ Pain	Improved muscle function and reduced pain with cherry supplementation.
Howatson *et al.* (2010)	Randomised, Parallel, Placebo Controlled 20 (13 male, 7 female) recreational marathon runners	237 mL twice daily for 8 days - Juice Food diary during supplementation phase to monitor compliance	Marathon run MVIC pre-post exercise	Faster isometric muscle strength recovery Post-Exercise: ↓ IL-6 ↓ CRP ↓ Uric Acid ↑ TAS ↓ TBARS	Cherry supplementation improved muscle function in a shorter timeframe and reduced inflammation, oxidative stress post-exercise coinciding with greater anti-oxidant status.
Bowtell *et al.* (2011)	Randomised, Crossover, Placebo Controlled, Double-Blinded 10 well-trained males	30 mL twice daily for 10 days – Juice concentrate Habitual diet for first 5 days of supplementation period. Participants recorded their diet for 48 h before the first main trial and then repeated this diet before the second trial.	10 x 10 single-leg knee extensions at 80% one-repetition maximum on the eighth day of supplementation	Faster isometric muscle strength recovery ↓ Protein oxidation	Cherry supplementation improved muscle function in a shorter timeframe with reduced protein oxidative stress.

Bell *et al.* (2014c)	Randomised, Parallel, Placebo Controlled, Double-Blinded 16 trained male cyclists	30 mL twice daily for 7 days – Juice concentrate Low-polyphenolic diet during loading phase	109 min cycling trial completed on days 5, 6 and 7 of the supplementation phase	↓ IL-6 ↓ hsCRP ↓ Lipid Hydroperoxides	Cherry supplementation reduced inflammation and lipid oxidation with repeated metabolically induced muscle damage.
Bell *et al.* (2015)	Randomised, Parallel, Placebo Controlled, Double-Blinded 16 trained male cyclists	30 mL twice daily for 8 days – Juice concentrate Low-polyphenolic diet during loading phase	109 minutes cycling trial on the fifth day of supplementation	Improved muscle function post-exercise ↓ IL-6 ↓ hsCRP	Cherry supplementation improved muscle function in a shorter timeframe and reduced inflammation with metabolically induced muscle damage.
Dimitriou *et al.* (2015)	Randomised, Parallel, Placebo Controlled 20 (13 male, 7 female) recreational marathon runners	2 x 236 mL on the day of exercise – Juice No dietary control	Marathon run	↓ CRP ↓ Incidence and severity of URTS post-exercise	Systemic inflammation and upper-respiratory tract symptoms significantly lower post-exercise with cherry supplementation.
Levers *et al.* (2015)	Randomised, Parallel, Placebo Controlled, Double-Blinded 23 healthy, resistance-trained males	480 mg.day^{-1} for 10 days – Powdered tart cherry capsules Monitor dietary intake for 4 days. No dietary restriction enforced	10 x 10 repetitions at 70% of a one-repetition maximum back squat exercise	↓ Muscle soreness ↓ Total Bilirubin ↓ ALT ↓ AST ↑ Serum Creatinine ↑ Total Protein	Powdered tart cherry significantly reduced muscle catabolism markers and muscle soreness after resistance exercise.

Levers *et al.* (2016)	Randomised, Parallel, Placebo Controlled, Double-Blinded 27 (18 male, 9 female) endurance trained or triathletes	480 mg.day^{-1} for 10 days – Powdered tart cherry capsules No dietary control reported	Half-marathon run on the eighth day of supplementation	Faster half-distance split and final finish time ↓ IL-12p70 ↓ IL-2 ↓ IL-5 ↑ IL-6	Powdered tart cherry significantly reduced inflammation after aerobic exercise.
Bell *et al.* (2016)	Randomised, Parallel, Placebo Controlled, Double-Blinded 16 semi-professional male soccer players	30 mL twice daily for 7 days – Juice concentrate Low-polyphenolic diet 48hr before each trial and during loading phase	12 x 20m sprints every 60s followed by an adapted LIST Protocol (6 x 15 min sections)	↑ Muscle function post-exercise ↓ Muscle soreness ↓ IL-6	Reducing the post-exercise inflammatory response significantly improved functional markers of muscle function after damaging exercise from prolonged intermittent sprinting.
McCormick *et al.* (2016)	Randomised, Crossover, Placebo Controlled, Double-Blinded 9 highly-trained Water Polo players	2 boluses of 30 mL and 60 mL – Juice concentrate No dietary control reported	Battery of swimming-based tests: in-water vertical jump test, 10 m sprint test, the repeat sprint test and WIST	No difference between conditions for: IL-6 CRP Uric Acid F_2-Isoprostanes DOMS Performance in Battery Test	6 consecutive days of tart cherry juice supplementation has no effect on athletic performance or recovery in highly-trained Water Polo athletes. Intermittent, non-weight bearing exercise may not induce a sufficient inflammatory response or oxidative stress; thereby negating any potential beneficial effects associated with tart cherry supplementation.
Hillman, Taylor and Thompkins (2017)	Randomised, Parallel, Placebo Controlled, Single-Blinded 16 healthy, recreationally active participants	2x240 mL twice daily for 10 days – Tart cherry and whey drink No dietary restriction enforced	5 x 20 Drop Jumps with 60s rest between intervals	↑ 67% ORAC	No change in markers of muscle damage, however increased ORAC with a tart cherry and whey blend. Indicates that addition of whey may limit the efficacy of tart cherry phytochemicals.

Brown, Stevenson and Howatson (2019)	Randomised, Parallel, Placebo Controlled, Double-Blinded 20 physically active females	30 mL twice daily for 8 days – Juice concentrate No dietary restriction enforced	Repeated sprint protocol 15 x 30m maximal sprints with 60s rest between bouts	↑ Muscle function post-exercise	In a female only cohort, tart cherry consumption improves muscle function and tends to lower muscle soreness after a damaging bout of exercise.
Morgan, Barton and Bowtell (2019)	Randomised, Crossover, Placebo Controlled, Double-Blinded 8 trained male cyclists	462.8 mg.day^{-1} for 7 days – Powdered tart cherry capsules No dietary restriction enforced	10 min steady-state at ~65% $\dot{V}O_2$peak. Followed by 15 km cycling TT	Faster TT completion time ↑ Blood Lactate after steady-state ↑ Resting Tissue Oxygenation Index No difference between conditions for: RER during steady state and TT exercise	7 consecutive days of Montmorency tart cherry capsule consumption increased muscle oxygenation and thus improved time trial performance. RER during moderate-intensity exercise was not different between conditions, however 10 minutes was likely to short a timeframe to observe significant changes.

ALT (Alanine aminotransferase); AST (Aspartate aminotransferase); CRP (C-reactive Protein); hsCRP (high-sensitivity C-reactive Protein); IL (Interleukin); LIST (Loughborough Intermittent Shuttle Test); MVIC (Maximal Voluntary Isometric Contraction); ORAC (Oxygen Radical Anti-oxidant Capacity); RER (Respiratory Exchange Ratio); TAS (Total Anti-oxidant Status); TBARS (Thiobarbituric Acid Reactive Substances); TT (Time Trial); URTS (Upper Respiratory Tract Symptoms); WIST (Water Polo Intermittent Shuttle Test).

The general design of these studies was randomised, crossover with a placebo control comparator. Most studies incorporated dietary control 3-7 days prior to and during the loading phase, which consisted of a low-polyphenolic diet monitored through food diaries (Table 2.3), to isolate the effects of cherry phytochemicals on outcome variables. This could be deemed a weakness as the addition of cherry phytochemicals may serve to only replace the polyphenol intake that would be lost after ceasing consumption of other polyphenol-rich sources. Consequently, any effects between placebo and cherry conditions cannot be differentiated from being due to an absolute loss of polyphenols or the addition of cherry phenolics. Secondly, cherry juice was commonly supplemented twice daily for a short loading phase, varying between 7-10 days (Table 2.3). Thus, physiological responses to long term supplementation (>10 days) in an exercise paradigm remain unknown.

In summary, Table 2.3 shows a clear trend for attenuating inflammation and oxidative stress with tart cherry supplementation in various forms including juice, juice from concentrate and encapsulated powder. Due to variations in inflammatory and oxidative stress biomarkers, supplementation strategy, exercise protocol and the lack of absolute data reported in studies, it is not possible to distinguish which form of cherry supplementation was most effective. The timing of supplementation pre-, during and post-exercise likely augmented total phenolic and anthocyanin concentrations in systemic circulation, regardless of the high metabolism and rapid elimination rates. A discernible relationship between greater bioavailability of cherry phytochemicals and effective mitigation of symptoms associated with EIMD was observed. Predictably, acute supplementation immediately before exercise was therefore effective at attenuating DOMS, inflammation and oxidative stress. Future studies are required where supplementation occurs at points where plasma concentrations of cherry phytochemicals are not maximal as a result of consumption immediately before, during or after exercise. An

understanding of the pharmacodynamics and pharmacokinetics of cherry anthocyanins and other phytochemicals is therefore required in an exercise paradigm.

2.1.6.5. Cherries and Health

A recent review analysed 29 (tart 20, sweet 7, unspecified 2) published studies, which assessed the effects of cherries (juice, powder, concentrate, capsules) on health markers in humans ranging from young athletes to older individuals with chronic conditions (Kelley, Adkins and Laugero, 2018). Nineteen studies employed a randomised, placebo-controlled design, with the remaining 10 only assessing pre-post responses to the cherry intervention. Study design varied considerably, with supplementation length commonly lasting <2 weeks (range 5 hours – 3 months) and daily equivalent dosages of 45-270 whole cherries (55–720 $mg.day^{-1}$ anthocyanins) provided in single or split doses (Kelley, Adkins and Laugero, 2018). Furthermore, other factors including habitual anthocyanin intake and metabolism of various forms of MTC affect bioavailability and thus bioefficacy of the intervention against health biomarkers; making comparisons between studies difficult.

Given the potent anti-oxidant and anti-inflammatory properties of cherries, Kelley, Adkins and Laugero (2018), found 8/10 and 11/16 studies reported reductions in oxidative stress and inflammatory biomarkers, respectively. Moreover, glycated haemoglobin (HbA_{1c}) was reduced in participants with T2D, VLDL and TG:HDL ratio were reduced in 2/5 studies, blood pressure in 5/7, arthritic pain and gout in 5/5 and sleep and cognitive function improved in 4/4 studies (Kelley, Adkins and Laugero, 2018). These findings were corroborated by Mayta-Apaza, Marasini and Carbonero (2017) and highlighted the impact of cherry interventions at improving health markers in humans; which is explored in more detail below.

2.1.6.5.1. Role of Cherries on Inflammation and Oxidative Stress

The health effects of cherries were first considered in *in vitro* experiments conducted by Wang, (1998) and Wang *et al.* (1999), who demonstrated their potent anti-oxidative and anti-inflammatory properties. Through *in vitro* analysis, it is thought cyanidin-3-glucosylrutinoside and cyanidin-3-rutinoside, exert their anti-inflammatory and anti-oxidative effects via inhibition of the cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) enzymes (Seeram *et al.*, 2001). The anti-inflammatory power of the cyanidin aglycone in tart cherries was shown to exceed that of the common non-steroidal anti-inflammatory drug aspirin (Wang *et al.*, 1999).

More recently, these results were corroborated by Kirakosyan *et al.* (2018) also in *in vitro* experiments. The COX-1, COX-2, lipoxygenase (LOX) and xanthine oxidase (XO) inhibitory activity of constituent phytochemicals within MTC were compared to MTC extract at physiologically relevant human dosages (Kirakosyan *et al.*, 2018). MTC extract inhibited COX-1, COX-2, LOX and XO by 65%, 38%, 64% and 26%, respectively, emphasising the anti-inflammatory and anti-oxidative properties of MTC. Strong LOX inhibition was observed with MTC compared to red raspberry, grape and blueberry extracts (Kirakosyan *et al.*, 2018). COX-1 and XO inhibition was greater with tart cherry extract compared to its constituent compounds, suggesting synergistic activity of the individual compounds. COX-2 inhibition showed greater effects with individual compounds (quercetin and isorhamnetin-3-rutinoside) of MTC compared to tart cherry extract itself. The inhibition of COX-1, COX-2, LOX and XO indicates MTC may be a suitable intervention for the management of MetS and its complications (Kirakosyan *et al.*, 2018).

Chronic low-grade inflammation is associated with a host of diseases (McCune *et al.*, 2011). Cherry consumption has been found to be beneficial at combating the inflammatory disease, gout in humans (Jacob *et al.*, 2003; Bell *et al.*, 2014c). Kelley *et al.* (2013) showed ingestion

of 45 whole sweet Bing cherries (280 g.day^{-1}) amongst healthy humans, for 28 days decreased plasma concentrations of extracellular newly identified receptor for advanced glycation end-products binding protein (EN-RAGE) (29%), CRP (20.1%), ferritin (20.3%), plasminogen activator inhibitor-1 (PAI-1) (19.9%), endothelin-1 (13.7%), IL-18 (8.1%) and increased that of IL-1 receptor antagonist (27.9%) compared with baseline values. The reduction of EN-RAGE, PAI-1, CRP and endothelin-1 as inflammatory markers influencing cardio-metabolic function provides support that cherry phenolics may be effective in regulating symptoms of MetS.

In healthy humans, MTCJ supplementation (240 mL twice daily for 14 days, 118 mg.day^{-1} anthocyanins) lowered the oxidative stress markers, F_2-isoprostanes and basal urinary excretion of oxidised nucleic acids (Traustadottir *et al.*, 2009). This provides preliminary evidence for a calorie restriction mimetic (CRM) effect of tart cherries through suppression of oxidative stress, as this mechanism has been cited by Speakman and Mitchell (2011) during calorie restriction (CR).

2.1.6.5.2. Role of Cherries on Cardio-Metabolic Health

Lastly and most importantly for this thesis, the role of cherries on cardio-metabolic parameters will be discussed from experiments performed *in vitro,* in animals and humans.

The positive influence of MTC on enzymes associated with MetS were mentioned above (Kirakosyan *et al.*, 2018). Angiotensin II is an enzyme involved in BP regulation but also stimulates COX-2 expression and induces oxidative stress in cardiovascular tissues (Quilley, 2011). Kirakosyan *et al.* (2018) showed a strong inhibitory potential (89% inhibition) of MTC extract on angiotensin-converting enzyme (ACE), signifying MTC may be a suitable treatment of pre-hypertension related to MetS through this mechanism. Moreover, impaired glycaemic function is also associated with MetS, and MTC extract was shown to inhibit α-amylase (IC50

3.46 mg.mL^{-1}) and α-glucosidase (IC50 11.64 mg.mL^{-1}) (Kirakosyan *et al.*, 2018). Although physiologically relevant concentrations were used, *in vivo* data is required to strengthen claims that MTC can prevent or ameliorate complications associated with MetS through inhibiting enzymes relevant to cardio-metabolic function (Kirakosyan *et al.*, 2018).

In vivo investigations into the effects of cherry consumption on cardio-metabolic markers were first conducted in rodents (Seymour *et al.*, 2008, 2009; Wu *et al.*, 2014). The ability to overcome insulin resistance is crucial for preventing and ameliorating MetS, CVD and diabetes (Jayaprakasam *et al.*, 2005). Cyanidin-3-glucoside and delphindin-3-glucoside in Cornelian cherries (*Cornus mas* L.) increased insulin secretion in rodents by 1.3-fold and 1.8-fold, respectively, in the presence of glucose at similar physiological levels in humans (4 mmol.L^{-1}) (Jayaprakasam *et al.*, 2005). In a follow-up study, mice were fed either a normal diet or high-fat diet for 4 weeks and thereafter a high-fat diet with Cornelian cherry anthocyanins (1 g.kg^{-1} of high fat diet) for a further 8 weeks (Jayaprakasam *et al.*, 2006). Mice fed anthocyanins exhibited decreased hepatic triglyceride accretion and a 24% decrease in weight gain compared to control mice. Insulin levels were extremely elevated with anthocyanin supplementation. However, islet of Langerhans structural integrity was the same in normal diet and anthocyanin-rich diet, signifying that these anthocyanins elevated insulin secretion and/or insulin sensitivity (Jayaprakasam *et al.*, 2006). Similarly, a human clinical trial with *Cornus mas* L. extract showed greater insulin concentrations, lower HbA_{1c} and triglycerides after consuming 600 mg.day^{-1} anthocyanins for 6 weeks in patients with T2D (Soltani *et al.*, 2015). The role of anthocyanin-mediated insulin secretion is equivocal, as *in vitro* and animal models primarily report anthocyanins to increase insulin secretion (Jayaprakasam *et al.*, 2005; Naseri *et al.*, 2018), whereas human studies indicate reduced secretion based on lower insulin concentrations (Udani *et al.*, 2011; Jennings *et al.*, 2013; Willems *et al.*, 2017).

Reductionist approaches to nutritional research have relevance in identifying the mechanisms of action of an intervention (Seymour *et al.,* 2008). However, these studies tend to supplement nutrients at supra-physiological dosages, thus studies that supplement at normal physiological levels are required to assess biological relevance (Seymour *et al.,* 2008). Seymour *et al.* (2008) therefore supplemented 1% MTC powder by weight of Dahl-Salt Sensitive rats for 90 days. This species of rat naturally exhibits hyperlipidaemia and insulin resistance where peroxisome proliferator-activated receptor (PPAR) agonist drugs have been shown to reverse these symptoms (Wilson, Alonso-Garcia and Roman, 1998). After supplementation, rats fed the cherry-rich diet presented significantly lower total cholesterol, triglycerides, fasting blood glucose, insulin and hepatic steatosis compared to controls (Seymour *et al.,* 2008). Mechanistically, this was thought to be induced by greater PPARα mRNA and hepatic PPARα target acyl-CoA oxidase mRNA expression, resulting in increased fatty acid oxidation (Seymour *et al.,* 2008). A follow-up study similar to Seymour *et al.* (2008) assessed abdominal gene transcription and adiposity in Zucker fatty rats (Seymour *et al.,* 2009). Cherry-fed rats had significantly greater PPARα mRNA and lower IL-6, TNF-α and NF-κB mRNA expression in retroperitoneal tissue, lower plasma IL-6 and TNF-α, percentage fat mass, retroperitoneal fat mass, total cholesterol, triglycerides, glucose and insulin (Seymour *et al.,* 2009). These findings were corroborated more recently in mice supplemented sweet cherries (Wu *et al.,* 2014). In addition to the above biomarkers, Wu *et al.* (2014) also reported smaller adipocytes, reduced leptin secretion and upregulation of endogenous anti-oxidant enzymes, SOD and GPx, with cherries.

These rodent studies provide exciting results that cherries may ameliorate symptoms associated with MetS, however human responses must be studied. Kelley *et al.* (2006) were the first to examine cherry supplementation with emphasis on cardio-metabolic markers. Healthy participants consumed 45 sweet cherries per day for 28 days and CRP, regulated upon

activation, normal T-cell expressed, and secreted (RANTES) and nitric oxide (NO) concentrations were reduced after 28 days. No significant differences were observed after cherry consumption for plasma total-, LDL-, HDL-, VLDL (very low-density lipoproteins) cholesterol, triglycerides, glucose and insulin. A number of reasons can be cited for these results, firstly, participants were healthy. Secondly, 45 cherries were unlikely to increase plasma phytochemical concentrations sufficiently to significantly improve the above markers; although the authors should be commended for providing realistic portion sizes. Finally, due to their lower pH, tart cherries are thought to be more bioavailable than sweet cherries and possess more phenolics (Seymour *et al.,* 2008). Similar weaknesses were apparent as with the other study (Kelley *et al.,* 2013) conducted by this research group, including the absence of a control comparator and the imbalance in male (n = 2) to female (n = 16) participant ratios. Secondly, both studies (Kelley *et al.*, 2006, 2013) restricted consumption of other polyphenol-rich sources. The lack of a control group means any differences observed over time cannot be differentiated from an absolute lack of polyphenols or the addition of cherry phenolics.

Garrido *et al.* (2013) examined responses to 5 days consumption of reconstituted freeze-dried sweet Jerte Valley cherry powder (27.85 g) mixed with 125 mL water in young (20-30 years), middle-aged (35-55 years) and older (65-85 years) participants. Urinary anti-oxidant capacity was increased in all age groups and remained 3-times higher than baseline in older participants, however returned to baseline in young and middle-aged participants after 5 days. Blood glucose between placebo and cherry conditions was not significantly different; potentially explained by normal baseline values for all age groups.

Vargas *et al.* (2014), also conducted a study using sweet cherries, and examined their effects on inflammatory and cardiac markers, in thirty-seven overweight men. Participants consumed three servings of 142 g cherries per day for four weeks, substantially greater than any previous whole cherry feeding study, to determine tolerance of regular sweet cherry intake and

biological benefit. Measurements of BMI, waist circumference, BP, heart rate (HR), serum high-sensitivity CRP (hsCRP), urinary prostaglandin E, urinary thromboxane B2 and serum homocysteine were obtained pre- and post-intervention. Tolerance to supplementation was high, with participants consuming a median daily intake of 415 g, equating to 6.77 $mg.day^{-1}$ of anthocyanins. No significant changes for inflammatory and functional markers were found, except waist circumference which interestingly increased despite a significant decrease in daily total dietary fat consumption. Although participants were overweight/obese, all were healthy therefore basal inflammation in these subjects may not have been elevated enough for sweet cherries to be effective.

Another study examined the effects of 6 weeks MTCJ supplementation on blood-based lipid biomarkers and arterial stiffness (Lynn *et al.*, 2014). Forty-seven, healthy participants were randomly allocated into an experimental (consumption of 30 mL MTC concentrate with 220 mL water) or control group (consumption of 250 mL lemonade). The timing of supplement consumption was at participants' discretion, therefore pharmacokinetics and plasma concentrations of MTC phytochemicals would likely have varied between individuals. Subsequently, this may have contributed to the non-significant results obtained between conditions for total cholesterol, HDL, arterial stiffness (brachial-knee PWV), SBP, diastolic blood pressure (DBP) and CRP. Additionally, participants were fasted overnight therefore bioavailability of cherry phenolics were likely not at peak levels. Similar to aforementioned studies, due to participants being healthy, short-term changes in blood-based biomarkers and arterial stiffness were likely more difficult to detect.

In contrast, daily 40 g (anthocyanins 720 $mg.day^{-1}$) tart cherry juice concentrate supplementation for 6 weeks significantly reduced body mass, SBP, DBP and HbA_{1c} in 20 women with T2D (Ataie-Jafari *et al.*, 2008). Moreover, in a dyslipidaemic cohort (n = 12), LDL was also significantly reduced after cherry consumption. However, this study did not

include a control group and only compared baseline to week 6 values, therefore it should be questioned whether participants were expecting an improvement in these variables with cherry juice consumption. Secondly, only female participants were recruited. Third, the anthocyanin dose was much higher than typically consumed from habitual diet; limiting ecological validity.

Martin *et al.* (2010) (only abstract published) also reported daily consumption of tart cherry juice (237 mL) for 4 weeks significantly reduced plasma triglycerides (10%), TG:HDL ratio (17%) and VLDL (15%), compared to a placebo in 10 individuals that were overweight or obese. Total cholesterol, LDL, HDL and homeostatic model assessment of insulin resistance (HOMA-IR) were not different between conditions. The significant differences observed may have been due to participants presenting high baseline hsCRP, signifying elevated chronic inflammation and risk of CVD.

A significant reduction in SBP (6 mmHg), DBP (6 mmHg) and HR (5 beats.min^{-1}) was observed in 13 patients (6 young and 7 older) with hypertension and dysphagia, 2 hours after consuming a bolus of 300 mL sweet Bing cherry juice (total anthocyanin 207 mg) compared to placebo (Kent *et al.*, 2015b). Values returned to baseline 6 hours after ingestion, coinciding with general anthocyanin and tart cherry pharmacokinetics (Seymour *et al.*, 2014). Interestingly, a split-dosage, 100 mL serving at 0, 1 and 2 hours (3 x 100 mL) did not result in significant alterations of SBP, DBP or HR (Kent *et al.*, 2015b). Perhaps indicating lower bioavailability of cherry metabolites after ingestion of multiple smaller servings of cherry juice than a large single bolus; although statistical analysis was not conducted to confirm this. Additionally, there were no significant differences for SBP, DBP and HR between younger and older participants highlighting the underlying pathology (hypertension) is of more importance for observing a positive effect with cherry supplementation than age. Furthermore, another study by the same authors (Kent *et al.*, 2015a) also reported a significant reduction in SBP, after 6 and 12 weeks, in older (70+ years) participants with dementia after consuming sweet

Bing cherry juice for 12 weeks (200 mL.day^{-1}) compared to a control group. These participants presented elevated baseline SBP (138 mmHg), whilst DBP was normal (79 mmHg), perhaps explaining only a trend towards significance for DBP (Kent *et al.,* 2015a). Similarly, Keane *et al.* (2016b) reported a single bolus of 60 mL MTC concentrate (total anthocyanins 547 mg) significantly reduced SBP up to 3-hours post-ingestion compared to placebo, in 15 early hypertensive males. Peak reductions (7 mmHg) at 2-hours post-ingestion coincided with elevated PCA and VA concentrations, suggesting these secondary metabolites may be responsible for the observed effects. No differences were observed for arterial stiffness or endothelial function (microvascular vasodilation by laser Doppler imaging). Keane *et al.* (2016c) also reported 60 mL MTC concentrate significantly reduced SBP up to 3 hours post-ingestion with peak reductions of 6 mmHg 1-hour post-consumption compared to placebo in 27 middle-aged (45-60 years) participants. Observations from Kent's *et al.* (2015ab) and Keane's *et al.* (2016bc) research demonstrated acute supplementation of cherry juice improved BP and HR in participants with elevated baseline values.

More recently, Chai *et al.* (2018) reported significant reductions in SBP (4 mmHg) and LDL (0.05 mmol.L^{-1}) compared to a control group, after 12 weeks MTCJ consumption in 34 older (65-80 years) adults. In alignment with previous studies (Ataie-Jafari *et al.*, 2008; Kent *et al.*, 2015a; Keane *et al.*, 2016bc), elevated baseline SBP in the MTCJ group facilitated improvements. Improvements in LDL with MTCJ may be contrived since the high fructose content in the placebo was thought to spike LDL concentrations (Chai *et al.*, 2018). However, Chai *et al.* (2018) explained MTCJ may have been able to bind bile acids thus dampening the rise in LDL concentrations. Compared to baseline, MTCJ also significantly lowered total cholesterol and increased fasting glucose, triglycerides and BMI. Moreover, there was no effect on body mass, HDL, DBP, fasting insulin or HOMA-IR, likely due to healthy baseline values.

A strength of the study was participants maintained habitual diet throughout the supplementation period, upholding ecological validity.

The only study to have examined responses to tart cherries in humans with MetS was conducted by Johnson *et al.* (2017) (only abstract published). In a randomised, parallel-arm, single-blind trial, participants consumed either 480 mL of MTCJ (n = 9, 5M/4F) or isocaloric placebo (n = 10, 5M/5F) daily for 12 weeks. Endothelial function markers, oxidised LDL and soluble vascular cell adhesion molecule-1 (sVCAM-1), were significantly lowered with MTCJ compared to placebo after 12 weeks and total cholesterol tended (P = 0.08) to be lower; no other differences in the lipid profile were observed. Similarly, no differences between conditions were observed for body composition, glucose, insulin, HOMA, brachial BP, central haemodynamics, arterial stiffness (PWA and PWV), FMD, inflammatory or oxidative stress markers. The limited information relating to specific biomarkers, participant baseline characteristics, dietary guidelines and anthocyanin content of MTCJ hinders explanation of non-significant results. Yet, in a MetS population, long-term MTCJ consumption attenuated biomarkers involved in accelerating atherogenesis.

Mechanisms for improved BP with cherry supplementation have yet to be confirmed. Modulation of vasomotor tone through proper functioning of vascular smooth muscle cell (Keane *et al.*, 2016a), increased NO bioavailability (Kent *et al.*, 2015b; Keane *et al.*, 2016a; Chai *et al.*, 2018) and/or inhibition of ACE (Hidalgo *et al.*, 2012; Kirakosyan *et al.*, 2018) have been proposed.

As mentioned in *section 2.1.5.2.2,* anthocyanins have been shown to positively modulate gut microbiota; potentially explaining health promoting effects. In relation to gut microbiota modulation by anthocyanin-rich cherries, research is very limited. Currently, only one study (Mayta-Apaza *et al.*, 2018) has assessed the gut microbiota profile of humans *in vivo,* after

supplementing tart cherries. After consumption of 230 mL MTCJ for 5 days, significant reductions in *Bacteroides* and increases in *Firmicutes* (*Ruminococcus*, Lachnospiraceae, *Clostridium* and *Clostridium* XI, *Lactobacillus* and *Streptococcus*) were observed (Mayta-Apaza *et al.,* 2018). Unexpectedly, *Bifidobacterium* and *Faecalibacterium*, microbiota generally hallmarked for healthy gut microbiome, were lower in abundance after MTCJ consumption; other health-promoting Actinobacteria were increased (Mayta-Apaza *et al.*, 2018). The change in gut microbiota profile was influenced by carbohydrates in MTCJ (Mayta-Apaza *et al.*, 2018). Also, PCA, caffeic and *p*-coumaric acids in MTCJ were metabolised by most *Lactobacillus* strains, explaining the greater bioavailability of phenolic acid derivatives from MTCJ after gut microbial metabolism (Filannino *et al.*, 2015); which may be responsible for tart cherries' beneficial health responses.

2.1.6.5.3. Cherries and Health Summary

In summation, the positive effect of cherries in humans with elevated pre-existing cardio-metabolic values seems to be more pronounced than in healthy individuals. There is sufficient evidence that cherries improve markers associated with MetS in humans; however, research is necessitated in a MetS population itself. The mechanisms of action through which cherry phenolics may prevent or ameliorate chronic disease are summarised in Figure 2.7. Speculation remains over the ideal supplementation strategy to improve cardio-metabolic function. Strategies previously implemented were not always economically viable or examined under ecologically valid conditions (AbuMweis, Jew and Jones, 2010). Hence, subsequent chapters of this thesis aim to address these research pitfalls by manipulating aspects of MTC supplementation strategy and observing subsequent responses in healthy and individuals and those with MetS. Ultimately, studies in **chapters 4-6** were designed to uphold ecological validity and ensure economic viability to maximise impact and ease of translating basic research into daily habitual practice.

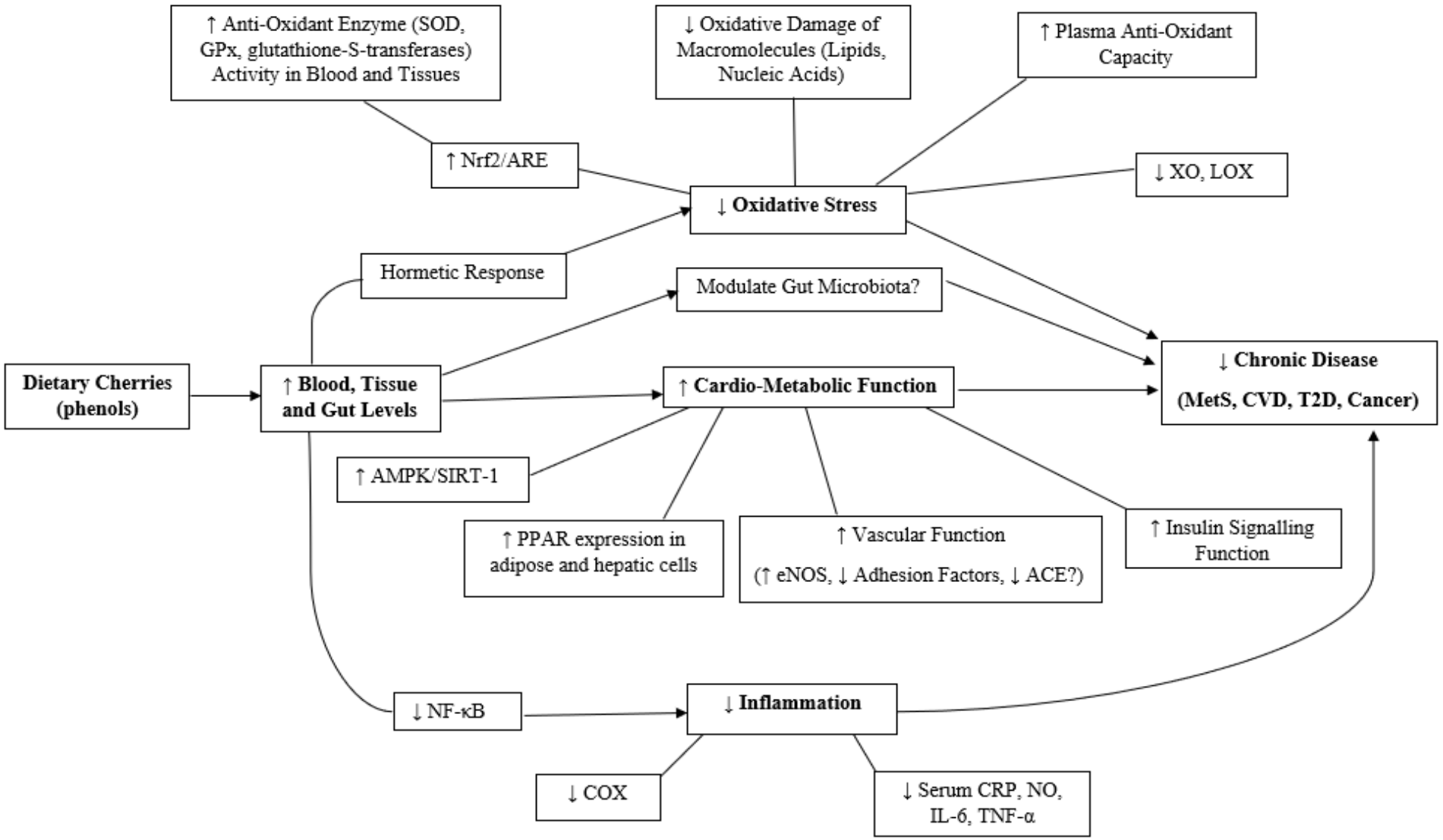


Figure 2.7. Proposed mechanisms of action through which cherry polyphenols act in the management of chronic disease. Adapted from Ferretti *et al.* (2010).

Part 2.2. Metabolic Syndrome

The second part of this literature review will assess the epidemiology, pathophysiology and interventions used to combat Metabolic Syndrome. A brief explanation and examination of the role of lipid metabolism in the aetiology of cardio-metabolic diseases will be presented, followed by a short commentary and analysis of calorie restriction and its mimetics. Finally, a detailed discussion of the effect of anthocyanins in humans with MetS will be provided.

2.2.1. Introduction

Obesity is generally recognised as storage of excessive body fat at various subcutaneous and visceral fat depots (Snel *et al.*, 2012); and global rates are rapidly rising annually (Ng *et al.*, 2014). In 1947, it was noted that obesity predisposed individuals to T2D and atherosclerosis, supporting observations from the 1920s of an association with hypertension, hyperglycaemia and hyperuricaemia (Kaur, 2014). The term 'Metabolic Syndrome' was first coined by Haller in 1977, when describing obesity, T2D, hyperlipoproteinaemia, hyperuricaemia and hepatic steatosis as risk factors associated with atherosclerosis (Haller, 1977). However, it was not until the 1988 Banting lecture, when Gerald Reaven proposed insulin resistance as the underlying cause of developing the cluster of risk factors previously stated, termed 'Syndrome X' (Reaven, 1988). Crucially however, Reaven omitted obesity as a risk factor for his 'Syndrome X' but it was later added. Many different terms have been used to describe the cluster of risk factors, although Metabolic Syndrome is now commonly used and has gained an International Classification of Diseases coding (E88.81) (Han and Lean, 2016).

A consequence of the distinctive cardio-metabolic features of MetS, is that over 5-10 years individuals with MetS have a 2- and 5-fold greater risk of developing CVD and T2D, respectively (Alberti *et al.*, 2009). Moreover, MetS patients have an increased risk of myocardial infarction (3- to 4-fold), stroke (2- to 4-fold) and dying from these events (2-fold)

than individuals without MetS (Alberti, Zimmet and Shaw, 2005), regardless of a previous history of CVD (Olijhoek *et al.*, 2004).

As it is increasingly apparent that MetS is a precursor of T2D and CVD (Wilson *et al.*, 2005), clinicians and researchers should be addressing MetS and its individual components with greater priority and importance; as a preventative measure to minimise the health, financial and social burden imparted on patients, health services and society in general.

2.2.2. Diagnosis

MetS is defined as a collection of interrelated physiological, biochemical, metabolic and clinical risk factors that directly augment the development of atherosclerotic CVD, T2D and all-cause mortality (Wilson *et al.*, 2005; Grundy *et al.*, 2006). Typically, an individual with MetS presents with abdominal obesity, pre-hyperglycaemia, pre-hypertension and atherogenic dyslipidaemia. However, this is often accompanied by endothelial dysfunction and a pro-inflammatory, pro-oxidant and pro-thrombotic state (Srikanthan *et al.*, 2016).

The diagnosis of MetS has proven to be an issue in clinical and epidemiological settings. Various organisations have attempted to outline criteria for diagnosing MetS (Table 2.4). All organisations agree on the core components for diagnosing MetS, however differences remain between thresholds of individual components and the specific combinations required to be met. Consequently, in 2009 a joint statement was issued (Alberti *et al.*, 2009), detailing obesity and insulin resistance need not be pre-requisites for MetS diagnosis, but any 3 of 5 criteria would suffice. The statement also detailed specific thresholds for waist circumference based on sex and ethnicity (Table 2.5). This extra stratification was important in delimiting the criteria used to diagnose MetS, particularly as ethnicity influences an individuals' predisposition to certain risk factors regardless of abdominal obesity severity (Han and Lean, 2016). Asians with a lower abdominal obesity cut-off are at greater risk of developing T2D than Europeans (Kaur, 2014).

Table 2.4. Criteria proposed for clinical diagnosis of MetS.

Clinical Measures	WHO (1998)	EGIR (1999)	NCEP-ATP III (2001)	AACE (2003)	IDF (2005)	Harmonised (Alberti *et al.*, 2009)
Insulin Resistance	High Insulin, IGT, IFG or T2D and 2 of the following:	High Fasting Insulin and 2 of the following:	No IR pre-requisite, any 3 of the following:	IGT or IFG (but not T2D) and any 2 of the following:	No IR pre-requisite	No IR pre-requisite, any 3 of the following:
Obesity	Waist-to-hip ratio Male: > 0.90; Female: > 0.85 and/or BMI > 30 $kg.m^{-2}$	Waist Circumference ≥ 94 cm (male) ≥ 80 cm (female)	Waist Circumference ≥ 102 cm (male) ≥ 88 cm (female)	BMI > 25 $kg.m^{-2}$	Ethnicity and sex specific Waist Circumference and any 2 of the following:	Ethnicity and sex specific Waist Circumference
Triglycerides	≥ 1.69 $mmol.L^{-1}$	≥ 2 $mmol.L^{-1}$	≥ 1.69 $mmol.L^{-1}$	≥ 1.69 $mmol.L^{-1}$	≥ 1.69 $mmol.L^{-1}$	≥ 1.69 $mmol.L^{-1}$
HDL	< 0.9 $mmol.L^{-1}$ (male) < 1 $mmol.L^{-1}$ (female)	< 1 $mmol.L^{-1}$	≥ 1.03 $mmol.L^{-1}$ (male) ≥ 1.29 $mmol.L^{-1}$ (female)	≥ 1.03 $mmol.L^{-1}$ (male) ≥ 1.29 $mmol.L^{-1}$ (female)	≥ 1.03 $mmol.L^{-1}$ (male) ≥ 1.29 $mmol.L^{-1}$ (female)	≥ 1.03 $mmol.L^{-1}$ (male) ≥ 1.29 $mmol.L^{-1}$ (female)
Blood Pressure	≥ 140/90 mmHg	≥ 140/90 mmHg or on anti-hypertensive medication	≥ 130/85 mmHg	≥ 130/85 mmHg	≥ 130/85 mmHg	≥ 130/85 mmHg
Glucose		IGT or IFG (but not T2D)	≥ 6.1 $mmol.L^{-1}$		≥ 5.6 $mmol.L^{-1}$ or T2D	≥ 5.6 $mmol.L^{-1}$
Other	Microalbuminuria: albumin: creatinine ratio of >30 $mg.g^{-1}$			Family history of T2D		

IGT (impaired glucose tolerance) defined as a 2 hour post glucose load of >7.8 $mmol.L^{-1}$ and <11.1 $mmol.L^{-1}$ (in the presence of a normal or elevated fasting glucose), and impaired fasting glucose (IFG) is defined as glucose levels >6.1 $mmol.L^{-1}$ and <6.9 $mmol.L^{-1}$. American Association for Clinical Endocrinology (AACE); Body Mass Index (BMI); European Group for the Study of Insulin Resistance (EGIR); Insulin Resistance (IR); International Diabetes Federation (IDF); National Cholesterol Education Program-Third Adult Treatment Panel (NCEP-ATP III); Type 2 Diabetes (T2D); World Health Organisation (WHO).

Table 2.5. Waist circumference thresholds based on ethnicity and sex (Alberti *et al.*, 2009).

Country/Ethnic Group	Waist Circumference Threshold (cm)	
	Male	Female
Europids	≥ 94	≥ 80
USA	≥ 102	≥ 88
Middle East and Eastern Mediterranean	Use Europid data until more specific data is available	
Sub-Saharan African	Use Europid data until more specific data is available	
South Asian (including Malay and Indian population)	≥ 90	≥ 80
Japanese	≥ 90	≥ 80
Chinese	≥ 90	≥ 80
South and Central American	Use South Asian data until more specific data is available	

2.2.3. Epidemiology

The prevalence and incidence rates of MetS by region, sex, ethnicity and age are difficult to ascertain given the variability in the diagnostic criteria used. It was estimated global prevalence rates of MetS ranges between <10% up to 84%, depending on the diagnosis criteria used and the population studied (region, sex, ethnicity and age) (Kaur, 2014). Approximately, a quarter of the global population are thought to have MetS, equating to 1.75 billion individuals (Saklayen, 2018).

Cross-sectional studies predominately from European countries approximate prevalence around 24.3%, where rates increase parallel with advancing age (Grundy, 2008; Scuteri *et al.*, 2015). Aguilar *et al.* (2015) estimated MetS prevalence rates in the United States increased by 1.8% from 2003-2004 to 2011-2012, with approximately 35% of all adults having MetS.

Of great concern is the increasing prevalence rates of MetS in children and adolescents. Prevalence in adolescents between National Health and Nutrition Examination Survey (NHANES) 1988-1994 and NHANES 1999-2000 increased from 4.2% to 6.4%, respectively (Kassi *et al.*, 2011). Prevalence between NHANES 2001-2006 was estimated to be 8.6%, indicating the upward trend in prevalence rates amongst adolescents (Johnson *et al.*, 2009).

The implications of such high prevalence rates are stark, as CVD is the leading cause of death worldwide, representing 31% of all deaths (World Health Organisation, 2017). Moreover, global prevalence of diabetes amongst individuals aged 18-99 years was 8.4% (451 million) as of 2017 and is expected to increase to 9.9% (693 million) by 2045, indicating a concomitant increase in CVD related deaths and placing evermore pressure on healthcare systems (Cho *et al.*, 2018). The global financial burden of treating T2D as of 2005 was thought to be in excess of 286 billion international dollars, rising to 396 billion international dollars by 2025;

accounting for 13% of the entire global healthcare budget (Yach *et al.*, 2006). Strikingly, these figures do not account for treating diabetes associated CVD complications.

Finally, it is important that past and future MetS prevalence statistics be generated based on the harmonised criteria set out by Alberti *et al.* (2009). This would help clinicians, epidemiologists and researchers detect trends and understand problems associated with MetS; in-turn assisting development of interventions to reduce MetS prevalence and incidence.

2.2.4. Pathophysiology

The aetiology of MetS has yet to be fully understood, however it is accepted that genetic and environmental factors are inherent to the underlying pathophysiology, suggesting epigenetic mechanisms (Kassi *et al.*, 2011). Specifically, visceral obesity and insulin resistance are considered to be central to MetS pathophysiology, with atherogenic dyslipidaemia, dysregulation of the renin-angiotensin-aldosterone system, endothelial dysfunction, hypercoagulable state and chronic stress contributing to its pathogenesis (Kaur, 2014) (Figure 2.8).

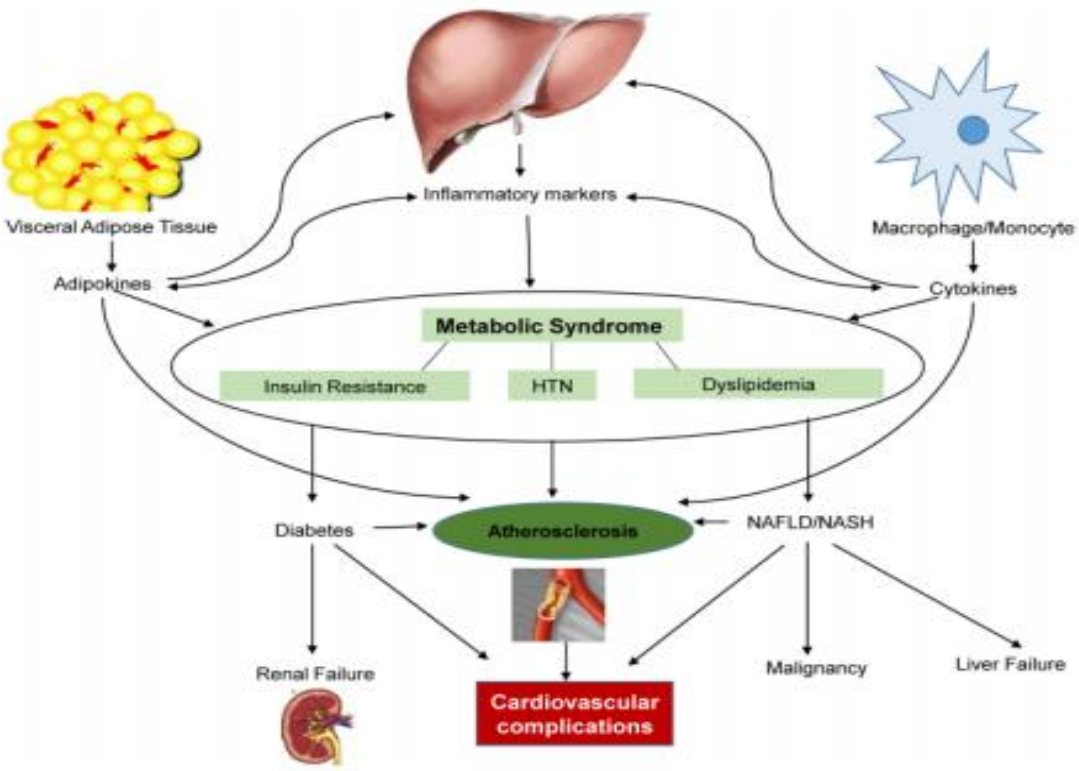


Figure 2.8. Schematic highlighting the interactions of adipokines, cytokines and inflammatory markers that contribute to the pathogenesis of MetS (Srikanthan *et al.*, 2016).

2.2.4.1. Obesity

Obesity manifests a state of chronic low-grade inflammation and oxidative stress, which underpins the development of insulin resistance; suggesting that at its core, MetS is a pro-oxidative and pro-inflammatory condition (Chiva-Blanch and Badimon, 2017).

Generally, adipose tissue, comprising of adipocytes and stromal vascular fraction, acts as the main site of lipid storage but also exerts an endocrine, inflammatory and oxidative stress response (Thompson, Pederick and Santhakumar, 2016). Such a response is mediated through secretion of adipokines, pro-inflammatory cytokines, free fatty acids (FFA), glycerol (Fernández-Sánchez *et al.*, 2011), ROS and species contributing to endothelial dysfunction from hypertrophied adipocytes (Table 2.6) (Thompson, Pederick and Santhakumar, 2016). The dysregulated secretion of FFAs, adipocytokines and thrombogenic factors facilitates the development of insulin resistance and all other components of MetS (Kaur, 2014).

Preventing adipocyte hypertrophy through augmenting fat mobilisation and oxidation would regulate adipocyte function and prevent ectopic accumulation in sites such as cardiac and skeletal muscle; thus, mitigate MetS related symptoms (Snel *et al.,* 2012). Ectopic accumulation is mediated by insulin-dependent suppression of white adipose tissue lipolysis and a concomitant reduction in FFA oxidation (Snel *et al.,* 2012); thus, dysregulating normal cellular function and inducing insulin resistance (Kwak, 2013). Therefore, normal lipid metabolism in skeletal muscle is crucial for ameliorating diseases associated with excessive lipid accumulation (Watt and Hoy, 2012). Skeletal muscle, accounting for ~40% of total body mass, is an adaptive organ with a plethora of physical and physiological functions, critical to maintaining whole-body homeostasis (Egan and Zierath, 2013). One physiological function is its role in lipid uptake, storage and oxidation (Watt and Hoy, 2012). Skeletal muscle is the most important target tissue for insulin action and is the main site of post-prandial glucose disposal (Roberts, Hevener and Barnard, 2014). Cellular signalling induced by ROS has been shown to be implicated in contraction-mediated increase in skeletal muscle glucose uptake (Merry and McConell, 2009). Hence, low concentrations of ROS, conforming to a hormetic response, produced during muscle contractions may be beneficial at preventing hyperglycaemia.

The ability of skeletal muscle to transition between fat and carbohydrate (CHO) oxidation under different physiological and environmental conditions is an important quality for maintaining homeostasis and is known as metabolic flexibility (Snel *et al.,* 2012). This transition is dependent on substrate availability where substrate oxidation dysfunction is associated with accumulation of intramuscular lipids and insulin resistance (Galgani, Moro and Ravussin, 2008). Due to their sedentary lifestyle, patients with MetS, obesity and T2D are unable to alter lipid oxidation rates to lipid availability resulting in intramuscular lipid accretion and metabolic inflexibility (Kelley *et al.*, 1999). Hence, reversing metabolic inflexibility by

facilitating fat oxidation at the skeletal muscle and whole-body levels would be an effective strategy to mitigate MetS (Roberts, Hevener and Barnard, 2014).

Table 2.6. Characteristic status of cardio-metabolic biomarkers in individuals with MetS.

Biomarker	Source	Status in MetS
Visceral Obesity		↑
Leptin	Adipocytes, VSM	↑
Adiponectin	Adipocytes	↓
Resistin	Adipocytes, SM, Stomach	↑
Visfatin	Adipocytes	↑
Ghrelin	Stomach	↓
Insulin Resistance		↑
Fasting Glucose	Liver	↑
HbA_{1c}	Erythrocytes	↑
Fasting Insulin	Pancreas	↑
Fasting C-peptide	Pancreas	↑
Dyslipidaemia		↑
Fasting FFAs	Adipocytes, Liver	↑
Fasting Triglycerides	Adipocytes, Liver	↑
Fasting Total Cholesterol	Liver, Intestines	↑
Fasting HDL	Liver, Intestines	↓
Small, dense LDL	Liver	↑
Fasting VLDL	Liver	↑
Hypertension		↑
Angiotensin II	Kidney	↑
Endothelial Dysfunction		↑
Nitric Oxide	Endothelium	↓
Adhesion Molecules	Endothelium	↑
ox-LDL	Adipocytes	↑
Pro-thrombotic State		↑
Fibrinogen	Liver	↑
PAI-1	Endothelium, Adipocytes	↑
Platelet Aggregation		↑
Pro-inflammatory State		↑
IL-6	Adipocytes, M1 macrophage	↑
TNF-α	Adipocytes, M1 macrophage	↑
CRP	Adipocytes	↑
IL-10	Monocytes, M2 macrophage	↓
Pro-oxidative State		↑
ox-LDL	Adipocytes	↑
Superoxide Dismutase	Cytosol and Mitochondria	↓
Glutathione Peroxidase	Mitochondria	↓

Catalase	Peroxisomes	↓
Malondialdehyde	Mitochondria	↑
Hyperuricaemia		↑
Uric Acid	Liver	↑
Microalbuminuria		↑
Urine Albumin	Kidney	↑

CRP (C-reactive protein); FFA (Free Fatty Acids); HbA_{1c} (Glycated Haemoglobin); HDL (High-density Lipoprotein); IL-6 (interleukin-6); IL-10 (interleukin-10); LDL (Low-density Lipoprotein); ox-LDL (oxidised LDL); PAI-1 (Plasminogen Activator Inhibitor-1); SM (Skeletal Muscle); TNF-α (Tumour Necrosis Factor Alpha); VLDL (Very low-density Lipoprotein); VSM (Vascular Smooth Muscle).

2.2.4.2. Insulin Resistance

Reaven proposed MetS pathophysiology stemmed from insulin resistance, based on the observed pleiotropic effects of insulin resistance on each component of MetS (Roberts, Hevener and Barnard, 2014). Insulin resistance defined by a state where secretion of normal insulin concentrations to a glucose load, does not produce the desired response of sufficient peripheral glucose uptake and suppression of hepatic glucose production; leading to hyperglycaemia, a component of MetS, and then hyperinsulinaemia (Lebovitz, 2001).

The development of insulin resistance in MetS manifests from excessive FFA production from visceral adipocytes. The ectopic deposition of FFAs particularly in hepatic and skeletal muscle parenchyma contributes to increased hepatic glucose production and impaired glucose uptake in skeletal muscle (de Luca and Olefsky, 2008) (Figure 2.9). Insulin resistance in these organs induces a state of hyperinsulinaemia which stimulates lipogenesis, inhibits lipolysis and further augments adipocyte hypertrophy in a vicious cycle (de Luca and Olefsky, 2008). Moreover, chronic hyperinsulinaemia due to a continuous insult on pancreatic β-cells leads to β-cell dysfunction, and prevents sufficient insulin production thus inducing hyperglycaemia, in another vicious cycle (Han and Lean, 2016).

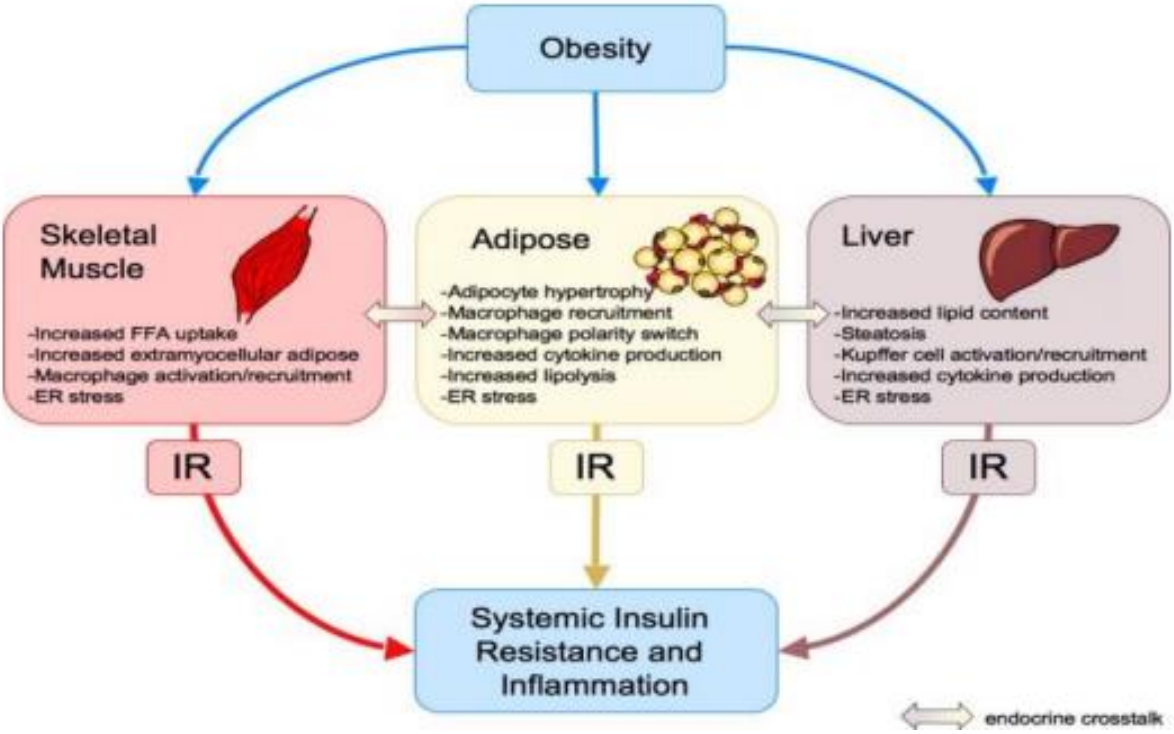


Figure 2.9. Manifestation of insulin resistance in various organs central to MetS (de Luca and Olefsky, 2008).

2.2.4.3. Dyslipidaemia

Dyslipidaemia in MetS is characterised by atherogenic hypertriglyceridaemia and hyperlipoproteinaemia (Kolovou, Anagnostopoulou and Cokkinos, 2005). Lipolysis and subsequent mobilisation of FFAs from adipose tissue is augmented under insulin resistant conditions, but also contributes to insulin resistance development (Kolovou, Anagnostopoulou and Cokkinos, 2005). Therefore, dyslipidaemia is not only a hallmark of MetS but also responsible for the underlying pathophysiology (Kaur, 2014). In addition to facilitating insulin resistance, the overproduction of FFAs acts as a substrate for hepatic triglyceride synthesis, eventually promoting LDL formation (Kolovou, Anagnostopoulou and Cokkinos, 2005). Triglyceride-rich LDL are hydrolysed into small, dense particles highly susceptible to oxidation, subsequently causing endothelial dysfunction and atherogenesis (Kolovou, Anagnostopoulou and Cokkinos, 2005).

2.2.4.4. Hypertension

Research indicates insulin resistance activates the sympathetic nervous and renin-angiotensin-aldosterone systems by expressing the potent vasoconstrictor angiotensin-II (Kaur, 2014).

The cellular cascade implicating insulin resistance and hypertension involves the mitogen-activated protein kinase (MAPK) pathway (Kaur, 2014). Under insulin resistant conditions, MAPK remains unaltered thus promotes production of the vasoconstrictor endothelin-1, expression of vascular cell adhesion molecules and proliferation of vascular smooth muscle cells (Zhou, Wang and Yu, 2014). Subsequently, this increases the risk of atheroma formation and arterial stiffness, which facilitates hypertension (Touyz *et al.*, 2018). Conclusive evidence has been provided showing the manifestation of arterial stiffness in individuals with MetS independent of age or sex (Schillaci *et al.*, 2005; Scuteri *et al.*, 2014).

2.2.5. Treatments

Given the pro-inflammatory and pro-oxidative pathophysiology of MetS, interventions which elicit anti-inflammatory and anti-oxidative responses are particularly sought after. Pharmacological drugs, exercise, calorie restriction and diet, especially polyphenols are interventions capable of exerting anti-inflammatory and anti-oxidative responses (Golbidi, Mesdaghinia and Laher, 2012). Lifestyle interventions are increasingly recommended as they are a safer and more tolerable alternative to pharmacological drugs. Significantly, diet and exercise interventions were found to reverse MetS, with these interventions being 87% more clinically effective than pharmacological therapy (Dunkley *et al.*, 2012).

2.2.5.1. Exercise and MetS

The importance of exercise in mitigating MetS has been repeatedly proven by numerous authors (Roberts, Hevener and Barnard, 2014). An inverse dose-response association was found between volume of exercise and MetS prevalence (Kaur, 2014). Individuals engaging in

moderate-intensity activity for 1 hour or less per week were at 60% greater risk of developing MetS than those exercising >3 hours per week (Kaur, 2014). Few studies have used exercise as an intervention in individuals with MetS *per se*, however these studies demonstrated various forms of exercise reversed MetS or reduced the number of MetS components (Roberts, Hevener and Barnard, 2014). Importantly, in a cohort of 19,223 males aged 20-83 years, this translated into lower mortality risk as evidenced by 5.18% mortality risk in healthy, unfit individuals, 5.15% in non-exercising individuals with MetS and 2.69% in exercising individuals with MetS (Katzmarzyk, Church and Blair, 2004). Notably, exercising individuals with MetS had a significantly lower mortality risk than apparently healthy, sedentary individuals.

The ability to transition from carbohydrate to fat oxidation when in a glucose-depleted state is impaired in metabolically inflexible subjects, thus individuals oxidise carbohydrate with and without insulin stimulation (Kelley *et al.*, 1999). Exercise reduces insulin resistance by activating adenosine monophosphate kinase (AMPK) and thus augmenting fat oxidation (Auwerx *et al.*, 2010). The ability to stimulate fat oxidation with exercise and thus reverse metabolic inflexibility and insulin resistance is an effective strategy to prevent MetS and/or treat its complications (Roberts, Hevener and Barnard, 2014).

2.2.5.1.1. FATMAX Exercise and Fat Oxidation

The development of cardio-metabolic risk factors is associated with impaired fat oxidation (Robinson *et al.*, 2016). Robinson *et al.* (2015) demonstrated maximal fat oxidation (MFO) rate during exercise is associated with 24-hour fat oxidation rate and insulin sensitivity as markers of long-term metabolic health. Hence, augmenting MFO rates may prove useful for optimising cardio-metabolic health; thus, researchers set out to determine optimal fat oxidation rates during exercise (Achten and Jeukendrup, 2004).

Romijn *et al.* (1993) were one of the first to assess fat oxidation rates over a range of exercise intensities. Fat oxidation rate was greatest at 65% $\dot{V}O_2$max compared to 25% and 85% $\dot{V}O_2$max, in trained athletes (Romijn *et al.,* 1993). Achten, Gleeson and Jeukendrup (2002) devised a protocol to more accurately determine MFO, using a greater range of intensities. The incremental protocol was conducted on a cycle ergometer amongst 18 moderately trained males and consisted of starting at 95 W with increments of 35 W every 5 minutes until respiratory exchange ratio (RER) was >1. This protocol allowed identification of MFO, FATMAX (the intensity of MFO conveyed as %$\dot{V}O_2$max), FATMAX zone (area where fat oxidation is within 10% of FATMAX) and regions of low fat oxidation rates (FATMIN) from a FATMAX curve (Figure 2.10). FATMAX occurred at 64% $\dot{V}O_2$max corresponding to 74% HR_{max} (maximal heart rate) with an MFO of 0.6 $g.min^{-1}$ and FATMIN at >89% $\dot{V}O_2$max (92% HR_{max}) (Achten, Gleeson and Jeukendrup, 2002).

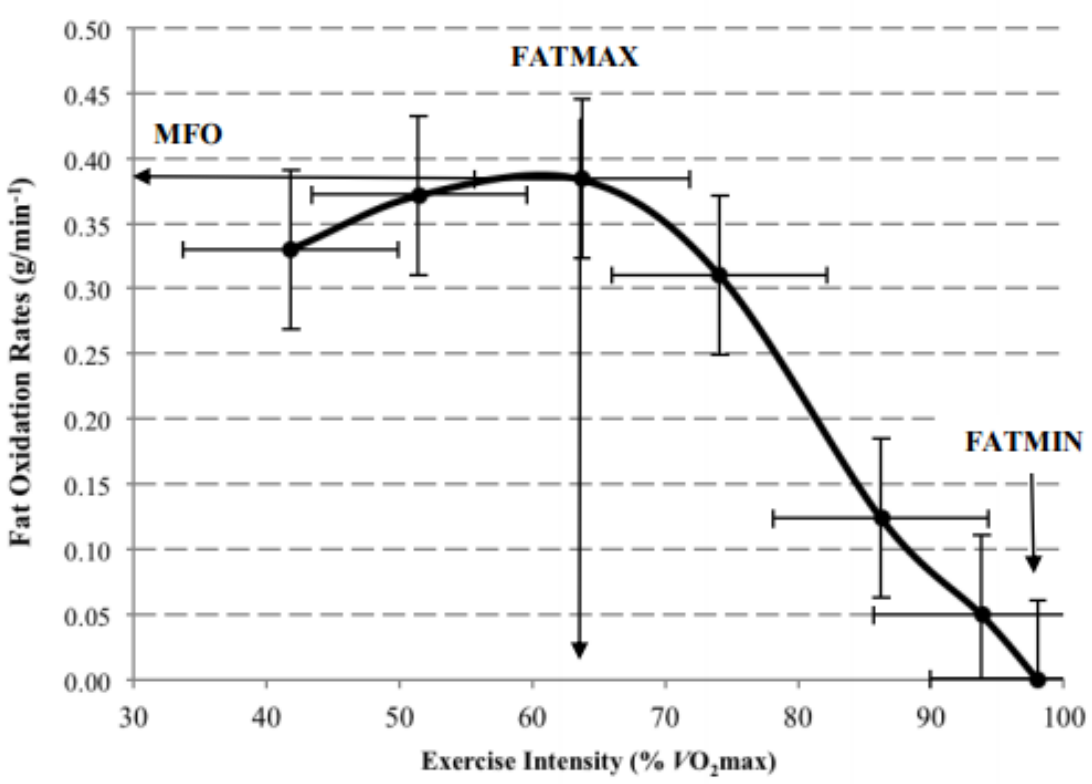


Figure 2.10. Graph depicting a typical fat oxidation curve determined from an incremental exercise protocol (Randell, 2013).

Venables, Achten and Jeukendrup (2005), showed individuals with markedly different $\dot{V}O_2max$ had similar fat oxidation rates; demonstrating high inter-individual variance in fat oxidation rates. Subsequently, studies implementing a protocol at a set percentage of $\dot{V}O_2max$ or W_{max} (percentage of maximal power output) should be criticised. Due to the large variation, identifying a universal exercise intensity for MFO is not possible. Therefore, identification of individual FATMAX using an incremental test with stringent control over diet and exercise at least 24-36 hours before the test, is considered the most reliable method of determining valid FATMAX intensity (Meyer, Gäßler and Kindermann, 2007).

2.2.5.1.2. Health Effects of Exercise at FATMAX

Individualised FATMAX exercise provides an opportunity to oxidise lipids, particularly from skeletal muscle, at accelerated rates thus potentially ameliorating symptoms associated with MetS (Brun, Romain and Mercier, 2011).

Sari-Sarraf *et al.* (2015) recommended FATMAX training in weight loss and health-related exercise programs for individuals with MetS. FATMAX training effectively reduced total body mass and fat mass, spared FFM and, reduced cholesterol and HbA_{1c} in patients with T2D (Brun, Romain and Mercier, 2011).

Continuous running at FATMAX (4 weeks, 5 days per week) for 1 hour induced 44% greater fat oxidation and improved insulin-sensitivity index by 27% compared to interval exercise at FATMAX in 8 males with obesity (Venables and Jeukendrup, 2008). Dumortier *et al.* (2003) reported similar findings with 8 weeks cycling at FATMAX compared to no exercise in 28 MetS patients. Compared to the controls, the training group lost significantly more body mass, fat mass and MFO increased from 0.12 to 0.18 $g.min^{-1}$ (Dumortier *et al.*, 2003). The transition from fat to carbohydrate oxidation (crossover point) occurred significantly later in the exercising group (31.5% v 52.6% $\dot{V}O_2max$) and FATMAX occurred at higher intensities

(27.7% vs 44.8% W_{max}). The increased fat oxidation rate and loss of fat mass may have contributed to the significantly improved insulin sensitivity and resistance indexes (HOMA) after FATMAX training compared to controls (Dumortier *et al.*, 2003).

Similar results were obtained when 136 women with obesity cycled at either FATMAX for 55 minutes, 60% $\dot{V}O_2max$ for 35 minutes or home-based moderate-intensity exercise for 30 minutes (4 times per week for 5 months) (Besnier *et al.*, 2015). In addition, all participants were provided with a diet rich in fruit and vegetables (5 pieces per day) throughout the study. Anthropometrics, BP and lipid profile were not significantly different between groups, likely due to the relatively low MFO rates (0.153 $g.min^{-1}$). However, all exercise interventions significantly reduced fat mass, HOMA-IR and augmented MFO after 5 months. Notably, MFO was significantly greater during FATMAX exercise resulting in improved glycaemic control, likely due to reduced insulin and HOMA-IR.

Mechanistically, the beneficial effect of fat oxidation during FATMAX exercise is thought to be accentuated by the release of adiponectin, leptin and the anti-inflammatory myokine IL-6, via phosphorylation and thus activation of AMPK (Brun, Romain and Mercier, 2011). Since exercise stimulates skeletal muscle contractions, expression of IL-6 is increased 100-fold during prolonged exercise (Roberts, Hevener and Barnard, 2014). The release of the myokine IL-6 has pleiotropic effects including enhancing lipolysis and glucose disposal, inhibiting pro-inflammatory molecules TNF-α and IL-1β, and expressing anti-inflammatory cytokines interleukin-1 receptor antagonist (IL-1ra) and IL-10 (Roberts, Hevener and Barnard, 2014). Mechanisms behind improved insulin resistance and sensitivity relate to increased glycogen synthase and GLUT-4 expression (Roberts, Hevener and Barnard, 2014). Activation of pathways emanating from improved skeletal muscle function, mediated by exercise, would be of significant benefit for the amelioration of insulin resistance and associated morbidities.

A meta-analysis of FATMAX training in patients with obesity, MetS and T2D confirmed a shift of fat oxidation to higher exercise intensities and reductions in body mass, fat mass, waist circumference, insulin sensitivity and total cholesterol (Romain *et al.*, 2012). Overall, exercise at individual FATMAX may ameliorate the symptoms associated with MetS, and prevent the onset of the syndrome in healthy, asymptomatic individuals (Brun, Romain and Mercier, 2011).

2.2.5.1.3. Polyphenolic Interventions, Exercise and Fat Oxidation

Given the benefits that increasing fat oxidation during exercise has on cardio-metabolic health, dietary interventions to further augment rates have been researched (Jeukendrup and Randell, 2011; Kim and Park, 2016). Table 2.7 details studies examining polyphenol-rich supplements in tandem with exercise on cardio-metabolic parameters. Polyphenols have been identified in *in vitro* and pre-clinical human trials to increase fat oxidation through a myriad of mechanisms including inhibition of catechol-*O*-methyltransferase and malonyl-coA and activation of sirtuin-1 (SIRT-1), AMPK, PPARα and carnitine palmitoyltransferase 1 (Rupasinghe *et al.*, 2016).

Table 2.7. Studies examining the effects of polyphenol supplementation in combination with exercise on substrate metabolism, particularly fat oxidation, and its effects on symptoms associated with cardio-metabolic diseases.

Study	Study Design, Sample Size and Cohort	Supplementation Strategy	Study Details	Results with Intervention compared to Placebo	Conclusion
Venables *et al.* (2008)	Randomised, Crossover, Placebo Controlled 12 healthy, males (50.9 mL.kg.min^{-1} $\dot{V}O_{2peak}$)	24 hours before participants consumed 3 capsules containing either decaffeinated GTE (total: 890 mg polyphenols and 366 mg EGCG) or placebo.	30 mins cycling at 60% $\dot{V}O_{2peak}$ 2-hour OGTT	↑ 17% Fat Oxidation ↑ EE from Fat ↑ Plasma glycerol ↓ Serum insulin ↑ 13% Insulin Sensitivity	Fat oxidation and lipolysis during exercise and insulin sensitivity at rest improves with GTE consumption.
Eichenberger, Colombani and Mettler (2009)	Randomised, Crossover, Placebo Controlled, Double-Blinded 10 endurance trained males (55 mL.kg.min^{-1} $\dot{V}O_{2peak}$)	Caffeinated GTE (total polyphenols: 160 mg.day^{-1}; EGCG: 70 mg.day^{-1}) for 3 weeks.	2 hours cycling at 50% W_{max}	↑ HDL at rest No difference: Fat Oxidation Body Fat % RER EE TG LDL Cholesterol	These non-significant findings may be a result of supplementing greatly reduced amounts of polyphenols daily compared to Venables *et al.* (2008).
Allgrove *et al.* (2011)	Randomised, Crossover, Placebo Controlled, Single-Blinded 10 healthy males (53.1 mL.kg.min^{-1} $\dot{V}O_{2peak}$)	40 g Dark Chocolate (catechins: 15.6 mg, epicatechins 38.7 mg) or placebo for 2 weeks and 2 hours before exercise bout.	90 mins cycle at 60% $\dot{V}O_2$max with intermittent 30 second sprints at 90% $\dot{V}O_2$max every 10 mins to simulate road race	↑ FFA ↓ F_2-isoprostanes ↓ Oxidised LDL Tendency for ↓ RER No difference: TG Glucose Insulin Lactate TAS Cortisol	Catechins and epicatechins from dark chocolate improve markers of oxidative stress and lipid metabolism after prolonged cycling exercise, thus highlighting the efficacy of polyphenols from various dietary sources on health markers after exercise.

Alkhatib (2014)	Randomised, Crossover, Placebo Controlled, Double-Blinded 14 (7 male, 7 females), healthy	1000 mg Yerba Maté (*Ilex paraguariensis*) or placebo. Consumed 1 hour before exercise.	Incremental cycling test Initiated at and increased by 0.5 $W.kg^{-1}$ of body mass	During exercise: ↓ RER ↑ Fat Oxidation at 40% and 50% $\dot{V}O_{2peak}$ ↑ EE from Fat	Yerba Maté increases fat oxidation at low exercise intensities primarily due to the thermogenic properties of supplement.
Randell *et al.* (2014)	Randomised, Crossover, Placebo Controlled, Double-Blinded 19 healthy, males (55.4 $mL.kg.min^{-1}$ $\dot{V}O_{2peak}$)	Decaffeinated GTE (total catechins 284 $mg.day^{-1}$; EGCG 156 $mg.day^{-1}$) or placebo for 1, 7 and 28 days.	30 mins cycle at 50% W_{max}	No difference: Fat Oxidation FFA Glycerol	No difference in fat oxidation and lipid markers between acute and long-term supplementation.
Alkhatib *et al.* (2015)	Randomised, Crossover, Placebo Controlled, Double-Blinded 12 recreationally active, male and female	4.5g of either placebo or multi-ingredient (caffeine, green tea, Yerba Maté, capsaicin and guarana) supplement. Consumed 150 mins before exercise.	150 mins rest before exercise 30 mins FATMAX cycling	↑ Fat Oxidation during rest ↓ RPE	Fat oxidation increased at rest through the thermogenic properties of the supplement.
Cook *et al.* (2015)	Randomised, Crossover, Placebo Controlled, Double-Blinded 14 healthy males (53 $mL.kg.min^{-1}$ $\dot{V}O_{2peak}$)	300 mg active cassis NZBE (containing 105 mg anthocyanins) per day for 7 days or placebo.	Cycle at 45, 55 and 65% $\dot{V}O_{2peak}$ for 10 mins at each intensity	↑ 27% Fat Oxidation at 65% $\dot{V}O_2max$ ↓ RER at 65% $\dot{V}O_{2peak}$ Tendency ↑ RER and Fat Oxidation at 45% and 55% $\dot{V}O_{2peak}$	Anthocyanin rich NZBE increases fat oxidation possibly via upregulation of PPARα.

Roberts *et al.* (2015)	Randomised, Parallel, Placebo Controlled, Double-Blinded 14 healthy, males (39 mL.kg.min^{-1} $\dot{V}O_{2peak}$)	Decaffeinated GTE (total catechins 571 mg.day^{-1}; EGCG 400 mg.day^{-1}) or placebo for 4 weeks.	1-hour cycle at 50% $\dot{V}O_{2peak}$ at baseline, weeks 2 and 4. Additional 1-hour cycle three times per week during supplementation period.	↑ 24.9% Fat Oxidation ↓ RER ↑ EE from Fat ↓ EE from CHO ↓ RPE ↓ Body Fat %	Long term supplementation of decaffeinated GTE is required to elevate fat oxidation rate.
Cook *et al.* (2017b)	Randomised, Crossover, Placebo Controlled, Double-Blinded 15 endurance-trained males (57 mL.kg.min^{-1} $\dot{V}O_{2peak}$)	300, 600 or 900 mg.day^{-1} active cassis NZBE (containing 105 mg anthocyanins per 300 mg capsule) for day for 7 days or placebo.	2-hour cycle at 65% $\dot{V}O_{2max}$	↓ RER with 600 and 900 mg ↑ 21.5% Fat Oxidation with 600 mg ↑ 24.1% Fat Oxidation with 900 mg	Dose-dependent increase in fat oxidation with anthocyanin-rich NZBE. However, only high anthocyanin doses exceeding habitually consumed levels alter substrate utilisation.
Solverson *et al.* (2018)	Randomised, Crossover, Placebo Controlled, Double-Blinded 17 overweight and obese males	High-fat (40% of energy from fat) diet which contained either 600 g.day^{-1} blackberries (1500 mg.day^{-1} flavonoids) or a calorie matched diet for 7 days.	30 minutes treadmill walking (3 mph)	↓ RER ↑ 12% Fat Oxidation during exercise ↑ 14% Fat Oxidation at rest ↓ Insulin AUC ↓ HOMA-IR	Blackberry anthocyanins increase fat oxidation rate at rest and during exercise in an overweight/obese population. Improved metabolic function in response to high CHO load.

AUC (Area Under Curve); CHO (Carbohydrate); EE (Energy Expenditure); EGCG (Epigallocatechin gallate); FFA (Free Fatty Acid); GTE (Green Tea Extract); HDL (High-density Lipoprotein); HOMA-IR (Homeostatic Model Assessment for Insulin Resistance); LDL (Low-density Lipoprotein); NZBE (New Zealand Blackcurrant Extract); OGTT (Oral Glucose Tolerance Test); PPARα (Peroxisome Proliferator-Activated Receptor Alpha); RER (Respiratory Exchange Ratio); RPE (Rating of Perceived Exertion); TAS (Total Anti-oxidant Status); TG (Triglycerides).

Table 2.7 suggests enhanced metabolic flexibility, through greater fat oxidation, may have contributed to the observed improvements in lipid, insulin and oxidative stress biomarkers. Cook *et al.* (2015, 2017b) provided encouraging results that fat oxidation rate increases with anthocyanin-rich New Zealand blackcurrant extract (NZBE) supplementation during exercise. The mechanism was hypothesised to relate to AMPK activation and thus inhibition of acetyl-coA carboxylase and upregulation of PPARα mRNA (Cook *et al.*, 2015; Cook *et al.*, 2017b). Furthermore, blackberries, containing a different anthocyanin profile (cyanidin-3-glucoside) compared to blackcurrants (delphinidin-3-rutinoside, cyanidin-3-rutinoside, delphinidin-3-glucoside, cyanidin-3-glucoside), increased fat oxidation during short-duration, low-intensity exercise in an overweight/obese but otherwise healthy population (Solverson *et al.*, 2018). Indicating various anthocyanins can increase fat oxidation during exercise in different populations. Although the literature is limited, these studies provide promising data that tart cherry anthocyanins may also augment fat oxidation rate during exercise and subsequently improve cardio-metabolic markers.

2.2.5.2. Diet and MetS

Various dietary strategies have been implemented against MetS, including calorie restriction, macronutrient manipulation, provision of vitamins, minerals, probiotics, prebiotics, synbiotics and polyphenols (de la Iglesia *et al.*, 2016; He and Shi, 2017). Some of these are discussed in greater depth below.

2.2.5.2.1. Calorie Restriction

Calorie restriction (CR) is an intervention requiring the reduction of daily calorie intake normally by 10-40%, but maintaining provisions of essential macronutrients and micronutrients, thus preventing malnutrition (Testa *et al.*, 2014). CR operates primarily on

reducing body mass and subsequently visceral adiposity; and has been practiced for centuries to prolong longevity and improve general wellbeing (Speakman and Mitchell, 2011).

Humans under CR tend to lower metabolic rate, energy expenditure and shift substrate metabolism towards greater fat oxidation (Martin *et al.*, 2007; Redman *et al.*, 2007). Improvements in MetS components such as body composition, fasting blood glucose, lipid profile, insulin sensitivity, BP, CRP, IL-6 and TNF-α have been noted in adults undergoing 30% CR (approximately 1800 kcal.day^{-1}) for periods between 2-15 years (Fontana *et al.*, 2004). Furthermore, 6 weeks calorie restriction (600-800 kcal.day^{-1}) in individuals with MetS resulted in rapid body mass loss (-8 kg) and significantly improved glucose, insulin, triglycerides and leptin concentrations compared to individuals without MetS (Xydakis *et al.*, 2004). It has been postulated that CR may regulate impaired substrate metabolism thus preventing MetS and other cardio-metabolic disease development (Speakman and Mitchell, 2011).

The mechanisms of action for these responses are similar to those exerted by exercise (Huffman, 2010) and dietary interventions, such as polyphenols (Testa *et al.*, 2014). Hence, exercise and polyphenols are considered CRMs.

2.2.5.2.1.1. Biological Mechanisms

A variety of biological mechanisms have been proposed to explain the effects of CR on longevity and wellbeing (Figure 2.11). The health effects of the most studied topic in the ageing/nutrition field, CR, are based on the hormetic concept (Martucci *et al.*, 2017). Other pathways through which CR may prolong lifespan include autophagy, nucleotide repair, anti-oxidant activity and metabolic shift involving sirtuins (Rattan, 2008).

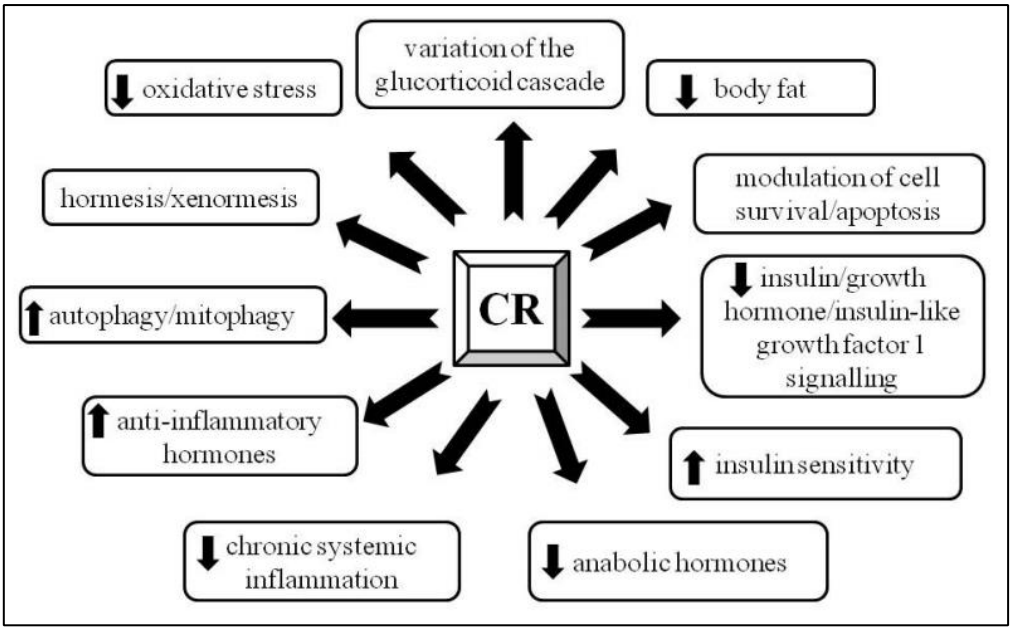


Figure 2.11. Potential biological mechanisms suggested to extend lifespan and healthspan via CR (Testa *et al.*, 2014).

2.2.5.2.1.2. Molecular Mechanisms

Researchers hypothesise regulation of physiological processes and lifespan extension in humans may be mediated via evolutionary conserved mechanisms (Lee and Min, 2013).

The insulin signalling pathway is key to the health benefits observed with CR (Testa *et al.*, 2014). Downregulation of insulin signalling increases forkhead box protein O1 (FOXO1) activity, a transcription factor involved in metabolic control (Testa *et al.*, 2014). Insulin via protein kinase B (Akt) induces irreversible phosphorylation and consequently nuclear exclusion of FOXO1 (Nakae *et al.*, 2001). Thus, dampening gluconeogenesis and glycogenolysis via downregulation of glucose-6-phosphatase transcription (Nakae *et al.*, 2001). Nuclear exclusion of FOXO1 also removes inhibition of PPARγ, thus enabling adipocyte maturation and therefore adipogenesis (Nakae *et al.*, 2003). This demonstrates the importance between the insulin signalling pathway and subsequent molecular cascades on glucose and lipid metabolism (Nakae *et al.*, 2003).

Sirtuin proteins are implicated in numerous cascades involving ageing, metabolism and stress resistance (Testa *et al.*, 2014). Sirtuins are classed as histone deacetylases and are therefore involved in epigenetic mechanisms (Mai *et al.*, 2008). SIRT-1 modulates inflammatory responses by inhibiting NF-κB and promotes fat oxidation in skeletal muscle by activating peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) and its coactivator PPARα (Salminen *et al.*, 2008). SIRT-1 also alters substrate metabolism by simultaneously inhibiting PPARγ and reducing glycolytic flux, therefore enhancing lipid mobilisation from white adipose tissue and subsequent oxidation (Picard *et al.*, 2004). SIRT-1 and AMPK are co-activated during CR, and both share common targets including PGC-1α, FOXO, NF-κB and endothelial nitric oxide synthase (eNOS) (Ruderman *et al.*, 2010). Dysregulation of SIRT-1 and AMPK has been attributed as a cause for the development of MetS (Ruderman *et al.*, 2010). AMPK is a critical enzyme implicated in substrate metabolism and plays a pivotal role during CR as it is activated during conditions of energy and nutrient depletion (Bordone and Guarente, 2005). AMPK alters substrate metabolism towards fatty acid oxidation and simultaneously inhibits cholesterol and triglyceride synthesis and lipogenesis (Canto and Auwerx, 2011). Additionally, AMPK improves insulin sensitivity and resistance to inflammation and oxidative stress (Canto and Auwerx, 2011).

Although adherence to prolonged CR (≥6 weeks) is difficult and discomforting for humans, the benefits of CR to improve cardio-metabolic health have been repeatedly observed (Larson-Meyer *et al.*, 2006). Biological and molecular cascades have been identified and therefore interventions which focus on these pathways should be examined to provide a targeted approach for maximising the benefits of CR. CRMs simulate the molecular and physiological effects of CR without reducing long-term caloric intake and its associated difficulties. Polyphenols are such compounds that activate similar mechanistic pathways as CR (Testa *et al.*, 2014).

2.2.5.2.1.3. Polyphenols as Calorie Restriction Mimetics

Polyphenols including resveratrol, EGCG (epigallocatechin-3-gallate), curcumin and quercetin have been identified as potential CRMs (Madeo *et al.*, 2014). Resveratrol, found in grape skins, has demonstrated the greatest effect as a CRM by activating and mimicking SIRT-1 (Li, Daniel and Tollefsbol, 2011). Resveratrol and quercetin allosterically modulate SIRT-1 and therefore suppress genes through epigenetic histone deacetylation (Bass *et al.*, 2007). Quercetin is present in tart cherry, therefore tart cherries may epigenetically modulate genes associated with CR, to improve cardio-metabolic function and extend lifespan.

2.2.5.2.2. Polyphenols and MetS

This section focusses on the effects of polyphenols and particularly anthocyanins on cardio-metabolic health in humans with MetS, although much work had been conducted in *in vitro* and animal models.

The effect of polyphenols on aspects of MetS has been studied extensively in various populations, with largely encouraging results (Chiva-Blanch and Visioli, 2012; Patti *et al.*, 2018). However, in comparison the influence of polyphenols in humans with MetS has received much less attention, with variable responses on certain components of MetS (Amiot, Riva and Vinet, 2016), thus more data is required to formulate conclusive recommendations in this population.

A negative association between MetS rates and polyphenol consumption has been observed based on epidemiological data (Chiva-Blanch and Badimon, 2017). Specifically, the highest tertile of flavonoid consumption was associated with a 31% lower risk in developing MetS, amongst 1265 participants after a 6-year follow-up (Sohrab *et al.*, 2018). Grosso *et al.* (2017) demonstrated the highest quartile of polyphenol intake amongst 8800 individuals presented

with significantly lower triglycerides, waist circumference and BP; highlighting the potential of polyphenols in combating MetS in various cohorts.

A brief summary of the effects of polyphenolic classes and sub-classes on cardio-metabolic, inflammatory and oxidative stress markers in humans with MetS is provided herein. Strong evidence from multiple studies demonstrated green tea catechins reduced waist circumference, BMI, body fat, LDL and malondialdehyde (Amiot, Riva and Vinet, 2016). Cocoa flavanols improved glucose, insulin resistance, HDL, LDL and BP but not CRP, total cholesterol, triglycerides or waist circumference (Shrime *et al.*, 2011). Polymers of flavanols, ellagitannins and proanthocyanidins beneficially modulated glucose (Selma *et al.*, 2018) and the lipid profile (Bladé, Arola and Salvadó, 2010) only. Citrus flavanone supplementation increased FMD by 2.48% and HDL, while significantly decreasing apolipoprotein B, total cholesterol and the inflammatory markers, CRP, amyloid A and E-selectin compared to placebo (Rizza *et al.*, 2011). Additionally, soy isoflavone supplementation improved LDL concentrations, body mass, body fat percentage and waist circumference but not BP, HDL or triglycerides (Amiot, Riva and Vinet, 2016). Lastly, the flavanol quercetin, was shown to reduce SBP, TNF-α and ox-LDL but not fasting blood glucose or triglycerides (Egert *et al.*, 2009), while the individual effects of flavones have yet to be examined in humans with MetS.

Of the non-flavonoid polyphenols, the stilbenoid, resveratrol has been associated with the most research, yet findings are equivocal in humans with MetS. Resveratrol was unable to change triglycerides, glucose (Fujitaka *et al.*, 2011; Dash *et al.*, 2013; van der Made, Plat and Mensink, 2015), HDL or inflammatory markers (Fujitaka *et al.*, 2011; van der Made, Plat and Mensink, 2015), however apoB lipoproteins (Dash *et al.*, 2013), BP (van der Made, Plat and Mensink, 2015) and endothelial function (Fujitaka *et al.*, 2011) were improved in individual studies. Lastly, high intake of lignans was associated with significantly lower waist circumference, but also higher fasting glucose and triglycerides (Grosso *et al.*, 2017).

In all, the effects of polyphenols in humans with MetS are largely positive. To strengthen this evidence, further research is required in larger cohorts to recommend polyphenols as a prophylactic or therapeutic intervention against MetS.

2.2.5.2.2.1. Anthocyanins and MetS

A major polyphenol sub-class not mentioned above were anthocyanidins and their glycosylated compounds, anthocyanins. Naseri *et al.* (2018) recently published a review of anthocyanins in the management of MetS. Findings indicated anthocyanins improved pathologies associated with MetS, including body composition, insulin resistance, insulin sensitivity, hyperglycaemia, dyslipidaemia and pre-hypertension (Naseri *et al.*, 2018). Anthocyanins also maintained endothelial function through heightened anti-inflammatory activity and mediated pancreatic β-cell protection from hyperglycaemia-induced oxidative stress (Naseri *et al.*, 2018). The mechanisms are purported to be through activation of AMPK and its downstream transcription factors (FOXO1, PPARs), modulation of adipokines, inhibition of NF-κB, induction of Nrf2 transcription and increased eNOS expression (Tsuda, 2008, 2016; Naseri *et al.*, 2018). However, much of the evidence was derived from *in vitro* and animal studies. So, what are the effects of anthocyanins on humans with MetS and are there any discernible trends? The following section provides a detailed review of the effects of supplementing various forms of anthocyanin-rich foods and extracts in humans with MetS.

Studies were selected based on specific criteria namely: intervention group included humans diagnosed with MetS; provision of whole foods, extracts or liquids predominately rich in anthocyanins; assessment of biomarkers relating to MetS criteria or associated pathophysiology (insulin resistance, inflammation, oxidative stress and endothelial function). In total 16 studies were included (Table 2.8), with a total sample size of 643 participants (healthy and MetS), of which 355 had MetS and consumed the anthocyanin-rich intervention.

Table 2.8. Overview of trials examining the effects of anthocyanin-rich interventions on health biomarkers in humans with MetS.

Form of Supplementation	Authors	Study Design	Participant Characteristics	Supplementation Strategy	Total Daily ACN Content	Dietary Control	Results (sig changes are effect of INT relative to PLA, unless specified)
Liquid	Basu *et al.* (2009)	Pre-Post Intervention Design	N=16 (all women) age 51 years; (BMI 38.6 $kg.m^{-2}$)	4 weeks INT: 240 mL juice twice daily (50 $g.day^{-1}$ freeze-dried strawberries reconstituted in water)	INT: 154 mg	Usual diet. Avoid products containing berries, cocoa, green tea and soy.	Post-supplement changes relative to baseline ↓ TC, LDL, MDA, HNE ↔ BM, WC, SBP, DBP, glucose, TG, HDL, VLDL, adiponectin, CRP, ox-LDL
	Basu *et al.* (2010a)	Randomised, Parallel, Placebo Controlled, Single-Blinded	N=48 (4 male, 44 female) INT: N=25 age 52 years; (BMI 38.1 $kg.m^{-2}$) CON: N=23 age 48 years; (BMI 37.5 $kg.m^{-2}$)	8 weeks INT: 240 mL juice twice daily (50 $g.day^{-1}$ freeze-dried blueberries reconstituted in water) (~350 g fresh blueberries). CON: 480 mL water twice daily	INT: 742 mg CON: 0 mg	Usual diet. Avoid products containing berries, cocoa, green tea and soy.	↓ SBP, DBP, ox-LDL, MDA, HNE ↔ BM, WC, HbA_{1c}, HOMA-IR, glucose, insulin, TG, TC, HDL, LDL, CRP, IL-6, sVCAM-1, sICAM-1, adiponectin
	Basu *et al.* (2010b)	Randomised, Parallel, Placebo Controlled	N=27 (2 male, 25 female) INT: N=15 age 48 years; (BMI 39 $kg.m^{-2}$) CON: N=12 age 45 years; (BMI 36.4 $kg.m^{-2}$)	8 weeks INT: 240 mL juice twice daily (50 $g.day^{-1}$ freeze-dried strawberries reconstituted in water) (~500 g fresh strawberries). CON: 480 mL water twice daily	INT: 154 mg CON: 0 mg	Usual diet. Avoid products containing berries.	↓ TC, LDL, small LDL particles, VCAM-1 ↔ WC, glucose, TG, HDL, VLDL, SBP, DBP, sICAM-1
	Basu *et al.* (2011)	Randomised, Parallel, Placebo Controlled, Double-Blinded	N=31 (all female); age 52 years; BMI 40 $kg.m^{-2}$	8 weeks INT: 240 mL cranberry juice twice daily. CON: 240 mL placebo twice daily	INT: 24.8 mg CON: 0 mg	Usual diet. Avoid products containing berries, cocoa, green tea and soy.	↓ ox-LDL, MDA, HNE ↑ TAC ↔ WC, SBP, DBP, glucose, TG, HDL, LDL, IL-6, CRP

	Simão *et al.* (2013)	Randomised, Parallel, Single-Blinded	N=56 (14 male, 42 female); age 50 years; BMI 32.5 kg.m^{-2}	8 weeks INT: 700 mL.day^{-1} cranberry juice. CON: Normal diet (no drink supplied)	INT: 66 mg CON: 0 mg	Usual diet.	↑ Folic Acid, Adiponectin ↓ Homocysteine, Lipoperoxidation and Protein Oxidation ↔ IL-1, IL-6, TNF-α
	Stull *et al.* (2015)	Randomised, Parallel, Placebo Controlled, Double-Blinded	N=44 (16 male, 28 female) INT: N=23; age 55 years; (BMI 35.2 kg.m^{-2}) CON: N=21 age 59 years; (BMI 36 kg.m^{-2})	6 weeks INT: 340 mL yoghurt and skim-milk smoothie twice daily (45 g.day^{-1} freeze-dried blueberries). CON: 340 mL placebo twice daily	INT: 290.3 mg CON: 0 mg	Usual diet. Avoid products containing berries.	↑ Endothelial Function ↔ BM, BF%, SBP, DBP, glucose, insulin, insulin sensitivity, TG, TC, HDL, LDL, 24-hr SBP, 24-hr DBP
	Johnson *et al.* (2017) * *Data obtained from abstract only.	Randomised, Parallel, Placebo Controlled, Single-Blinded	N=19 (10 males, 9 females) age 20-60 years INT: N=9 CON: N=10	12 weeks INT: 480 mL Montmorency tart cherry juice CON: 480 mL isocaloric placebo	INT: NR CON: NR	NR	↓ ox-LDL, sVCAM-1 ↔ TC, SBP, DBP, aortic haemodynamics, arterial stiffness
Capsule	Barona *et al.* (2012a)	Randomised, Crossover, Placebo Controlled, Double-Blinded	N=25 (all male); age 51 years; BMI 31.5 kg.m^{-2}	4 weeks INT: 46 g.day^{-1} grape powder (2 servings/day grapes = 160 g) CON: 46 g.day^{-1} placebo powder	INT: 35.42 mg CON: 0 mg	Usual diet. Avoid products containing berries, tea, grapes and wine.	↑ Peak FMD ↓ SBP, sICAM-1 ↔ BM, WC, DBP, TG, HDL, glucose, NOx, sVCAM-1
	Barona *et al.* (2012b)	Randomised, Crossover, Placebo Controlled, Double-Blinded	N=24 (all male); BMI 31.9 kg.m^{-2} N=11 MetS with dyslipidaemia; age 48 years N=13 MetS without dyslipidaemia; age 54 years	4 weeks INT: 46 g.day^{-1} grape powder (equivalent 252 g grapes) CON: 46 g.day^{-1} placebo powder	INT: 35.42 mg CON: 0 mg	Usual diet. Avoid products containing berries, tea, grapes and wine.	↑ Adiponectin, IL-10, iNOS mRNA in MetS without dyslipidaemia ↓ SBP ↔ IL-6, IL-8, TNF-α, SOD mRNA, GPx mRNA, ox-LDL, 8-isoprostanes

Broncel *et al.* (2010)	Randomised, Parallel, Single-Blinded	N=47 (15 men, 32 female); age 42-65 years N=22 healthy controls (BMI 24.15 $kg.m^{-2}$) N=25 MetS patients (BMI 31.05 $kg.m^{-2}$)	8 weeks INT: 100 mg Chokeberry Extract three times daily. CON: Normal diet (no capsules supplied)	INT: NR CON: 0 mg	NR	↓ SBP, DBP, Endothelin-1, TC, LDL, TG, TBARS, Catalase ↑ HDL, SOD, GPx, Fibrinogen ↔ BM, WC, CRP
Gurrola-Díaz *et al.* (2010)	Randomised, Parallel, Placebo Controlled, Single-Blinded	N=124 (42 males, 82 female); age 49 years N=73 healthy controls N=51 MetS patients (INT 1: n=27) (INT 2: n=26) (INT 3: n=20)	4 weeks INT 1: MetS on 100 $mg.day^{-1}$ Hibiscus sabdariffa extract powder INT 2: MetS on preventative diet INT 3: Preventative Diet + 100 $mg.day^{-1}$ Hibiscus CON: Non-MetS individuals + 100 $mg.day^{-1}$ Hibiscus	INT 1 and 3: 19.24 mg CON: 19.24 mg	Diet individually adjusted for each subject in INT 2, providing 30% energy from fat (~7% of saturated fat), 55% from carbohydrates, and 15% from protein, with ~200 mg cholesterol/day. Fibre content ranged from 20 to 30 g.	INT 1: ↓ TC, TG:HDL, glucose, LDL ↑ HDL ↔ SBP, DBP, TG, VLDL INT 2: ↑ HDL ↔ SBP, DBP, LDL, VLDL, glucose, TC, TG INT 3: ↓ TG, VLDL, glucose, SBP, DBP ↑ HDL ↔ TC CON: ↓ TG ↔ TC, TG, HDL, LDL, VLDL, glucose, SBP, DBP
Jeong *et al.* (2014)	Randomised, Parallel, Placebo Controlled, Double-Blinded	N=73 (36 males, 37 females) INT: N=38; age 58 years (BMI 26.3 $kg.m^{-2}$) CON: N=35; age 60 years (BMI 25.1 $kg.m^{-2}$)	12 weeks INT: 750 $mg.day^{-1}$ Black raspberry extract CON: 750 $mg.day^{-1}$ placebo powder	INT: 26 mg CON: 0 mg	NR	↓ TC, TC:HDL, IL-6, TNF-α ↑ brachial FMD, adiponectin ↔ TG, HDL, LDL, apoA1, apoB, CRP, sVCAM-1, sICAM-1

	Jeong *et al.* (2016)	Randomised, Parallel, Placebo Controlled, Double-Blinded	N=50 (23 males, 27 females) INT: N=25; age 55 years (BMI 24.7 kg.m^{-2}) CON: N=25; age 61 years (BMI 25.9 kg.m^{-2})	12 weeks INT: 750 mg.day^{-1} black raspberry extract CON: 750 mg.day^{-1} placebo powder	INT: 26 mg CON: 0 mg	All participants were instructed to follow a diet based on ''Dietary Approaches to Stop Hypertension'' (DASH) diet for controlling MetS. Also avoid any berry species.	↑ Adiponectin ↓ AIx, IL-6 and TNF-α ↔ SBP, DBP, central SBP, HR, CRP, sVCAM-1, sICAM-1
	Sikora *et al.* (2012)	Randomised, Parallel, Controlled, Single-Blinded	N=52 (42-65 years) INT: N=38; MetS CON: N=14; Healthy	8 weeks INT: 100 mg three times daily of chokeberry extract CON: No treatment	INT: 60 mg CON: 0 mg	Low fat diet. 30% calories from fat. Avoid products with chokeberry.	↓ TC, TG, LDL, platelet aggregation and coagulation ↔ WC, HDL
Whole Food	Puupponen-Pimiä *et al.* (2013)	Randomised, Parallel, Double-Blinded	N=32 (13 males, 19 females) INT: N=20 age 52 years (BMI 31.8 kg.m^{-2}) CON: N=12 age 50 years (BMI 32.9 kg.m^{-2})	8 weeks INT: Berry Mix 100 g strawberry puree, 100 g frozen raspberries and 100 g frozen cloudberries CON: Normal diet (no berries supplied)	INT: 70.7 mg CON: 0 mg	Berry consumption substituted evenly with the other carbohydrates in habitual diet for berry mix group.	↑ Leptin ↔ SBP, DBP, TC, LDL, HDL, resistin, 8-isoprostane
	Kolehmainen *et al.* (2012)	Randomised, Parallel, Placebo Controlled, Single-Blinded	N=27 (8 males, 19 females) INT: N=15 age 53 years (BMI 31.4 kg.m^{-2}) CON: N=12 age 50 years (BMI 32.9 kg.m^{-2})	8 weeks INT: 200 g bilberry puree, 40 g dried bilberries (Equivalent to 400 g fresh bilberries) CON: Normal diet (80 g.day^{-1} berry consumption allowed)	INT: 1381 mg CON: NR	Berry consumption substituted evenly with the other carbohydrates in habitual diet for bilberry group. Avoid all other berry product	↓ IL-12, Inflammation score (combination of CRP, IL-6, IL-12 and LPS) ↔ BM, WC, %BF, SBP, DBP, glucose, insulin, insulin sensitivity, TC, TG, HDL, LDL, apoA1, apoB, IL-6, CRP, adiponectin, leptin, LPS

Augmentation Index (AIx); Apolipoprotein A1 (apoA1); Apolipoprotein B (apoB); Body Fat Percent (%BF); Body Mass (BM); Body Mass Index (BMI); C-reactive protein (CRP); Control (CON); Diastolic Blood Pressure (DBP); Flow-mediated Dilatation (FMD); Glutathione Peroxidase (GPx); Glycated Haemoglobin (HbA_{1c}); Heart Rate (HR); High-density Lipoprotein (HDL); Hydroxynonenal (HNE); Homeostatic Model Assessment of Insulin Resistance (HOMA-IR); Interleukin (IL); Inducible Nitric Oxide (iNOS); Intervention (INT); Lipopolysaccharide (LPS); Low-density Lipoprotein (LDL); Malondialdehyde (MDA); Metabolic Syndrome (MetS); Nitric Oxide (NOx); Oxidised LDL (ox-LDL); Superoxide Dismutase (SOD); Soluble Intercellular Cell Adhesion Molecule (sICAM); Soluble Vascular Cell Adhesion Molecule (sVCAM); Systolic Blood Pressure (SBP); Total Anti-oxidant Capacity (TAC); Thiobarbituric Acid Reactive Substances (TBARS); Total Cholesterol (TC); Triglycerides (TG); Tumour Necrosis Factor alpha (TNF-α); Vascular Cell Adhesion Molecule (VCAM); Very Low-density Lipoprotein (VLDL); Waist Circumference (WC).

Overall, it is possible to discern trends from the studies above. Anthropometric changes in body mass and waist circumference were not significantly improved with any anthocyanin intervention. This may be due to short supplementation length and/or parallel research design where responses were compared in separate individuals, thus succumbing to inter-individual variance in genetics and behavioural patterns (habitual diet and physical activity). SBP improved in more studies than DBP, however BP responses remained unchanged in most studies. Of note, BP improvements were only observed in participants with elevated baseline levels.

Glucose levels remained unchanged in 6/7 studies, likely due to healthy baseline values and/or provision of high daily anthocyanin dosages. Metabolism of the sugar moieties from anthocyanins may have contributed to elevating glucose; masking any bioactive effects of phytochemicals (Faria *et al.*, 2009). This may explain parallel glycaemic and insulinaemic responses in the three studies to measure these markers, as daily anthocyanin dosages were the three highest of all studies.

In accordance with Wallace, Slavin and Frankenfeld (2016) and Yang *et al.* (2017), anthocyanin supplementation improved total cholesterol and LDL, but not other aspects of the lipid profile (triglycerides, HDL, VLDL and apolipoproteins). Improvements manifested only in individuals who were dyslipidaemic at baseline (Vendrame *et al.*, 2016; Wallace, Slavin and Frankenfeld, 2016).

Improvements in cardio-metabolic markers are thought to be due to the anti-inflammatory and anti-oxidative properties of anthocyanins (Vendrame *et al.*, 2016). In the MetS population, 4/9 studies improved inflammatory biomarkers and 6/8 studies improved oxidative stress biomarkers. The wide inter-individual variability in inflammatory markers may have limited the anti-inflammatory effect of anthocyanin supplementation and the ability to observe

differences against the control group. However, 3 of 5 studies did report upregulation of the anti-inflammatory adipocytokine, adiponectin (Tsuda, 2008), despite no changes in systemic pro-inflammatory markers (CRP and cytokines). Subsequently, anthocyanin-rich interventions in 7/9 studies, altered inflammatory milieu towards an anti-inflammatory state in individuals with MetS. Hence, a strong effect of anthocyanin-rich interventions on endothelial function markers was observed; where 9/11 studies improved. The anti-oxidant potential of anthocyanins was evidenced by a clear trend towards reductions in oxidative stress biomarkers. Given that inflammation and oxidative stress contribute to the underlying pathophysiology of MetS, anthocyanin-rich foodstuffs are a promising intervention for this population based on the evidence above.

2.2.5.2.2.1.1. Influence of Anthocyanin Supplementation Strategy

Previous reviews of anthocyanin-rich interventions on cardio-metabolic markers have mentioned the inability to determine the effective form (matrix), dosages and duration of supplementation, due to high variability between research designs, a lack of dose-response studies and standardised clinical biomarkers (Basu and Lyons, 2012; Reis *et al.*, 2016). This variability is highlighted in Table 2.8; however, some trends are discernible, which may influence the design of future clinical trials with MetS populations.

As previously mentioned in *section 2.1.4.1*, the form of supplementation influences the bioavailability of anthocyanins and other phytochemicals (Bohn, 2014). Accordingly, Table 2.8 indicated greater benefits with capsules over liquids and whole foods for SBP and lipid profile, despite on average fewer total anthocyanins being provided with capsules over the duration of supplementation. This is the first time such a trend has been noted in any population with supplementation of anthocyanin-rich sources. A possible reason may be due to the targeted release of anthocyanins afforded by the capsule shell; augmenting bioavailability and enabling anthocyanins to exert their bioactive functions more effectively.

Equally, the lack of effect from whole foods may be due to poor bioavailability despite consumption of high daily doses. Liquid supplements had no effect on glycaemic, insulinaemic and some lipid profile variables, possibly due to the high sugar content inducing a glycaemic stress that outweighed the benefits of the phytochemicals (Vendrame *et al.*, 2016). However, total cholesterol and LDL were reduced with liquid supplements as they normally possess the highest anti-oxidant content of any matrix (Basu and Lyons, 2012). The most common trend with liquid supplements was its consistent reduction of oxidative stress and subsequent improvement in endothelial function.

Each food source has a unique anthocyanin profile producing different phenolic acid metabolites (Fang, 2015). Analysis of the type of food and its effect on particular biomarkers revealed some trends. Blueberries and bilberries containing delphinidin were unable to significantly modulate body mass, waist circumference or the lipid profile, however endothelial function was improved with blueberries (Vendrame *et al.*, 2016). Moreover, foods rich in delphinidin have been purported to have positive metabolic effects (Overall *et al.*, 2017), however only hibiscus extract was found to lower plasma glucose concentration. Malvidin-based anthocyanins exert positive cardiovascular effects (Overall *et al.*, 2017), which complies with improvements in SBP and endothelial function from grape interventions. Pelargonidin has been shown to decrease lipid accumulation (Belwal *et al.*, 2017), explaining the reduction of total cholesterol and LDL with strawberries. The radical scavenging and inhibitory lipid peroxidation effects of cyanidin and pelargonidin (Tsuda *et al.*, 1996) likely explain the reduction in oxidative stress with cranberries, chokeberries and strawberries. Consequently, the improvements in endothelial function with cranberries, chokeberries, strawberries, tart cherries and black raspberries may be attributed to the anti-oxidant activity of these anthocyanidins. Furthermore, hypolipidaemic effects of cyanidin-rich extracts (Valcheva-Kuzmanova *et al.*, 2007) may explain subsequent improvements in endothelial function and BP with chokeberries.

Significant anti-inflammatory effects were observed with foods containing mainly cyanidin and peonidin glycosides, such as black raspberries and bilberries; induced by their phenolic acid metabolites PCA and VA (Fang, 2015). Furthermore, cyanidin and peonidin glycosides can be metabolically transformed to each other by methylation and demethylation (Fang, 2014), thus further contributing to PCA and VA production.

Differences in supplement strategies (dosage and duration) between studies make deciphering trends difficult (Reis *et al.*, 2016; Wallace, Slavin and Frankenfeld, 2016), particularly without dose-response data. Studies providing <100 $mg.day^{-1}$ anthocyanins, improved SBP more than >100 $mg.day^{-1}$ dosages. Prolonged supplementation beyond 8 weeks was ineffective on BP. A trend for reducing total cholesterol and LDL was observed for anthocyanin dosages <154 $mg.day^{-1}$. Lipid profile alterations tended to be influenced more by the presence or absence of dyslipidaemia at baseline. Pro-inflammatory and oxidative stress markers were inclined to greater reductions at anthocyanin dosages between 26-35 $mg.day^{-1}$ and ≥154 $mg.day^{-1}$, respectively. Lastly, dosage seemed not to affect endothelial function, however prolonged supplementation corresponded to greater improvements.

Humans consume anthocyanins commonly from their diet (Wallace, Slavin and Frankenfeld, 2016), thus dietary restrictions in human trials require examination to provide ecologically valid recommendations. Anthocyanin bioactivity is influenced by its interactions with fibre, lipids, complex carbohydrates and other anti-oxidants (Yang *et al.*, 2017). Surprisingly, only one study maintained usual diet resulting in reduced lipid peroxidation and greater adiponectin (Simão *et al.*, 2013). This design is the most ecologically valid, thus more data is required when mimicking normal daily-living conditions to accurately assess the effect of anthocyanin-rich interventions.

Bohn (2014) mentioned polyphenol bioavailability may be augmented when various polyphenols are consumed together since they synergistically facilitate uptake, absorption and distribution pathways. Perhaps this may explain non-significant improvements for BP, glucose, insulin, triglycerides and HDL with polyphenol and berry restricted diets. Furthermore, improvements in total cholesterol, LDL, inflammation and endothelial function may be a consequence of other polyphenols consumed from the habitual diet. Endothelial function and BP effects from black raspberry anthocyanins were likely masked by the incorporation of the DASH diet in addition to berry restriction (Jeong *et al.*, 2016). Total cholesterol, oxidative stress and endothelial function tended to improve with polyphenol-restricted diets indicating an effect primarily of anthocyanins in humans with MetS. Low fat diets were found to improve the lipid profile, primarily total cholesterol and LDL. The studies to substitute dietary carbohydrates with berries both provided whole food anthocyanin-rich interventions. These interventions were ineffective on all biomarkers, except inflammation and oxidative stress, potentially due to the fibre content of whole foods which has been shown to attenuate polyphenol bioavailability (Bohn, 2014).

2.2.5.2.2.2. Summary of Effects of Anthocyanins on MetS

Overall, there is sufficient evidence to suggest anthocyanin-rich interventions may help ameliorate Metabolic Syndrome in this population. Specifically, anthocyanin-rich interventions in a human MetS population improved biomarkers central to MetS pathophysiology namely, total cholesterol, LDL, inflammation, oxidative stress and endothelial function. Other biomarkers displayed more equivocal findings, due to differences in study design, high baseline inter-individual variability of biomarkers and habitual diet; potentially masking any benefits of the anthocyanin-rich interventions.

A recurring theme from Table 2.8 indicated individuals benefitted more from anthocyanin-rich interventions if they had developed MetS features, leading to abnormal baseline values. The

efficacy of polyphenol interventions in individuals at risk of developing MetS compared to those with MetS remains unknown (Cherniack, 2011).

In relation to practical implications, depending on the biomarkers requiring improvement consideration of the matrix used is recommended. More studies are required to judge the most effective form of supplementation for improving cardio-metabolic, inflammatory and oxidative biomarkers in humans with MetS. Future studies are also required with purified anthocyanins compared against whole foods, to determine whether dietary interactions with anthocyanins influence their bioactivity. This would also enable ecologically valid assessments of whether a whole food anthocyanin-rich diet is more beneficial for individuals with MetS, compared to an anthocyanin supplemented diet.

Despite these interventions possessing a high anthocyanin content, the synergistic influence of other polyphenols, micronutrients and/or fibre on the health-promoting responses should be considered. Consequently, these interventions may be incorporated as part of a healthy, varied diet and not a magic-bullet solution for ameliorating complications in humans with MetS.

Finally, the question is posited, can anthocyanin-rich tart cherries improve biomarkers associated with MetS? Johnson *et al.* (2017) reported tart cherry juice improved markers of endothelial function (ox-LDL and sVCAM-1) and tended to lower total cholesterol after 12 weeks consumption compared to a placebo drink, in individuals with MetS (*section 2.1.6.5.2*). Despite this solitary study in humans (Johnson *et al.*, 2017), observations from *in vitro* (Seeram, 2001; Keane *et al.*, 2016b; Kirakosyan *et al.*, 2018) and rodent (Seymour *et al.*, 2008, 2009) studies suggest tart cherries are an intervention with great promise for preventing and ameliorating MetS and its complications. Further research examining responses to tart cherries in humans with MetS is clearly warranted and will be addressed as part of this thesis.

Part 2.3. Application of *Caenorhabditis elegans* for Human Health

The final section encompasses a brief review and commentary of *Caenorhabditis elegans* and its use as a model organism to understand the responses and mechanisms of action through which dietary interventions, particularly MTCJ, act and how they may be applied to human health. The literature reviewed here informs the original research conducted in **chapter 7**.

2.3.1. Introduction to *Caenorhabditis elegans*

In 1965, Sydney Brenner was the first to exploit the soil nematode *Caenorhabditis elegans* as a model organism (Brenner, 1974); for which his work on genetic regulation of organ development and programmed cell death in *C. elegans* won him, Robert Horvitz and John Sulston the Nobel Prize for Physiology or Medicine in 2002 (Dijkstra, 2002).

The nematode worm, *C. elegans*, has a short life-cycle with the egg-to-egg cycle lasting 3-4 days (Figures 2.12 and 2.13) at 20°C. The egg-laying adult is 1.3-1.5 mm long and has a lifespan of 2-3 weeks under suitable living conditions (Markaki and Tavernarakis, 2010). Adult hermaphrodites have 959 somatic cells, forming different organs and tissues including muscle, hypodermis (skin), intestine, reproductive organs, glands, and a nervous system containing 302 neurons (Kaletta and Hengartner, 2006) (Figure 2.14). Adults exist predominately as self-fertilising hermaphrodites each capable of producing ~300 genetically identical offspring, although male worms appear at a rate of <0.2% in the population (Corsi, Wightman and Chalfie, 2015). The body of *C. elegans* is transparent making it is easy to track cells, observe internal organs and follow biological processes such as fat metabolism with fluorescent dyes and reporters (Kaletta and Hengartner, 2006).

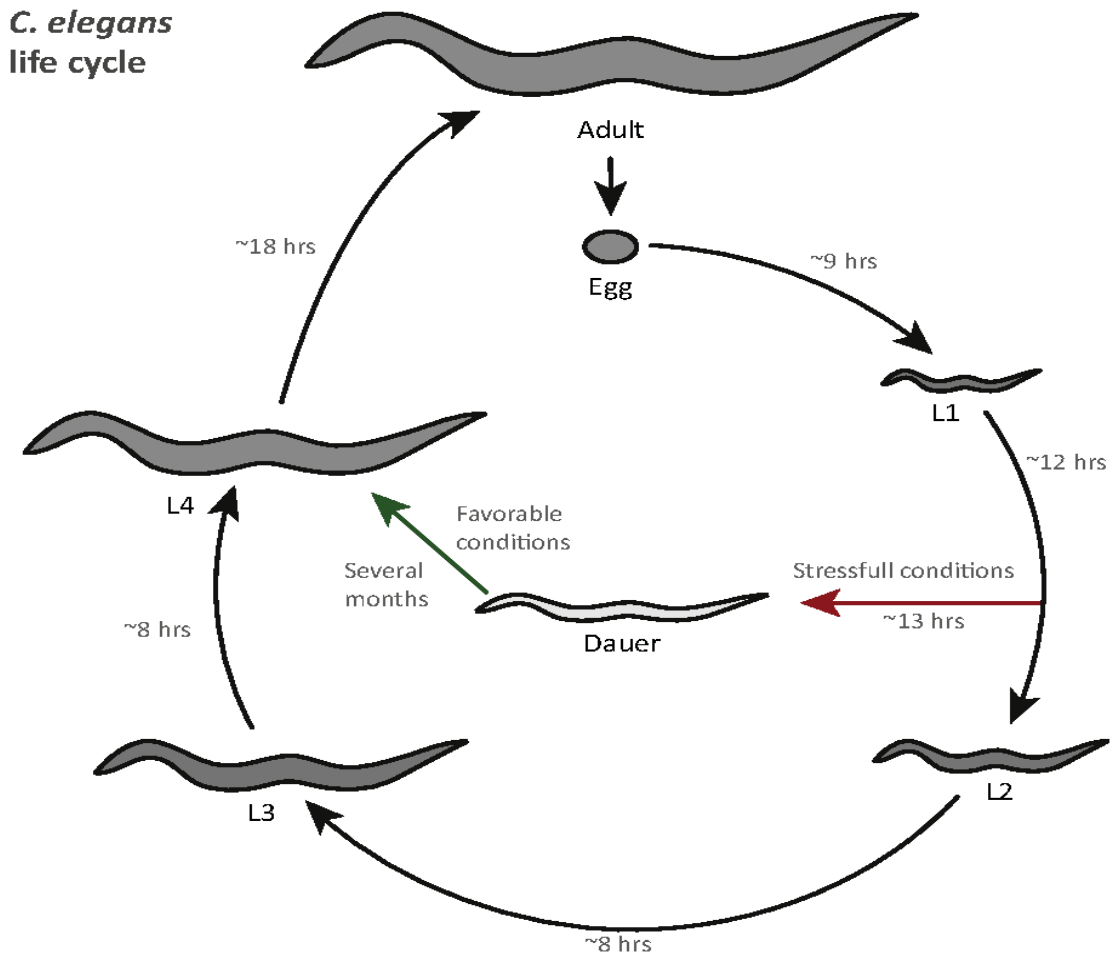


Figure 2.12. Life cycle of *C. elegans* at 22°C. Numbers along the arrows represent hours (h) necessary to transition from one stage to the next. Stressful conditions include lack of food, high population density and environmental conditions (thermal, hypoxic, osmotic and oxidative stress).

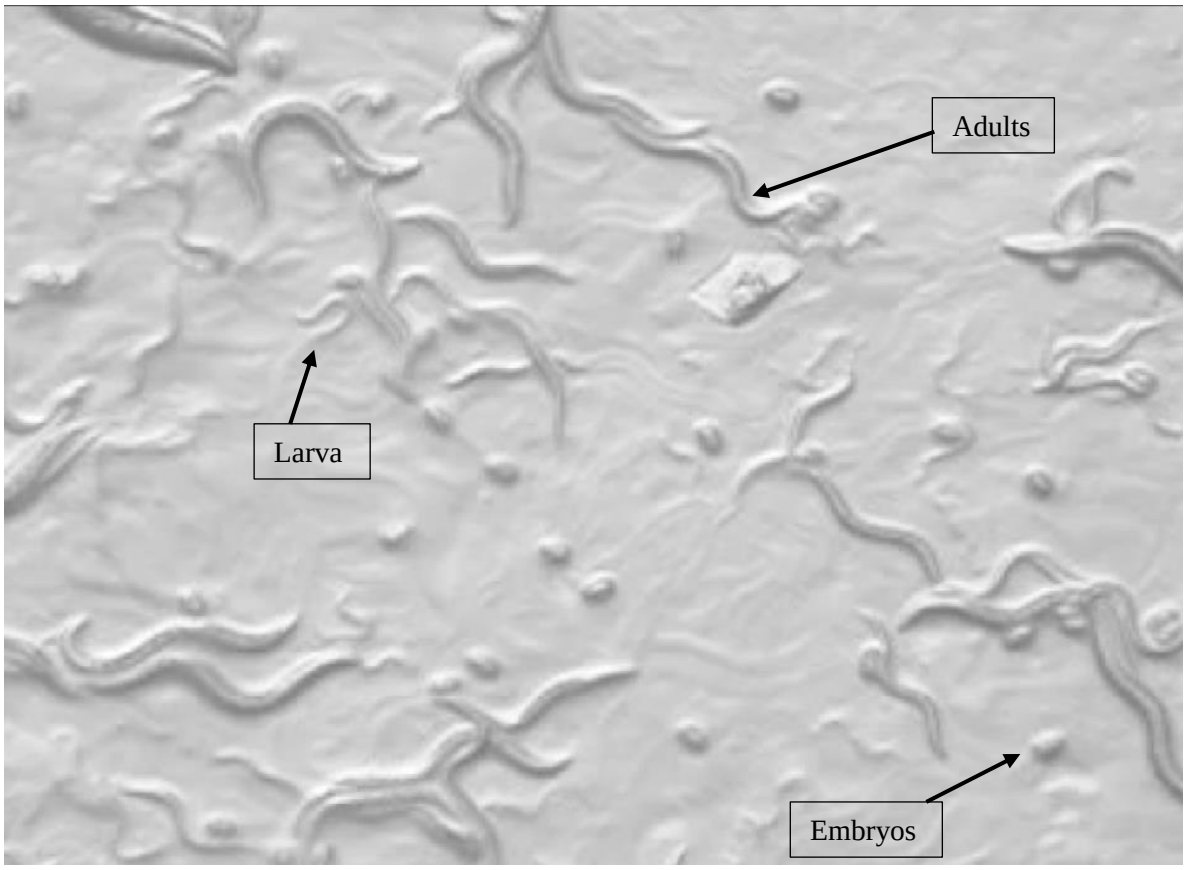


Figure 2.13. *C. elegans* at different developmental stages as viewed under microscope. Tracks in the plate indicate where animals have travelled on the bacterial lawn. Image courtesy of Dr Samantha Hughes.

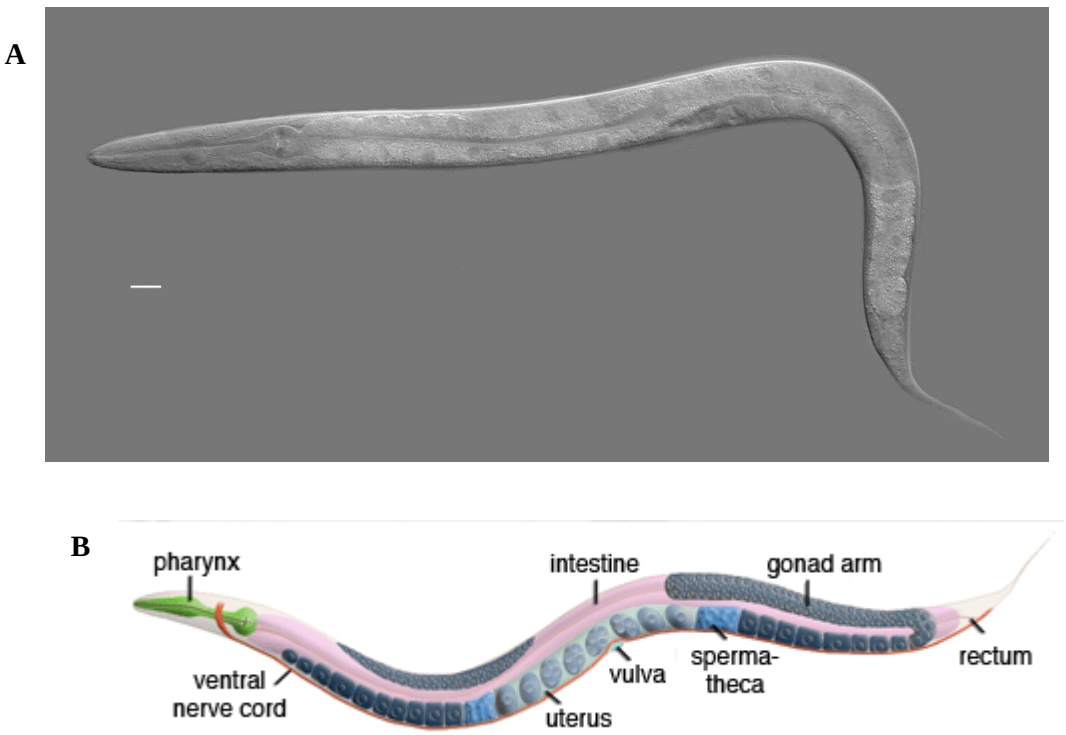


Figure 2.14. (A) *C. elegans* as viewed through a high-power microscope. The head is on the left, with the intentional cells clearly visible towards the tail, some oocytes can be seen. Scale bar is 100 µm. Image courtesy of Dr Samantha Hughes. (B) Stylised image of the *C. elegans* basic anatomy. The pharynx (shown in green) is the head of the animal. The worm is a self-fertilising hermaphrodite, with both male (blue) and female (grey) reproductive organs. The intestine runs the length of the animal, in pink (Altun and Hall, 2009).

There is strong, evolutionary conservation of molecular and cellular pathways between *C. elegans* and mammals (Shaye and Greenwald, 2011; Kim *et al.*, 2018); 12 out of 17 known signal transduction pathways are conserved in both humans and *C. elegans* (Leung *et al.*, 2008). The human and *C. elegans* genomes have significant homology, approximately 80% of human genes have orthologues in *C. elegans* (Kaletta and Hengartner, 2006) and 40% of genes known to be associated with human disease also have orthologues in *C. elegans* (Corsi, Wightman and Chalfie, 2015). The *C. elegans* genome has been fully sequenced and mapped, containing ~20,000 coding genes and ~25,000 non-coding genes (Howe *et al.*, 2016). Moreover, at least 83% of the *C. elegans* proteome has human homologues (Lai *et al.*, 2000).

There are several other advantages to those listed above which endorse the use of *C. elegans* as a model of human disease. *C. elegans* can be grown easily and cheaply in a controlled, low

maintenance environment on a diet of *Escherichia coli* (Kaletta and Hengartner, 2006). Due to its rapid life-cycle and prolific reproductive rates, high-throughput screening of interventions (such as drugs, chemicals or nutrients) can be observed in multiple generations of animals, using different disease models (Markaki and Tavernarakis, 2010). Furthermore, its small size facilitates high-throughput analysis as assays can be performed with ~100 animals per well of a 96-well plate (Kaletta and Hengartner, 2006). Lastly, it is a cost-effective model to conduct molecular and cellular research relating to human diseases as high-throughput experiments can be performed quickly (Kaletta and Hengartner, 2006) in accordance with the 3R rule (replacement, refinement and reduction) of conducting humane animal research (Russell and Burch, 1959).

As the genome has been fully sequenced and mapped, genome manipulation in *C. elegans* is relatively simple and is amenable to unbiased forward and reverse genetic screens (Markaki and Tavernarakis, 2010). Gene function can be simply and quickly knocked down by the process of RNA interference (RNAi) (Corsi, Wightman and Chalfie, 2015). Furthermore, mutant strains that either overexpress a gene or inhibit gene function can be efficiently generated and the resulting phenotypes identified (Markaki and Tavernarakis, 2010). Subsequently, many phenotypes modelling human disease are available, which facilitates the understanding of pathways and molecular mechanisms that underpin human diseases (Corsi, Wightman and Chalfie, 2015). Consequently, it is possible to use the *C. elegans* model to design effective mechanism-based therapies against human disease (Silverman *et al.*, 2010). Thus, the ability to develop therapies which specifically target the source of disease pathogenesis results in more efficient and effective prevention or treatment. Moreover, the efficacy of prophylactic and therapeutic interventions can be rapidly assessed for their ability to confer health benefits in a live model.

2.3.2. *C. elegans* as a Model for Human Disease

C. elegans provides a unique model to assess the genetic and phenotypic basis of human disease *in vivo* (Silverman *et al.*, 2010). Additional advantages over an *in vitro* model include the study at a multicellular, whole-organism level compared to that of tissue culture which focuses on a single cell; detection of organism-level end points (feeding, reproduction, lifespan and motility) (Leung *et al.*, 2008) and monitoring of behavioural responses. Importantly, dissection of both *in vivo* targeted and non-targeted responses from an intervention can be observed. Together, this allows a more informed mechanistic understanding of disease when applied to humans (Silverman *et al.*, 2010). Mammalian animal models offer better understanding and is the superior preclinical model for therapeutic interventions. However, such models are complex, labour-intensive and expensive to isolate mechanisms of action (Kaletta and Hengartner, 2006). Indeed, *C. elegans* is an ideal model for bridging the gap between *in vitro* assays and mammalian animal models (Markaki and Tavernarakis, 2010).

Worms are a standard model system for a wide-range of research; and are used as a model of various human diseases including cancer (Kyriakakis, Markaki and Tavernarakis, 2015), diabetes (Moreno-Arriola *et al.*, 2014), obesity (Zheng and Greenway, 2012) and neurological diseases (Calahorro and Ruiz-Rubio, 2011). Many notable discoveries relating to basic biology and medicine were first made in *C. elegans,* such as the cell death abnormality pathway involved in apoptosis (Ellis and Horvitz, 1986). *C. elegans* is particularly useful as a model for assessing responses and mechanisms pertaining to lifespan (Markaki and Tavernarakis, 2010), ageing (Tissenbaum, 2015), obesity (Elle *et al.*, 2008), diabetes (Markaki and Tavernarakis, 2010), and calorie restriction (Lakowski and Hekimi, 1998).

As eluded to in *section 2.2.5.2.1*, calorie restriction has been practiced for centuries to improve cardio-metabolic health and prolong longevity (Masoro, 2000). Positive preliminary results have been presented from studies incorporating CR in microorganism (yeast) (Lin *et al.*, 2004)

and animal models (Heilbronn and Ravussin, 2003). Lee *et al.* (2006) reported extended lifespan and improved resistance to oxidative stress amongst adult *C. elegans* worms that were totally deprived of food. Two possible mechanisms through which CR may increase lifespan in *C. elegans* include a slower metabolic rate and reduced insulin signalling (Walker *et al.*, 2005). The latter as demonstrated in *C. elegans*, is purported to be inherently linked with nutrient-sensing pathways that mediate CR, such as target of rapamycin (TOR), MAPK and AMPK (Narasimhan, Yen and Tissenbaum, 2009; Templeman and Murphy, 2018). CR regulates glucose metabolism and insulin signalling, thus reducing the risk of developing cardio-metabolic disease and mediating lifespan extension (Walker *et al.*, 2005). Therefore, since nematodes and mammals have common responses to glucose homeostasis and insulin signalling with CR, results could potentially be applied to humans (Partridge, 2010).

C. elegans is a useful tool for understanding the interactions between nutrients and cardio-metabolic diseases as key evolutionary pathways involving energy homeostasis are conserved between worms and mammals (Hashmi *et al.*, 2013). Understanding the effects of nutrients on the insulin signalling pathway is important for dissecting molecular mechanisms associated with diabetes, obesity and ultimately lifespan (Ashrafi *et al.*, 2003). *C. elegans* has an insulin-like signalling (ILS) pathway that regulates glycogenesis, lipogenesis, lipid homeostasis, lipid storage and lifespan (Markaki and Tavernarakis, 2010). RNAi knockdown of acetyl-coenzyme A carboxylase (ACC), fatty-acid synthase (FAS) and fatty-acid desaturase (SCD, *fat-7*) orthologues in *C. elegans* were found to reduce body fat (Ashrafi *et al.*, 2003), mimicking responses observed in calorie restricted mice (Bruss *et al.*, 2009). *daf-2* (mammalian insulin receptor homologue) mutants with reduced insulin signalling have been observed to accumulate high amounts of body fat (Ashrafi *et al.*, 2003). Moreover, RNAi knockdown of *daf-16* (forkhead box O orthologue, FOXO) restored insulin signalling, thus accelerated ageing and reduced body fat to wild-type levels (Ashrafi *et al.*, 2003). Interestingly, inactivation of the

ILS pathway and subsequent activation of DAF-16 in *C. elegans* (Figure 2.15), leads to a metabolic shift from glucose to lipid oxidation and is associated with increased longevity (van Heemst, 2010). Similarly, as mentioned in *section 2.2.5.2.1.2* under conditions of limited cellular energy status during CR in humans, AMPK is activated prompting PGC-1α activation through direct phosphorylation, which predisposes PGC-1α to deacetylation by SIRT-1 (Canto and Auwerx, 2011). Subsequently, insulin signalling is reduced, and substrate metabolism shifts in favour of lipid oxidation (Apfeld *et al.*, 2004). In *C. elegans, sir-2.1* (SIRT-1 orthologue) (Tissenbaum and Guarente, 2001), *aak-2* (AMPK orthologue) and *daf-16/*FOXO function simultaneously to mediate lifespan extension (Apfeld *et al.*, 2004).

In addition to a switch towards lipid oxidation, greater DAF-16 activity upregulates anti-oxidant enzyme (catalase and manganese superoxide dismutase) synthesis and prevents the manifestation of oxidative stress and NF-κB mediated inflammation (van Heemst, 2010). Therefore, normal lipid storage and metabolism has become tightly coupled to suppression of oxidative stress and inflammation (van Heemst, 2010). Furthermore, FOXO activation through reduced ILS may extend lifespan via the anti-oxidative effects of PGC-1α activation, responsible for mitochondrial biogenesis and shifting substrate utilisation towards fat from carbohydrate (van Heemst, 2010). CR has been associated with greater mitochondrial number and thus more efficient mitochondrial activity, utilising less oxygen, producing less ROS and eventually extending lifespan (van Heemst, 2010). Similarly, the anti-oxidative, anti-inflammatory and CRM properties of MTC may operate through the ILS pathway to increase lifespan.

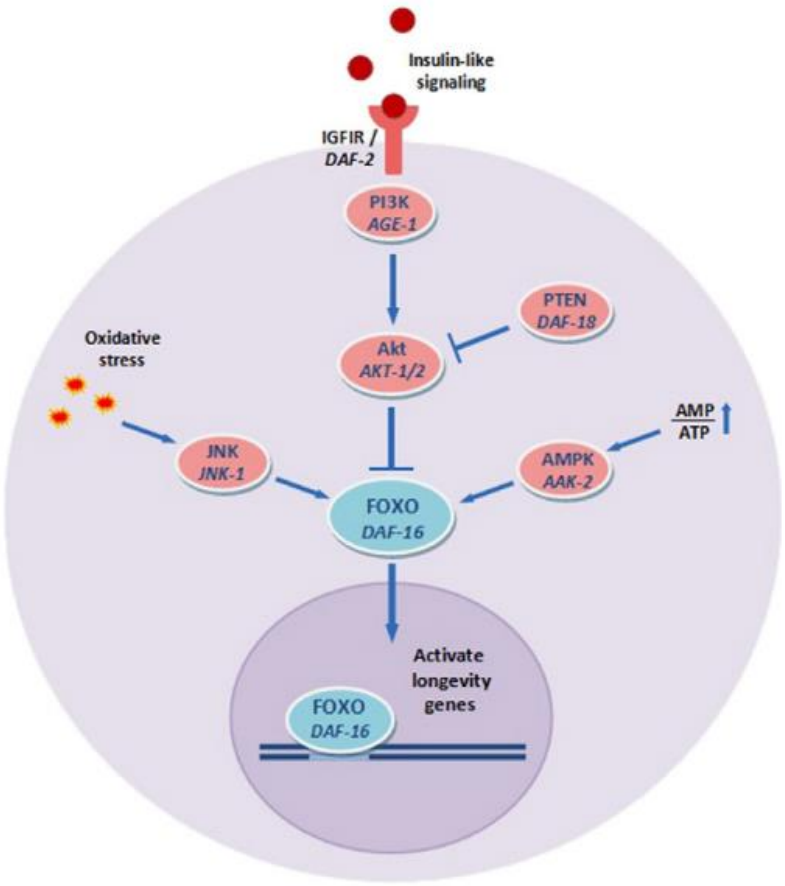


Figure 2.15. Schematic representation of regulation of FOXO activity (Hesp, Smant and Kammenga, 2015). Insulin-like signalling leads to PI(3)K activation, which induces Akt to inhibit FOXO by phosphorylation. The human tumour suppressor PTEN inhibits Akt activity, possibly by phosphorylation of PIP3 (not shown). When cells are under oxidative stress, JNK activity increases, while AMPK is activated by high AMP/ATP ratios. Both kinases activate FOXO by phosphorylation. When FOXO is active, it relocates to the nucleus and promotes the expression of genes that promote longevity. *C. elegans* homologues of mammalian proteins are shown in italics.

Overall, the evolutionary conserved ILS pathway in *C. elegans* is central to the relationship between metabolism, calorie restriction and longevity. DAF-16/FOXO repression accelerates ageing, reduces body fat but does not extend lifespan (Silverman *et al.*, 2010). This indicates the *daf-2/daf-16* pathway to be involved in extending lifespan through expression of genes related to oxidative stress, inflammation and substrate utilisation (Elle *et al.*, 2008). Analysis of the molecular responses to the ILS pathway in *C. elegans* provides a model to elucidate mechanisms of action of dietary interventions against human diseases such as diabetes and

obesity. Hence, the study conducted in **chapter 7** assessed responses to MTC within the insulin signalling pathway.

2.3.3. Dietary Interventions on Lifespan and Cardio-Metabolic Responses in *C. elegans*

Given the complexity of cellular and molecular interactions in humans, understanding the mechanisms of action in response to dietary interventions is very difficult. Comparatively, the *C. elegans* model is simpler and smaller yet evolutionarily conserved with high genetic homology to humans, therefore suitable for understanding the mechanisms of action in response to dietary interventions.

Metabolic Syndrome is underpinned by insulin resistance and thus improvements in insulin sensitivity would delay or prevent the development of cardio-metabolic disease in humans. Understandably, the insulin signalling pathway is key to modulating insulin sensitivity, where cross-talk between insulin signalling and PPAR pathways mediates improved insulin sensitivity through regulating lipid metabolism in humans (Leonardini *et al.*, 2009). *nhr-49* is considered to be a functional homologue of mammalian PPARα in *C. elegans* (Elle *et al.*, 2008). *nhr-49* is a central regulator of lipid and glucose metabolism gene transcription in response to low nutritional status as found during CR (van Gilst *et al.*, 2005; Elle *et al.*, 2008).

2.3.3.1. Polyphenols as Calorie Restriction Mimetics

Polyphenols including resveratrol, EGCG (epigallocatechin-3-gallate), curcumin, quercetin and anthocyanins, have been identified as potential calorie restriction mimetics (CRMs) (Madeo *et al.*, 2014; Willcox and Willcox, 2014). Of these, resveratrol, found in grape skins, has demonstrated the greatest effect as a CRM by activating SIRT-1 (Li, Daniel and Tollefsbol, 2011). Likewise, activation of SIR-2.1 protein by resveratrol in *C. elegans* was found to extend lifespan, thus mimicking CR pathways (Wood *et al.*, 2004). Zhang *et al.* (2009) demonstrated EGCG to significantly extend longevity in *C. elegans* under stressful conditions through

upregulation of *daf-16, sod-3* (human superoxide dismutase-3 orthologue, *SOD3*), and *skn-1* (human Nrf2 orthologue, *NFE2L2*), all associated with anti-oxidant and anti-inflammatory functions. Moreover, the PPAR pathway involved in mediating the benefits of CR has been shown to be activated by polyphenols in *C. elegans* (Sun *et al.*, 2016; Machado *et al.*, 2018). Polyphenols from Yerba Maté (*Ilex paraguariensis*) were shown to reduce fat storage and stimulate fatty acid oxidation through increased ATGL-1 and NHR-49 activity in *C. elegans* (Machado *et al.*, 2018). Increased *hosl-1* (hormone-sensitive lipase orthologue, *LIPE*) expression was also found in worms after exposure to polyphenols from Yerba Maté and cranberries, explaining reduced fat accumulation due to activation of NHR-49 and SBP-1 pathways which increase β-oxidation and regulate fat/sterol synthesis, respectively (Sun *et al.*, 2016; Machado *et al.*, 2018).

The *C. elegans* model has been used to determine the mechanistic relationship between polyphenolic supplementation, cardio-metabolic function and longevity. Viswanathan *et al.* (2005) observed that lifespan extension in the presence of resveratrol was independent of *daf-16*/FOXO. Likewise, blueberry extract, particularly proanthocyanidins, extended lifespan through the p38 MAPK signalling cascade and not via *sir-2.1* or ILS pathway (Wilson *et al.*, 2006). Thus, upregulation of SKN-1 via p38 MAPK signalling may explain lifespan extension through greater oxidative stress resistance (Pang *et al.*, 2014). Furthermore, Grünz *et al.* (2012) showed flavanols induced DAF-16 nuclear exclusion, thus suggesting that lifespan extension was independent of *daf-16*/FOXO. In contrast, nuclear localisation of DAF-16 with the polyphenol myricetin elicited lifespan extension (Büchter *et al.*, 2013). Guha *et al.* (2013) also suggested lifespan extension was mediated through *daf-16*/FOXO, as shown with cranberry extract. Cocoa flavanols (Martorell *et al.*, 2011) and purple wheat anthocyanins (Chen *et al.*, 2013) increased oxidative stress resistance and extended lifespan through *sir-2.1* and DAF-16 in *C. elegans*. This is in agreement with murine models where anthocyanin-rich blueberry

(Seymour *et al.*, 2011) and Montmorency tart cherry (Seymour *et al.*, 2008, 2009) extracts were shown to increase PPARα and PPARγ activity and PPARα, PPARγ and PGC-1α mRNA expression at the muscle and adipose tissue levels thus reversing MetS phenotype. This highlights the action of polyphenols on the PPAR pathway to modulate lipid and glucose metabolism since activation of the ILS pathway initiates phosphorylation and thus nuclear exclusion of FOXO, subsequently removing repression of PPARγ activity.

Interestingly, the findings above may indicate that structural differences in polyphenols influence the pathway through which lifespan extension occurs (Grünz *et al.*, 2012). Ultimately, lifespan extension through improved cardio-metabolic function has repeatedly been observed with polyphenols in *C. elegans* through mimicking similar changes in signalling pathways induced by CR.

2.3.4. Significance for MTC Research – Could MTC concentrate be the elixir of life?

There is an inherent lack of understanding relating to mechanisms of action in the field of polyphenols and health, which extends to MTC interventions. The ability to delineate mechanisms allows the development of mechanism-based therapies against diseases. This chapter has briefly discussed molecular mechanisms of action pertaining to cardio-metabolic function and lifespan extension with polyphenols in *C. elegans.* As this thesis emphasises the effect of MTC on cardio-metabolic function, a study was conducted in *C. elegans* to determine whether dilutions of MTC concentrate induced comparable responses and operated through similar pathways as other polyphenols **(Chapter 7)**.

The simple anatomy, physiological traits, similarity to humans (at the genetic, molecular and tissue level) together with the ease of genome manipulation, demonstrates how *C. elegans* is a convenient yet powerful model to research the innate biological responses to dietary interventions and dissect the molecular pathways through which they operate. As eluded to

previously, the *C. elegans* model has several distinct advantages which enables cost- and time-effective high-throughput screening of interventions and subsequent phenotypic traits. Most importantly, using unbiased forward and reverse genetic screening the *C. elegans* model allows the identification of mechanisms of action related to prophylactic and therapeutic interventions against human disease (Silverman *et al.,* 2010). Subsequently, this enhances understanding of human disease processes and enables the formation of mechanism-based interventional strategies to effectively mitigate disease development and progression (Silverman *et al.,* 2010).

Literature has shown a beneficial effect of polyphenols in dietary interventions on cardio-metabolic function, longevity and the pathways through which these occur in *C. elegans* (Viswanathan *et al.*, 2005; Wilson *et al.*, 2006; Martorell *et al.*, 2011; Chen *et al.*, 2013; Guha *et al.*, 2013; Sun *et al.*, 2016; Machado *et al.*, 2018). Hence, related to this section a similar study was performed with MTC as this had not been done previously **(Chapter 7)**. This study specifically observed development, lifespan and fat content responses to various dilutions of MTC concentrate in the worms. Then, using developmental delay at a certain MTC dilution as a read-out, a targeted small-scale RNAi screen of genes associated with lipid and glucose metabolism was performed. From this, specific genes were identified which were key in regulating the MTC response. Furthermore, the lifespan assay enabled determination of whether MTC could operate as a CRM. The molecular effects of MTC consumption on cardio-metabolic function have previously been examined in a rodent model (Seymour *et al.,* 2008, 2009), as discussed in *section 2.1.6.5.2*. However, *C. elegans* was chosen for this thesis as it enabled targeted genetic screening. Mechanisms elucidated from the study in **chapter 7** may be translated to humans and thus this study serves to potentially inform future clinical research with MTC in human participants.

2.4. Aims and Hypotheses

The literature review has identified numerous areas where further research is required to determine the efficacy of Montmorency tart cherries on cardio-metabolic health parameters, in healthy and MetS human populations. Subsequently, these gaps are addressed in the following experimental chapters.

2.4.1. Aims

2.4.1.1. General Aim

The overarching aim of the series of studies that comprise of this thesis was to establish whether Montmorency tart cherry (*Prunus cerasus* L.) supplementation with and without FATMAX exercise could improve biomarkers associated with cardio-metabolic health.

2.4.1.2. Specific Aims

The four experimental chapters within this thesis addressed four separate research objectives:

i. Assess the effects of Montmorency tart cherry juice supplementation and individualised FATMAX exercise on cardio-metabolic biomarkers in healthy humans.
ii. Determine the effects of a single, acute bolus of Montmorency tart cherry juice and capsules on acute cardio-metabolic responses in humans with MetS. Furthermore, identify whether capsules are more efficacious than juice at improving cardio-metabolic function. Lastly, establish whether a dose-response effect is observed with consumption of Montmorency tart cherry juice.
iii. Examine acute and prolonged cardio-metabolic responses to short-term, continuous supplementation of Montmorency tart cherry juice in humans with MetS. Also, ascertain whether 6 days prior loading of Montmorency tart cherry juice can enhance acute cardio-metabolic function compared to a single, acute bolus.

iv. Elucidate the molecular mechanisms of action through which Montmorency tart cherries influence cardio-metabolic function, using a *C. elegans* model.

2.4.2. Hypotheses

Hypotheses relating to the overall thesis are outlined below. Specific hypotheses pertaining to individual studies are presented in their respective chapters.

H_1: Montmorency tart cherry supplementation will significantly improve resting cardio-metabolic biomarkers compared to the placebo-controlled condition, in healthy and MetS human populations.

H_2: Montmorency tart cherry supplementation with FATMAX exercise will improve cardio-metabolic biomarkers compared to the placebo-controlled condition, in a healthy human cohort.

H_3: A single, acute bolus of Montmorency tart cherry juice and capsules will improve acute cardio-metabolic biomarkers compared to the placebo-controlled condition, in humans with MetS.

H_4: Short-term, continuous supplementation of Montmorency tart cherry juice will improve cardio-metabolic biomarkers compared to the placebo-controlled condition, in humans with MetS.

H_5: Montmorency tart cherry acts as a calorie restriction mimetic and exerts its beneficial cardio-metabolic responses through evolutionary conserved mechanisms related to calorie restriction, in *C. elegans*.

Chapter 3

General Methodology

This chapter outlines common methodological procedures that were used and serves to function as a reference for experimental chapters presented later in this thesis. Specific methodologies pertaining to studies can be found in the relevant chapters.

3.1. Participant Recruitment

Chapters 4-6 recruited male and female human participants aged between 18-65 years using convenience sampling. **Chapter 4** recruited healthy participants (*section 4.2.1*), **chapters 5** (*section 5.2.1.1 and 5.2.2.1*) and **6** (*section 6.2.1*) recruited participants with Metabolic Syndrome.

All participants were recruited through methods including posters, email and word of mouth from the target populations previously described. Ethical approval for each study was gained prior to recruitment and all participants were aware of the risks and benefits of participating in studies. Prior to inclusion in studies, participants were also made aware of the dietary and exercise restrictions associated with the studies. Before research commenced, all participants provided written informed consent, completed health screen questionnaires and underwent a screening procedure to determine suitability for inclusion in each study. All studies **(Chapters 4-6)** were conducted in accordance with the Declaration of Helsinki (2013).

3.2. Dietary and Exercise Guidelines

Participants were provided with specific dietary and exercise guidelines to adhere to for each study. All participants arrived at the laboratory between 7–10 am, after an overnight fast of a minimum of 10 hours, to account for circadian variation as previously shown by Bell *et al.* (2014b) after MTC supplementation in humans. Participants were instructed to cease consumption of any other supplementation two weeks before and during the course of each study.

Participants were instructed to maintain their habitual polyphenol intake, particularly anthocyanins, as opposed to complete restriction throughout the study. This was to ensure that the polyphenols provided by MTC supplementation were supplementary to the existing habitual polyphenol intake of each participant, rather than replacing the habitual provisions which would occur by enforcing a total restriction of polyphenol-rich (particularly anthocyanin) dietary sources. Another reason for maintaining habitual polyphenol consumption was to assess the effects of MTC supplementation on outcome variables in conditions representing normal daily activity, therefore upholding ecological validity (Meyer, Gäßler and Kindermann, 2007).

Total energy, macronutrient and polyphenol intake of participants' 'Western' habitual diet was assessed through food diaries. This was to assess compliance of replicating dietary intake for the 3 days prior to each testing session (Alkhatib *et al.*, 2015; Nordby *et al.*, 2015; Roberts *et al.*, 2015). Instructions on completing food diaries and information regarding portion sizes were provided. Participants were instructed to record all dietary items consumed during two weekdays and a weekend day of the same week in order to analyse habitual diet (Stull *et al.*, 2010, 2015; Chai *et al.*, 2018). Fluids or food were not provided during testing sessions in **chapters 5** and **6**, but participants were able to drink water *ad libitum*, where volume of consumption was monitored during the first session and repeated in subsequent sessions (Keane *et al.*, 2016b). Participants also refrained from consuming water 3 hours prior to arrival at the laboratory to limit variations in body composition analysis.

Food diaries were analysed using dietary analysis software (Dietplan 7.0, Forestfield Software, UK), to monitor adherence of total energy, macronutrient and polyphenol intake compared to the habitual diet and the 3-day period before testing sessions. If dietary items were not found in the database software, manual entry of nutrient composition of the item was conducted.

3.3. Supplementation

The series of studies presented in this thesis used various forms of MTC supplementation. For details on composition of placebo, refer to the methodology section of relevant chapters. GraphPad software (GraphPad, QuickCalcs, San Diego, California, USA) was used to randomise supplementation order in **chapters 4-6**.

Chapter 4 (*section 4.2.1.1*) supplemented Montmorency Tart Cherry Juice (MTCJ), composed of a commercially available MTC concentrate (30 mL) (Montmorency Tart Cherry Concentrate, Cherry Active, Active Edge Ltd, Hanworth, UK) mixed with 100 mL water and a placebo drink, twice daily for 20 days. In **chapter 5, part A** (*section 5.2.1.2.3*), participants were acutely supplemented with a single dose of MTCJ (30 mL concentrate with 100 mL water), 10 MTC capsules (Montmorency Tart Cherry Capsules, Cherry Active, Active Edge Ltd, Hanwell, UK) and a placebo drink. During **chapter 5, part B** (*section 5.2.2.2.1*), participants were also supplemented a single, acute dose of double concentrated MTCJ (60 mL concentrate with 100 mL water). **Chapter 6** (*section 6.2.2.3*) supplemented MTCJ (30 mL concentrate with 100 mL water) and a placebo drink for 7 consecutive days. Participants consumed each dose within 3 minutes of commencing ingestion of the supplement.

Each 30 mL serving of MTC concentrate provided a total anthocyanin content of 270 mg (9 $mg.mL^{-1}$) (Howatson *et al.*, 2012) and juice from approximately 90-110 whole Montmorency tart cherries; whilst 1 capsule was made from approximately 10 whole Montmorency tart cherries.

The total anti-oxidant status of 30 mL MTCJ and 60 mL MTCJ was found to be 2.32 ± 0.03 and 2.51 ± 0.02 $mmol.L^{-1}$, respectively.

3.4. Testing Protocol

All testing sessions in **chapters 4-6** were conducted in a temperature-controlled laboratory maintained between 21-24ºC, 38-45% relative humidity (dry-bulb) and 99.8-102.4 kPa atmospheric pressure (AWS888N, Oregon Scientific, USA). Testing protocols are described further in their relevant chapters.

3.5. Measures and Equipment

3.5.1. Anthropometrics

Stature (m) was measured on a stadiometer (Seca 217 Stadiometer, Seca, Hamburg, Germany) to the nearest 1 mm with subjects standing without shoes, heels and back touching the stadiometer, head in the Frankfort horizontal plane and after holding a maximal inhalation. Body mass (kg) (Seca 799, Seca, UK) was measured on a flat, uncarpeted surface with as minimal clothing as possible ensuring repeated measurements were performed with the same clothing. Based on stature and body mass data, body mass index (BMI) was calculated using the formula of Keys *et al*. (1972).

Waist circumference (cm) was measured at the end of normal expiration between the top of the iliac crest and the lower (10^{th}) costal (Stewart and Marfell-Jones, 2011) using a handheld ergonomic tape measure (Seca 201, Seca, Hamburg, Germany).

3.5.2. Body Composition

Segmental body composition analysis (Body Composition Analyser BC-418, Tanita, Japan) was performed in all studies according to manufacturer's specifications. The principle of the analyser was based on bioelectrical impedance, therefore only measurements with total body water between 50-70% of total body mass were considered for analysis. Prior to measurement, all participants were required to fulfil criteria per manufacturer's recommendations, in addition to the dietary and exercise guidelines outlined for each study. These included abstentions from

caffeine and alcohol ingestion 24 hours before measurement and urination before measurement. To ensure validity and reliability, all measurements were conducted at the same time of day to avoid circadian variation influencing results. Values for fat mass, fat free mass (FFM) and body fat percentage (whole body and trunk) were monitored.

A previous study using the Tanita BC-418 analyser reported a high correlation, no significant mean bias and narrow limits of agreement for body fat percentage measurements between the analyser and Dual-Energy X-ray Absorptiometry (Pietrobelli *et al.*, 2004), suggesting the analyser is a valid tool for measuring body fat percentage. Additionally, a test-retest CV of 1.4% was obtained for body fat percentage by Kelly and Metcalfe (2012).

Intraclass Correlation Coefficient (ICC) estimates and their 95% confidence intervals were calculated using SPSS v22 (IBM, Chicago, USA) based on a single-measures (type), absolute agreement (definition), two-way mixed-effects model for within- and between-day test-retest reliability. ICC was chosen as it is a common statistical test for test-retest reliability which accounts for the correlation and agreement between measurements (Koo and Li, 2016). ICC values were measured on a scale between 0 and 1, and classified as poor (0.00 – 0.50), moderate (0.50 – 0.75), good (0.75 – 0.90) and excellent (0.90 – 1.00) (Koo and Li, 2016).

Test-retest reliability of the analyser for fat mass, FFM and body fat percentage (whole body and trunk) were assessed from a sample of 5 healthy participants (age 28 ± 7 years; body mass 74.9 ± 14 kg; stature 1.77 ± 0.47 m), as previously conducted by Pietrobelli *et al.* (2004). Participants were not on any weight loss or weight gain regimen during this period. The within-day intra-individual reliability for the same variables was determined by measuring each participant on 10 occasions within 1 hour (Pietrobelli *et al.*, 2004). Between-day intra-individual reliability was calculated by taking three measurements per day on three different days each separated by 10 days to mimic the study design of **chapter 4**. All variables

demonstrated good to excellent reliability according to 95% confidence intervals for within-day and between-day reliability (Table 3.1).

Table 3.1. Within-day and between-day mean, standard deviation, range, ICC and Coefficient of Variation (%) for body composition variables measured with bioelectrical impedance body composition analyser.

	Mean ± SD (Range)		ICC (95% CI)		CV (95% CI)	
	Within-Day	Between-Day	Within-Day	Between-Day	Within-Day	Between-Day
Fat Mass (kg)	12.1 ± 0.2 (11.7 – 12.4)	11.8 ± 0.3 (11.1 – 12.1)	0.93 (0.88 – 0.97)	0.90 (0.88 – 0.91)	2 (1 – 4)	3 (2 – 7)
FFM (kg)	58.6 ± 0.2 (58.3 – 58.8)	57.5 ± 0.2 (57.1 – 57.9)	0.97 (0.89 – 0.99)	0.92 (0.88 – 0.96)	1 (0 – 3)	3 (1 – 6)
Whole-body Fat (%)	17.2 ± 0.4 (16.7 – 17.6)	17.0 ± 0.5 (16.7 – 17.4)	0.91 (0.89 – 0.94)	0.92 (0.84 – 0.97)	2 (1 – 5)	3 (2 – 5)
Trunk Fat (%)	21.0 ± 0.7 (20.3 – 22.1)	20.8 ± 0.9 (19.9 – 21.3)	0.89 (0.87 – 0.94)	0.88 (0.86 – 0.93)	3 (1 – 6)	4 (2 – 8)

FFM (Fat Free Mass)

3.5.3. Cardiovascular Function

3.5.3.1. Cardiac Haemodynamics

Beat-to-beat resting cardiac haemodynamic parameters including heart rate (HR), cardiac output (CO), stroke volume (SV), mean arterial pressure (MAP) and total peripheral resistance (TPR) were measured non-invasively (Finometer MIDI Model-2, Finapres Medical Systems BV, Amsterdam, The Netherlands), using the arterial volume clamp method (Penaz, 1985). An appropriately sized finger-cuff was applied around the middle phalanx of the middle finger of the hand and a height correction unit was subsequently fixed to the finger-cuff. All measurements were obtained in the seated position. To avoid data selection, recordings were averaged over 10 consecutive beats with the lowest values for each parameter taken for statistical analysis (Cook *et al.*, 2017a).

3.5.3.2. Blood Pressure

Brachial BP was measured using an automated sphygmomanometer (Omron MX3, Omron, Japan) in **chapters 4-6**. Four measurements were recorded in an upright seated position, on the arm not used for venepuncture, with an average of the final three being taken as BP (Cook *et al.*, 2015). The cuff was placed around the upper arm, 2-3 cm above the antecubital fossa, by the same researcher each time. The first reading was not included due to the defence mechanism (a rise in BP as a consequence of increased anxiety which diminishes with subsequent measurements) affecting results (Naismith and Braschi, 2003). Prior to the first measurement, participants rested for 10 minutes, with 2 minutes rest between subsequent measurements (Rosenkilde *et al.*, 2010).

3.5.3.3. Pulse Wave Analysis

Pulse wave analysis (PWA) was used to monitor arterial stiffness non-invasively using applanation tonometry (SphygmoCor, ScanMed Medical, UK) as there is a strong association between PWA and cardiovascular disease incidents, independent of traditional risk factors (Choi *et al.*, 2007; Keane *et al.*, 2016b). A high-sensitivity tonometer was placed over the radial artery at the wrist, which was slightly extended and rested on a pillow (Doupis *et al.*, 2016). Aortic (central) systolic and diastolic BP, pulse pressure (PP), augmentation pressure (AP), augmentation index (AIx), corrected augmentation index at heart rate 75 bpm (AIx at HR75) and subendocardial viability ratio (SEVR) were determined by the software using generalised transfer functions. Gallagher, Adji and O'Rourke (2004) and Pauca, O'Rourke and Kon (2001) have previously validated these functions at rest. High reproducibility of AIx determined from PWA, using SphygmoCor, has been shown in various populations suggesting suitability of use in clinical interventional studies (Wilkinson *et al.*, 1998). Recordings with a quality-index ≥80% only, as suggested by the manufacturer, were selected for statistical analysis.

3.5.3.4. Cardiac Haemodynamics, PWA and Brachial Blood Pressure Reliability

Waldron *et al.* (2017) found intra-trial measurements of cardiac haemodynamic parameters were reliable at rest in healthy, normotensive, males, using the Finapres system; although stroke volume was associated with larger errors. However, in individuals with MetS, intraclass correlation coefficients (ICC) were used to assess test-retest reliability of resting cardiac haemodynamic (Finapres), PWA (SphygmoCor) and brachial BP parameters during pilot testing, prior to initiating the second study **(Chapter 5)**. ICC estimates and their 95% confidence intervals were calculated, using SPSS v22.0 (IBM, Chicago, USA), based on a single-measures (type), absolute agreement (definition), two-way mixed-effects model for intra-trial and inter-trial test-retest reliability. ICC was chosen as it is a common statistical test for test-retest reliability, which accounts for the correlation and agreement between measurements (Koo and Li, 2016). Five participants with MetS (age 45 ± 10 years, body mass 89.9 ± 12.1 kg, stature 1.72 ± 0.51 m) participated in the pilot testing where intra-trial (7 repeated measurements during the same testing session, corresponding to the 7 time points) and inter-trial (one measurement obtained on three different days, each separated by 2 weeks, corresponding to the 3 conditions) reliability was determined in the same format as the protocol used in **chapter 5**. Interpretation was based on ICC classifications outlined in *section 3.5.2.* Intra-trial reliability for AP, SV and TPR demonstrated moderate to excellent reliability, all other variables in Table 3.2 demonstrated good to excellent reliability according to 95% confidence intervals. Inter-trial reliability for aortic SBP, aortic DBP, SEVR, HR, brachial SBP and brachial DBP demonstrated good to excellent reliability according to 95% confidence intervals. All other variables in Table 3.2 demonstrated moderate to excellent reliability. Consequently, based on this data and similar research design, it was deemed suitable to measure cardiac haemodynamic and PWA parameters in individuals with MetS for **chapter 6.**

Table 3.2. Intra-trial and inter-trial mean, standard deviation, range, ICC and Coefficient of Variation (%) for resting PWA (SphygmoCor), cardiac haemodynamic (Finapres) and brachial blood pressure parameters measured during pilot testing.

	Mean ± SD (Range)		ICC (95% CI)		CV (95% CI)	
	Intra-Trial	Inter-Trial	Intra-Trial	Inter-Trial	Intra-Trial	Inter-Trial
Aortic SBP (mmHg)	125 ± 10 (113 – 144)	122 ± 13 (110 – 140)	0.90 (0.81 – 0.96)	0.88 (0.86 – 0.91)	8 (5 – 14)	11 (7 – 17)
Aortic DBP (mmHg)	83 ± 4 (69 – 93)	83 ± 7 (69 – 95)	0.96 (0.86 – 0.98)	0.91 (0.88 – 0.97)	5 (2 – 7)	9 (3 – 11)
Augmentation Pressure (mmHg)	12 ± 1 (7 – 18)	15 ± 1 (12 – 17)	0.92 (0.66 – 0.98)	0.92 (0.72 – 0.98)	8 (4 – 12)	7 (4 – 10)
AIx (%)	28 ± 2 (21 – 38)	36 ± 3 (26 – 38)	0.93 (0.77 – 0.94)	0.86 (0.57 – 0.96)	7 (5 – 9)	10 (4 – 12)
AIx at HR75 (%)	26 ± 2 (20 – 33)	24 ± 2 (21 – 30)	0.96 (0.85 – 0.99)	0.91 (0.69 – 0.97)	7 (4 – 9)	8 (2 – 11)
SEVR (%)	193 ± 20 (166 – 219)	184 ± 30 (157 – 217)	0.88 (0.78 – 0.93)	0.84 (0.80 – 0.90)	10 (7 – 14)	16 (8 – 20)
Cardiac Output ($L.min^{-1}$)	6.62 ± 0.51 (5.81 – 7.23)	7.14 ± 0.62 (6.24 – 7.84)	0.93 (0.78 – 0.98)	0.85 (0.52 – 0.96)	8 (3 – 11)	9 (6 – 13)
Stroke Volume (mL)	106 ± 12 (98 – 110)	108 ± 13 (101 – 124)	0.88 (0.60 – 0.97)	0.81 (0.56 – 0.95)	11 (5 – 16)	12 (7 – 19)
MAP (mmHg)	93 ± 6 (89 – 98)	109 ± 7 (98 – 115)	0.94 (0.79 – 0.96)	0.91 (0.70 – 0.91)	6 (2 – 9)	6 (5 – 15)
Heart Rate ($beats.min^{-1}$)	62 ± 3 (60 – 67)	66 ± 3 (64 – 70)	0.92 (0.84 – 0.93)	0.92 (0.84 – 0.97)	5 (4 – 10)	5 (2 – 12)
TPR ($mmHg \cdot min^{-1} \cdot L$)	1.44 ± 0.11 (1.05 – 1.49)	1.42 ± 0.13 (1.31 – 1.55)	0.87 (0.52 – 0.97)	0.84 (0.53 – 0.92)	7 (3 – 13)	9 (4 – 15)
Brachial SBP (mmHg)	130 ± 12 (125 – 149)	132 ± 13 (122 – 148)	0.92 (0.83 – 0.97)	0.87 (0.80 – 0.94)	9 (4 – 12)	10 (6 – 12)
Brachial DBP (mmHg)	72 ± 5 (66 – 77)	72 ± 6 (68 – 78)	0.94 (0.85 – 0.98)	0.89 (0.85 – 0.95)	7 (4 – 10)	8 (2 – 11)

AIx (Augmentation Index); DBP (Diastolic Blood Pressure); MAP (Mean Arterial Pressure); SBP (Systolic Blood Pressure); SEVR (Subendocardial Viability Ratio); TPR (Total Peripheral Resistance).

3.5.4. Indirect Calorimetry

Through indirect calorimetry, breath-by-breath measurements of $\dot{V}O_2$ consumption and $\dot{V}CO_2$ production during pulmonary gas exchange were used to determine whole-body energy expenditure, fat oxidation rate, carbohydrate oxidation rate and RER at rest and during exercise. The contribution of protein to total energy expenditure at rest and during exercise was considered negligible (Jeukendrup and Wallis, 2005).

Resting metabolic rate (RMR) was assessed in **chapters 4-6**, to determine resting energy expenditure, substrate oxidation rates and RER. Determination of RMR was conducted in a temperature-controlled annexe of the main laboratory to ensure a thermoneutral environment. This was to prevent results being affected by inducing a thermoregulatory effect on heat generation (Henry, 2005). Participants arrived at the laboratory according to the dietary and exercise restrictions outlined for each study. RMR was measured based on an open-circuit indirect calorimetry system (GEM Nutrition Ltd, Cheshire, UK). Prior to use, the unit was calibrated against a mixture of gases with known concentrations (1.1% CO_2, 21% O_2). Participants lay supine for 30 minutes with data averaged for the final 20 minutes only, to achieve steady-state and account for any initial short-term variances in respiration (Kelly *et al.*, 2013). A ventilated hood was placed over the head with a flexible plastic seal around the neck and shoulders to prevent air inside and outside the hood from mixing excessively. Participants were instructed to remain silent and lay as still as possible, whilst music was played to prevent sleeping. Values for $\dot{V}O_2$ ($L.min^{-1}$), $\dot{V}CO_2$ ($L.min^{-1}$), RER and EE ($kcal.day^{-1}$) were obtained every minute during the half hour period.

Resting EE was determined by application of the Weir equation (Weir, 1949) below.

$$\textit{Energy Expenditure}\ (\text{kcal.day}^{-1}) = [(3.94 * \dot{V}O_2) + (1.106 * \dot{V}CO_2)] * 1.44 \quad (1)$$

Equations 2 and *3* outlined by Frayn (1983), were used to determine fat oxidation and carbohydrate oxidation rates at rest.

$$\textit{Fat Oxidation Rate}\ (\text{g.min}^{-1}) = (1.67 * \dot{V}O_2) - (1.67 * \dot{V}CO_2) \quad (2)$$

$$\textit{Carbohydrate Oxidation Rate}\ (\text{g.min}^{-1}) = (4.55 * \dot{V}CO_2) - (3.21 * \dot{V}O_2) \quad (3)$$

RER or respiratory quotient (RQ) can be used as an alternative indirect indicator of substrate oxidation based on stoichiometry. The oxidation of 1 mol of glucose requires 134 L of O_2 and produces 134 L of CO_2 and therefore an RQ of 1 (134 L / 134 L = 1), representing predominately carbohydrate oxidation. Equally, an RQ of 0.7 suggests primarily oxidation of fat as 1 mol of palmitic acid requires 515 L of O_2 and produces 358 L of CO_2 (358 L / 515 L = 0.7) (Jeukendrup and Wallis, 2005).

$$\textit{RER (or RQ)} = \dot{V}CO_2 / \dot{V}O_2 \quad (4)$$

Intraclass Correlation Coefficient (ICC) estimates and their 95% confidence intervals were calculated using SPSS v22 (IBM, Chicago, USA) based on a single-measures (type), absolute agreement (definition), two-way mixed-effects model for within- and between-day test-retest reliability. ICC was chosen as it is a common statistical test for test-retest reliability which accounts for the correlation and agreement between measurements (Koo and Li, 2016). ICC values were measured on a scale between 0 and 1, and classified as poor (0.00 – 0.50), moderate (0.50 – 0.75), good (0.75 – 0.90) and excellent (0.90 – 1.00) (Koo and Li, 2016).

RMR reliability was measured using the same people as reliability testing for body composition with healthy participants. Within-day intra-individual reliability was not measured as

participants were only measured once a day during the testing protocol. Between-day intra-individual reliability was taken by taking a measurement on three different days each separated by 10 days to mimic the study design of **chapter 4**.

Between-day ICC and Coefficient of Variation (%) for RMR measurements, showed good to excellent reliability in healthy individuals (Table 3.3). ICC values were measured on a scale between 0 and 1, and classified as poor (0.00 – 0.50), moderate (0.50 – 0.75), good (0.75 – 0.90) and excellent (0.90 – 1.00) (Koo and Li, 2016).

Table 3.3. Mean, standard deviation, range, ICC and Coefficient of Variation (%) for RMR variables measured with an open-circuit indirect calorimetry system.

	Mean ± SD (Range)	ICC (95% CI)	CV (95% CI)
EE (kcal.day^{-1})	1776 ± 218 (1507 – 2053)	0.89 (0.86 – 0.92)	12 (6 – 16)
RER (AU)	0.84 ± 0.08 (0.77 – 0.88)	0.94 (0.92 – 0.96)	10 (6 – 12)
$\dot{V}O_2$ (L.min^{-1})	264 ± 30 (190 – 365)	0.91 (0.87 – 0.91)	11 (7 – 15)
$\dot{V}CO_2$ (L.min^{-1})	220 ± 20 (164 – 318)	0.95 (0.91 – 0.97)	9 (6 – 13)

EE (Energy Expenditure); RER (Respiratory Exchange Ratio); $\dot{V}CO_2$ (Volume of Carbon Dioxide Production); $\dot{V}O_2$ (Volume of Oxygen Uptake).

Within-day, intra-individual reliability was measured in the fasted state in individuals with MetS (using the same participants as those for cardiac haemodynamic/PWA reliability testing), but not using the same design as **chapter 5**, as most measurements during testing were taken in the post-prandial state. Hence, measurements were taken twice separated by one hour (to mimic the length between measurements during the testing protocol).

Between-day, intra-individual reliability was measured by obtaining a measurement on three different days each separated by 14 days (washout) to mimic the study design of **chapter 5**.

Within-day and between-day ICC and Coefficient of Variation (%) for RMR measurements, showed good to excellent reliability in MetS individuals (Table 3.4). ICC values were measured on a scale between 0 and 1, and classified as poor (0.00 – 0.50), moderate (0.50 – 0.75), good (0.75 – 0.90) and excellent (0.90 – 1.00) (Koo and Li, 2016). As the data showed good to excellent reliability, it was deemed suitable to take RMR measurements in **chapter 6** also.

Table 3.4. Within-day and between-day mean, standard deviation, range, ICC and Coefficient of Variation (%) for RMR variables measured with an open-circuit indirect calorimetry system.

	Mean ± SD (Range)		ICC (95% CI)		CV (95% CI)	
	Within-Day	Between-Day	Within-Day	Between-Day	Within-Day	Between-Day
EE (kcal.day^{-1})	1742 ± 236 (1531 – 2497)	1992 ± 230 (1534 – 2224)	0.93 (0.90 – 0.96)	0.91 (0.89 – 0.93)	11 (8 – 15)	12 (6 – 13)
RER (AU)	0.85 ± 0.05 (0.82 – 0.90)	0.86 ± 0.09 (0.84 – 0.91)	0.95 (0.91– 0.97)	0.93 (0.90 – 0.94)	6 (3 – 8)	10 (8 – 13)
$\dot{V}O_2$ (L.min^{-1})	247 ± 28 (190 – 336)	246 ± 31 (176 – 350)	0.91 (0.89 – 0.94)	0.88 (0.86 – 0.91)	11 (7 – 16)	13 (6 – 15)
$\dot{V}CO_2$ (L.min^{-1})	291 ± 18 (177 – 301)	286 ± 23 (166 – 322)	0.94 (0.87 – 0.94)	0.93 (0.86 – 0.93)	6 (4 – 9)	8 (4 – 10)

EE (Energy Expenditure); RER (Respiratory Exchange Ratio); $\dot{V}CO_2$ (Volume of Carbon Dioxide Production); $\dot{V}O_2$ (Volume of Oxygen Uptake).

3.6. Blood Sampling and Analysis

3.6.1. Blood Sampling

Venous blood was sampled using the venepuncture butterfly method (BD Vacutainer Safety-Lok Blood Collection Set 21G with Luer Adapter, Becton Dickinson and Co., Oxford, UK) in **chapters 4-6**. Whole blood (8-12 mL), sampled from veins located in the antecubital fossa region, was collected into either ethylenediaminetetraacetic acid (EDTA), lithium heparin or serum separator tubes (Vacutainer, Becton Dickinson and Co., Oxford, UK). The serum separator tube was left to stand for a minimum of 30 minutes at room temperature (Tuck *et al.*, 2009), then all tubes were centrifuged for 10 minutes at 4000 rev.min^{-1} (Sorvall ST 8R, Thermo

Fisher Scientific, USA). Serum and plasma supernatants were aliquoted into cryogenic vials (2 mL Cryogenic Vials, Fisherbrand, Leicestershire, UK) and stored in a freezer (MDF-U53V, VIP Series, Sanyo, Japan) at -80ºC for later analysis.

3.6.2. Glucose

Glucose concentrations were determined through reaction of glucose oxidase with glucose to form hydrogen peroxide and gluconic acid. The amount of hydrogen peroxide released indicated glucose concentration via amperometric hydrogen peroxide determination.

Immediately after centrifugation, serum samples were assessed for glucose (range 0.5-50 $mmol.L^{-1}$, CV ≤1.5%) (Biosen C-Line, EKF Diagnostics, Cardiff, UK) in duplicates, by pipetting 10 µL of sample into a pre-filled reaction cup (Safe-Lock Mixing Cup, EKF Diagnostics, Cardiff, UK) containing 500 µL of haemolysing solution. Each reaction cup was then vortexed for 5 seconds before being analysed. For purposes of enhanced accuracy, serum was chosen over plasma and whole blood as serum lacks erythrocytes, leukocytes and clotting factors such as fibrinogen. Furthermore, metabolite concentrations are higher in serum than plasma thus less prone to background noise affecting accuracy, with glucose tending to be more accurate by 5% in sera (Ladenson *et al.*, 1974; Yu *et al.*, 2011).

3.6.3. General Assay Methodology

Prior to analysis, serum samples were allowed to thaw and were then vortexed (Whirlimixer, Fisherbrand, Leicestershire, UK). Serum samples were directly analysed for ACE (angiotensin-I-converting enzyme), HDL, insulin, TAS, total cholesterol and triglycerides. Indirect calculation of LDL was performed after determination of the other lipid metabolites using the formula suggested by Ahmadi *et al.* (2008) in **chapter 4**, and by the Friedewald, Levy and Fredrickson (1972) formula outlined below in **chapters 5-6**. The use of the Friedewald formula (*Equation 5*) in **chapter 5-6** was based on data showing a strong, significant correlation

between predicted LDL concentrations and direct LDL measurement in individuals with MetS (Knopfholz *et al.*, 2014).

$$\text{LDL (mmol.L}^{-1}\text{)} = \text{(Total Cholesterol) - (HDL) - (}\frac{\text{Triglycerides}}{2.2}\text{)} \quad (5)$$

A semi-automated spectrophotometer (RX monza, Randox Laboratories Ltd, Antrim, UK) was used to measure the absorbency of the sample at a specific wavelength and temperature relevant to each assay (TAS, triglycerides, total cholesterol and HDL only). A two-point calibration of the spectrophotometer was conducted for each assay using a reagent blank and a standard to generate a calibration curve, against which total anti-oxidants, triglycerides, total cholesterol and HDL within samples were compared.

All samples were measured in duplicates, with intra-assay CV determined by analysing all serum samples. Each test run was assessed for validity and test-retest reliability prior to determination of biomarkers in serum samples by analysing quality control samples (analytical variance), relevant to each assay, 10 times. With regards to lipid metabolites, samples exhibiting pathological (Lipid Control Level 3 LE2663, Randox Laboratories Ltd, Antrim, UK) and borderline (Lipid Control Level 2 LE2662, Randox Laboratories Ltd, Antrim, UK) values were determined to provide improved reliability and accuracy of results. The overall assay CV was determined from values obtained from each duplicate across all time points for all participants.

All directly assayed biomarkers were determined colorimetrically, where the colour change induced by the relevant indicator for each assay correlated linearly to the concentration of the target metabolite within serum, according to Beer-Lambert's law (Swinehart, 1962). Lipid assays were associated with a positive linear correlation, where a greater concentration of the target metabolite was related to a greater concentration of the indicator thus intensifying the

pink colour produced (Figure 3.1). According to the manufacturer's guidelines, all samples were assessed within 3 months of blood sampling for lipid metabolites.

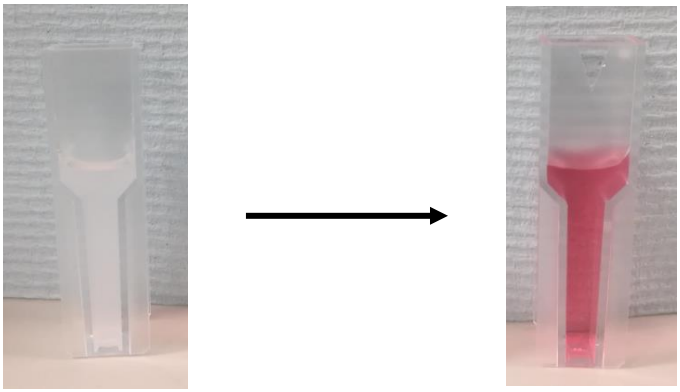


Figure 3.1. Example of colour change from clear (left) to pink (right) for lipid assays.

3.6.4. Triglycerides Assay

Serum triglycerides (Triglycerides TR210, Randox Laboratories Ltd, Antrim, UK) were determined after enzymatic hydrolysis with lipases, where values were corrected for free glycerol by subtracting 0.11 mmol.L^{-1} (Stinshoff *et al.*, 1977), according to the manufacturer's guidelines. The assay standard curve (Figure 3.2) and principle are shown below.

$$\text{Triglycerides} + H_2O \xrightarrow{\text{lipases}} \text{glycerol} + \text{fatty acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{Glycerol kinase}} \text{glycerol-3-phosphate} + \text{ADP}$$

$$\text{Glycerol-3-phosphate} + O_2 \xrightarrow{\text{Glycerol-3-phosphate oxidase}} \text{dihydroxyacetone-phosphate} + H_2O_2$$

$$2H_2O_2 + \text{4-aminophenazone} + \text{4-chlorophenol} \xrightarrow{\text{peroxidase}} \text{quinoneimine} + HCl + 4H_2O$$

Thawed samples (5 µL) were reacted with enzyme reagent (500 µL) and then incubated in a dry heating block (Rx Monza Heat Block DB-10C, Randox Laboratories Ltd, Antrim, UK) for

5 minutes at 37ºC. Samples were then analysed in the spectrophotometer at 37ºC and a wavelength of 546 nm. Analytical variance based on quality control levels 2 and 3 CV were 5.18% and 6.22%, respectively, suggesting the spectrophotometer and assay were reliable. All quality control values were within the quality control low (level 2: 0.92 – 1.39 mmol.L^{-1}) and high (level 3: 2.52 – 4.48 mmol.L^{-1}) concentration ranges, suggesting the spectrophotometer and the assay were also valid. Assay accuracy, sensitivity and linearity were 95.35%, 0.26 mmol.L^{-1} and 13.23 mmol.L^{-1}, respectively, falling within the range of measurement for experimental samples obtained in **chapters 4-6**. Intra- and inter-assay precision for level 2 quality control was 2.90% and 5.32%, respectively, and 2.48% and 3.82%, respectively for level 3 quality control. All lipid biomarkers were analysed within 3 months of blood sampling.

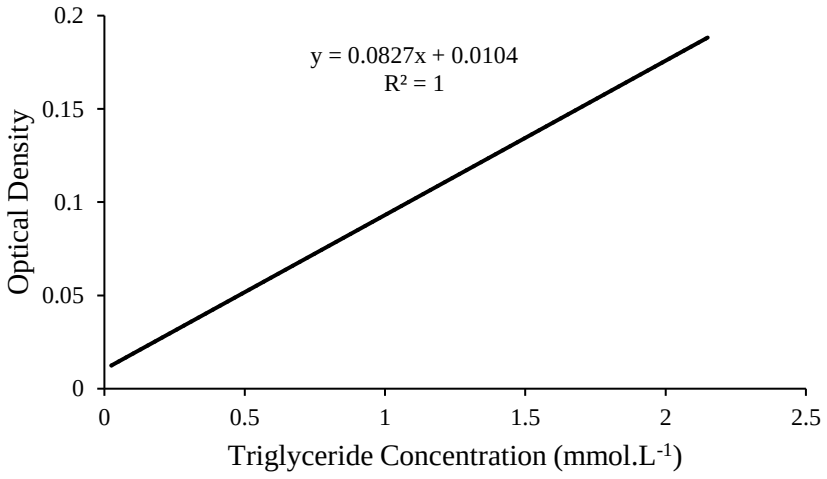
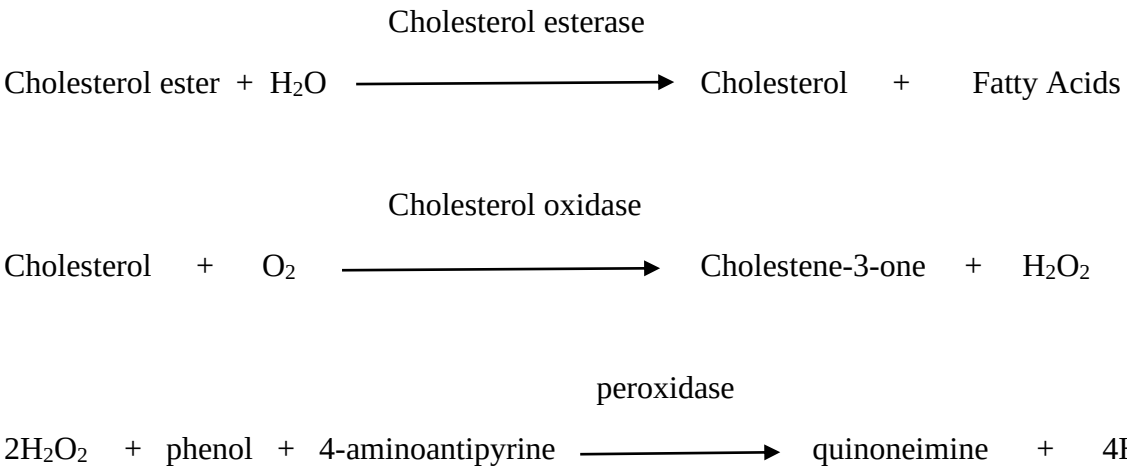


Figure 3.2. Representative two-point standard curve calibration used to determine triglyceride concentrations from serum samples.

3.6.5. Total Cholesterol Assay

Total cholesterol (Cholesterol CH200, Randox Laboratories Ltd, Antrim, UK) was determined from serum samples after enzymatic hydrolysis and oxidation. The formation of the indicator quinoneimine from hydrogen peroxide and 4-aminoantipyrine in the presence of phenol and peroxidase induced a colour change linearly correlating to the concentration of cholesterol within the sample. The assay standard curve (Figure 3.3) and principle are shown below.



Similar to triglycerides, samples (5 µL) were reacted with 500 µL of reagent, containing peroxidase, phenol, cholesterol esterase, cholesterol oxidase and 4-aminoantipyrine, then incubated at 37ºC for 5 minutes. Absorbency of the sample was then analysed in the spectrophotometer at 25ºC and a wavelength of 510 nm. The spectrophotometer and assay were valid as values for quality control samples were within the low (level 2: 3.31 – 4.29 $mmol.L^{-1}$) and high (level 3: 5.14 – 6.68 $mmol.L^{-1}$) concentration ranges. Analytical variance for quality control level 2 (CV – 5.92%) and level 3 (CV – 6.29%) suggested the spectrophotometer and assay were reliable. Assay accuracy, sensitivity and linearity were 92.08%, 0.35 $mmol.L^{-1}$ and 17 $mmol.L^{-1}$, respectively, falling within the range of measurement for experimental samples obtained in **chapters 4-6**. Intra- and inter-assay precision for level 2 quality control was 2.74% and 3.92%, respectively, and 1.23% and 4.64%, respectively, for level 3 quality control.

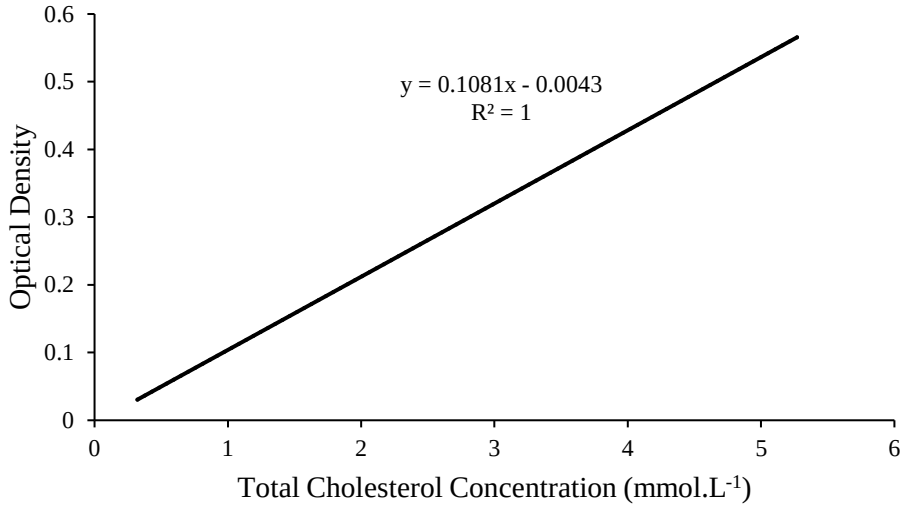


Figure 3.3. Representative two-point standard curve used to ascertain total cholesterol concentrations in serum samples.

3.6.6. HDL Assay

According to manufacturer's guidelines, in order to isolate HDL from other lipid metabolites (LDL and chylomicrons) in serum, samples were pre-treated with a buffer containing phosphotungstic acid and magnesium chloride. Samples were then centrifuged at 12000 rev.min^{-1} for 2 minutes (Hyspin 16K, Anachem, Luton, UK) to leave a supernatant which was then assessed using the same procedure as total cholesterol (*section 3.6.5*). Values obtained when assessing assay validity were within the low (level 2: 1.35 – 1.83 mmol.L^{-1}) and high (level 3: 2.57 – 3.49 mmol.L^{-1}) concentration ranges, suggesting the spectrophotometer and the assay were valid. Quality control levels 2 and 3 CV were 3.93% and 2.53%, respectively suggesting the spectrophotometer and assay were also reliable. Assay accuracy, sensitivity and linearity were 95.41%, 0.07 mmol.L^{-1} and 23.70 mmol.L^{-1}, respectively, falling within the range of measurement for experimental samples obtained in **chapters 4-6**. The high (level 2) and low (level 3) concentration quality controls, intra- and inter-assay precision were reported as 10.5% and 10.2% (level 2), respectively, and 8.8% and 11.7% (level 3), respectively. An example of the assay standard curve (Figure 3.4) is shown below.

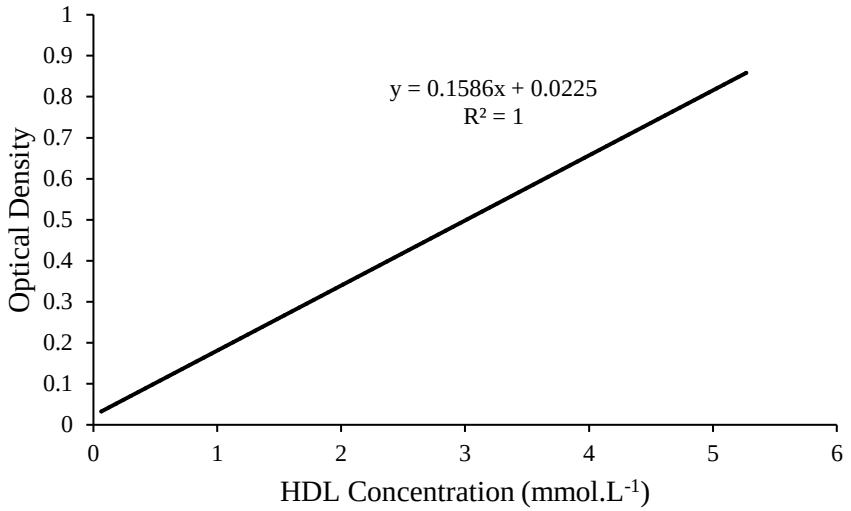


Figure 3.4. Representative two-point calibration standard curve used to determine HDL concentrations in serum samples.

3.6.7. Total Cholesterol:HDL Ratio

Ratios between total cholesterol and HDL (TC:HDL) were calculated by dividing total cholesterol values by their corresponding HDL values for each time point measured in **chapters 4-6**, to determine atherogenic (Castelli) index (Millán *et al.*, 2009). TC:HDL has been shown to be a better predictive indicator of cardiovascular risk than either parameter in isolation (Millán *et al.*, 2009). Furthermore, TC:HDL is also a good predictor of the degree of benefit from lipid-lowering treatments (Millán *et al.*, 2009).

3.6.8. Insulin Assay

Serum insulin samples were measured in duplicates using a human 96-well insulin enzyme-linked immunosorbent assay (ELISA) (Insulin Human ELISA KAQ1251, Invitrogen, Thermo Fisher Scientific, USA), with a common sample measured on each plate for determining inter-plate CV. Ready-made standards (concentrations: 0.0, 4.9, 14.0, 42.0, 98.0, 172.0 µIU) were reconstituted with 2 mL distilled water for the 0.0 standard and with 1 mL for all other standards, in order to generate a six-point standard curve. The ELISA was based on the 'sandwich immunoassay' principle where 50 µL of standards/controls/samples were added to

wells pre-coated with anti-insulin capture antibodies to enable selective binding for insulin. Following this, 50 µL anti-insulin horseradish peroxidase (HRP) conjugate was added to each well to bind to capture antibodies and form an antibody-antigen complex. To ensure complete binding, the plate was incubated for 30 minutes at room temperature and then washed with wash buffer solution to remove unbound antibodies. Chromogen substrate (tetramethylbenzidine, TMB), was added (100 µL) to each well within 15 minutes following the washing step and then the plate was incubated in the dark for 15 minutes. A detectable colour change from colourless to blue ensued upon addition of chromogen substrate due to conversion by HRP (Figure 3.5). After incubation, the reaction was stopped with the addition of 100 µL stop solution resulting in a second colour change from blue to yellow (Figure 3.5).

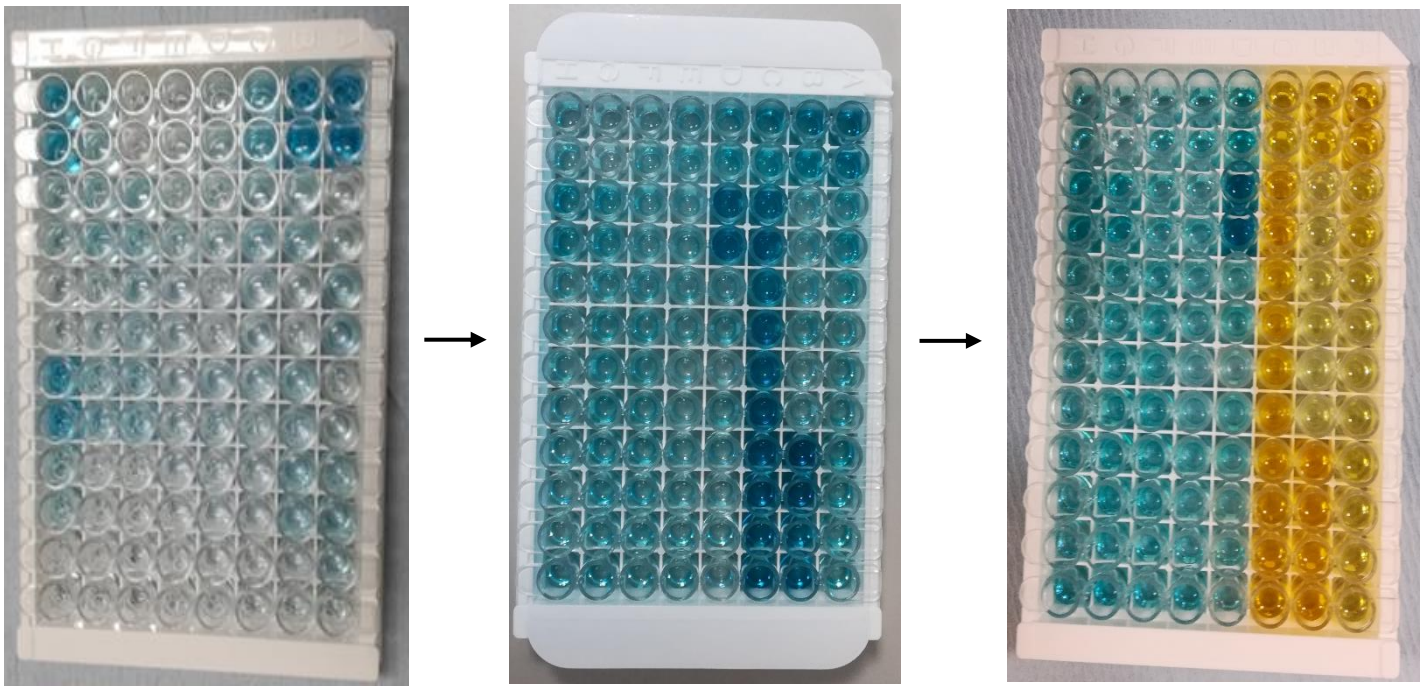


Figure 3.5. Representation of colour change from colourless (left) to blue (middle) upon addition of chromogen substrate, and colour change from blue (middle) to yellow (right) upon addition of stop solution.

The plate was then immediately read on a plate reader (Multiskan™ FC Microplate Photometer, Thermo Scientific, USA) at 450 nm to obtain absorbances of each well and insulin concentrations (pmol.L^{-1}) were then calculated using the standard curve according to Beer-

Lambert's law. A four-parameter algorithm standard curve was generated (Figure 3.6) using standard curve fitting software (SigmaPlot, Systat Software Inc, San Jose, USA), where values were converted from μIU.mL^{-1} to pmol.L^{-1} by multiplying by 6.945 (Bos *et al.*, 2010). The kit inter-assay using normal and high quality controls and intra-assay precision, range and sensitivity were 8.1% (normal quality control) and 9.0% (high quality control), 5.4%, 35.42-1736.25 pmol.L^{-1} and 1.18 pmol.L^{-1}, respectively. Intra-plate precision was 8.8% (normal quality control) and 9.6% (high quality control) and 7.69%, respectively.

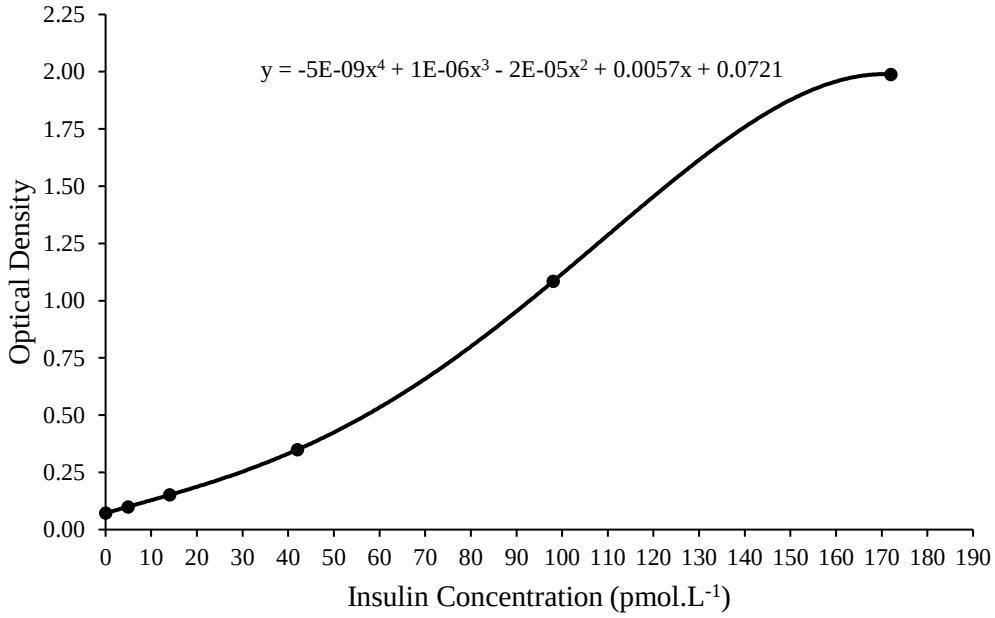


Figure 3.6. Representative four-parameter algorithm standard curve generated using standard curve fitting software to determine insulin concentrations in pmol.L^{-1}.

3.6.8.1. Insulin Resistance and Sensitivity Indexes

In **chapters 5-6**, Homeostatic Model Assessment (HOMA) was used to estimate fasting steady-state pancreatic β-cell function (HOMA2-β), insulin resistance (HOMA2-IR index) and insulin sensitivity (HOMA2-%S) through the HOMA2 model (HOMA2-IR, available from https://www.dtu.ox.ac.uk/homacalculator/) (Levy, Matthews and Hermans, 1998). *Equations 6, 7 and 8* outline the determination of these variables (Matthews *et al.*, 1985). The HOMA model estimates insulin sensitivity and pancreatic β-cell function based on fasting glucose and insulin concentrations (Wallace, Levy and Matthews, 2004). The relationship between these

concentrations reflects the effect of hepatic glucose production and insulin secretion; maintained by a feedback loop between the liver and β-cells (Wallace, Levy and Matthews, 2004).

$$\text{HOMA2-}\beta\ (\%) = \frac{\left(20 * \text{Fasting Insulin}\ \left(\mu\text{IU.mL}^{-1}\right)\right)}{\left(\text{Fasting Glucose}\ \left(\text{mmol.L}^{-1}\right)\text{-}3.5\right)} * 100 \quad (6)$$

$$\text{HOMA2-\%S}\ (\%) = \left(\frac{1}{\text{HOMA2-IR}}\right) * 100 \quad (7)$$

$$\text{HOMA2-IR} = \frac{\left[\text{Fasting Glucose}\ \left(\text{mmol.L}^{-1}\right) * \text{Fasting Insulin}\ \left(\mu\text{IU.mL}^{-1}\right)\right]}{22.5} \quad (8)$$

As recommended by Wallace, Levy and Matthews (2004), HOMA2-%S was calculated to assist in the correct interpretation of HOMA2-β. Classifications where HOMA2-β = 100% and HOMA2-IR = 1 indicate normal pancreatic β-cell function and lack of insulin resistance in healthy individuals (Wallace, Levy and Matthews, 2004). A value lower than the threshold for HOMA2-β indicates dysfunctional pancreatic β-cell function and a value greater than the threshold for HOMA2-IR indicates increased insulin resistance.

Statistically significant, strong, positive correlations of HOMA2-%S and HOMA2-β against other insulin sensitivity and β-cell function assessment methods have been observed in various populations, as summarised by Wallace, Levy and Matthews (2004). Additionally, strong, positive correlations have been detected in healthy participants and those with hypertension and T2D between HOMA2-IR and the hyperinsulinaemic-euglycaemic clamp method (Wallace, Levy and Matthews, 2004; Sarafidis *et al.*, 2007), suggesting suitability of using the HOMA2 model to determine pancreatic β-cell function and insulin resistance. Consequently, the HOMA2 model has also been utilised in various studies examining responses in humans with MetS (Vittone *et al.*, 2007; Saadi *et al.*, 2008; Yeap *et al.*, 2010; Beltrán-Debón *et al.*, 2015).

Chapter 4

Effects of Montmorency Tart Cherry Juice Supplementation and FATMAX Exercise on Fat Oxidation and Cardio-Metabolic Markers in Healthy Humans

Publication arising from this Chapter (Appendix 10): Desai, T., Bottoms, L. and Roberts, M. (2018). The effects of Montmorency tart cherry juice supplementation and FATMAX exercise on fat oxidation rates and cardio-metabolic markers in healthy humans. *European Journal of Applied Physiology, 118* (12), pp. 2523-2539.

4.1. Introduction

Cardiovascular disease, T2D and associated diseases combined are the leading health burden and cause of mortality worldwide (Danaei *et al.*, 2014), therefore the necessity for an intervention is paramount. Dietary interventions to improve cardio-metabolic health are highly sought after as they pose less risk than pharmacological drugs (Vendrame *et al.*, 2016). Substantial evidence now exists demonstrating the relationship between high consumption of vegetables and fruits, improvements in disease symptoms and the reduced risk of disease development (Li *et al.*, 2017). However, concerns remain over the feasibility of maintaining a high fruit and vegetable intake over a prolonged time period, therefore daily dietary supplementation containing health-promoting phytonutrients is appealing. Subsequently, the present study supplemented small volumes of highly concentrated MTCJ, to boost consumption of phytonutrients and thus provide sufficient amounts at physiologically relevant concentrations in a more practical and efficient method (Zheng *et al.*, 2017).

Tart cherries and thus tart cherry juice possess a high phytochemical content particularly rich in anthocyanins, flavonols and phenolic acids (Seymour *et al.*, 2009; Kirakosyan *et al.*, 2010). These phytochemicals are thought to contribute to effectively combating oxidative stress, inflammation and repairing muscle damage post-exercise (Bell *et al.*, 2014c; Bell *et al.*, 2015). In rodents, tart cherries modulated peroxisome proliferator-activated receptor (PPAR) signalling pathways, thus percentage fat mass, hyperinsulinaemia, hyperlipidaemia and inflammation were all reduced (Seymour *et al.*, 2008, 2009). Observations in humans have been more equivocal, with tart cherry supplementation showing no effect on blood pressure and blood-based cardio-metabolic markers in healthy participants (Lynn *et al.*, 2014). More favourable findings have been reported in studies (Ataie-Jafari *et al.*, 2008; Martin *et al.*, 2010; Keane *et al.*, 2016bc) examining ‘at risk’ or diseased populations, indicating amelioration of cardio-metabolic function with tart cherry juice.

Skeletal muscle is a critical organ involved in lipid metabolism and dysfunction of cellular and molecular cascades which are implicated in the manifestation of CVD, insulin resistance, inflammation and oxidative stress (Stump *et al.*, 2006). During sub-maximal exercise at FATMAX intensity, skeletal muscle primarily oxidises lipids (Romijn *et al.*, 1993). In healthy participants, Robinson *et al.* (2015) demonstrated a significant positive correlation between MFO and both 24-hour fat oxidation and insulin sensitivity. As eluded to in *section 2.2.5.1.2* findings suggest FATMAX exercise encourages enhancements in fat oxidation rate leading to improved body composition, total cholesterol and insulin sensitivity (Brun, Romain and Mercier, 2011). Consequently, the purpose of specifically incorporating aerobic FATMAX exercise in the present study were twofold. Firstly, an individualised approach to achieve maximal fat oxidation rates during moderate-intensity exercise has been suggested as the best method to reduce HbA_{1c}, insulin-dependent glucose, fat mass and total cholesterol (Brun, Romain and Mercier, 2011). Secondly, it is an appropriate methodological test to measure the effects of an intervention on fat oxidation rates during exercise and when combined with a potential CRM, such as MTCJ, may induce additional improvements on cardio-metabolic pathways and therefore overall health (Besnier *et al.*, 2015).

Rationale for the present study was based on previous work demonstrating the benefits of anthocyanin-rich blackcurrants (Cook *et al.*, 2015, 2017b) and green tea polyphenols (Venables *et al.*, 2008) on fat oxidation rates during exercise. Also, the positive results regarding the effects of tart cherry supplementation against cardio-metabolic dysfunction in rodents (Seymour *et al.*, 2008, 2009) and humans (Ataie-Jafari *et al.*, 2008; Martin *et al.*, 2010; Keane *et al.*, 2016bc). However, to date, no research has explored the cardio-metabolic responses to tart cherry supplementation and exercise in tandem, thus healthy participants were recruited to assess for any adverse effects prior to investigating responses in clinical populations. Subsequently, this study set out to examine the physiological responses of MTCJ

supplementation with FATMAX exercise on fat oxidation rates, body composition and cardio-metabolic markers in healthy participants. It was hypothesised that MTCJ supplementation would augment fat oxidation rates at rest and during exercise, thus proving more efficacious at improving body composition, functional and *in sera* cardio-metabolic markers than previous research conducted with tart (Martin *et al.*, 2010; Lynn *et al.*, 2014) and sweet (Kelley *et al.*, 2006) cherry supplementation at rest in healthy participants.

4.2. Methods

4.2.1. Participants

Eleven (7 males and 4 females) healthy, recreationally active (≥150 minutes moderate-intensity aerobic exercise per week), participants (mean ± SD; age 30 ± 10 years, stature 1.76 ± 0.09 m, body mass 76.4 ± 13.2 kg, BMI 24.43 ± 3.23 $kg.m^{-2}$, $\dot{V}O_{2peak}$ 35.87 ± 4.78 $mL.kg^{-1}.min^{-1}$) volunteered for the study. All participants were non-smokers, BMI <30, injury-free and not diagnosed with any cardio-metabolic or renal diseases at the time of testing but had a family history of cardio-metabolic disease. Participants were instructed to cease consumption of any other supplementation two weeks before and for the duration of the study. All participants provided written informed consent to participate in the study and completed health screen questionnaires before the study commenced. Ethical approval was obtained from the University of Hertfordshire Health and Human Sciences Ethics Committee. The study was registered as a clinical trial on clinicaltrials.gov (NCT02999256).

As this was the first study to examine fat oxidation with cherries, it was difficult to confidently predict a sample size using power analysis. Previous studies that had researched the effects of fat oxidation (Cook *et al.*, 2015; Roberts *et al.*, 2015) or cherry supplementation (Bowtell *et al.*, 2011; Bell *et al.*, 2014bc) had a total sample size between 10-16 participants.

4.2.2. Procedures

4.2.2.1. Research Design

This study utilised a single-blind (blinded to participant), placebo-controlled, randomised, crossover design, where each participant acted as their own control. Participants were required to complete two conditions over 10 weeks, differing only in supplementation, Montmorency tart cherry juice (MTCJ) and placebo (PLA). Participants were randomised to start consumption of either MTCJ or PLA first, followed by a 14-day washout period (Howatson *et al.*, 2012; Cook *et al.*, 2015; Keane *et al.*, 2016b) and then consumption of the reverse supplement to the first condition.

Both conditions were identical in terms of design and testing procedures and comprised of 5 sessions each, with sessions lasting approximately 2.5 hours. A timeline for testing sessions and the supplementation period is shown in Figure 4.1.

Baseline measurements were obtained during the first FATMAX/$\dot{V}O_2$max session in the first condition. Other than blood sampling and the type of exercise itself, all other testing procedures pre- and post-exercise were identical across each session. Blood sampling was conducted pre- and post-exercise in order to ascertain the acute differences induced by one-hour sub-maximal FATMAX exercise; and pre-, mid- and post-supplementation to assess the longer-term effects of supplementation on cardio-metabolic biomarkers, within and between conditions.

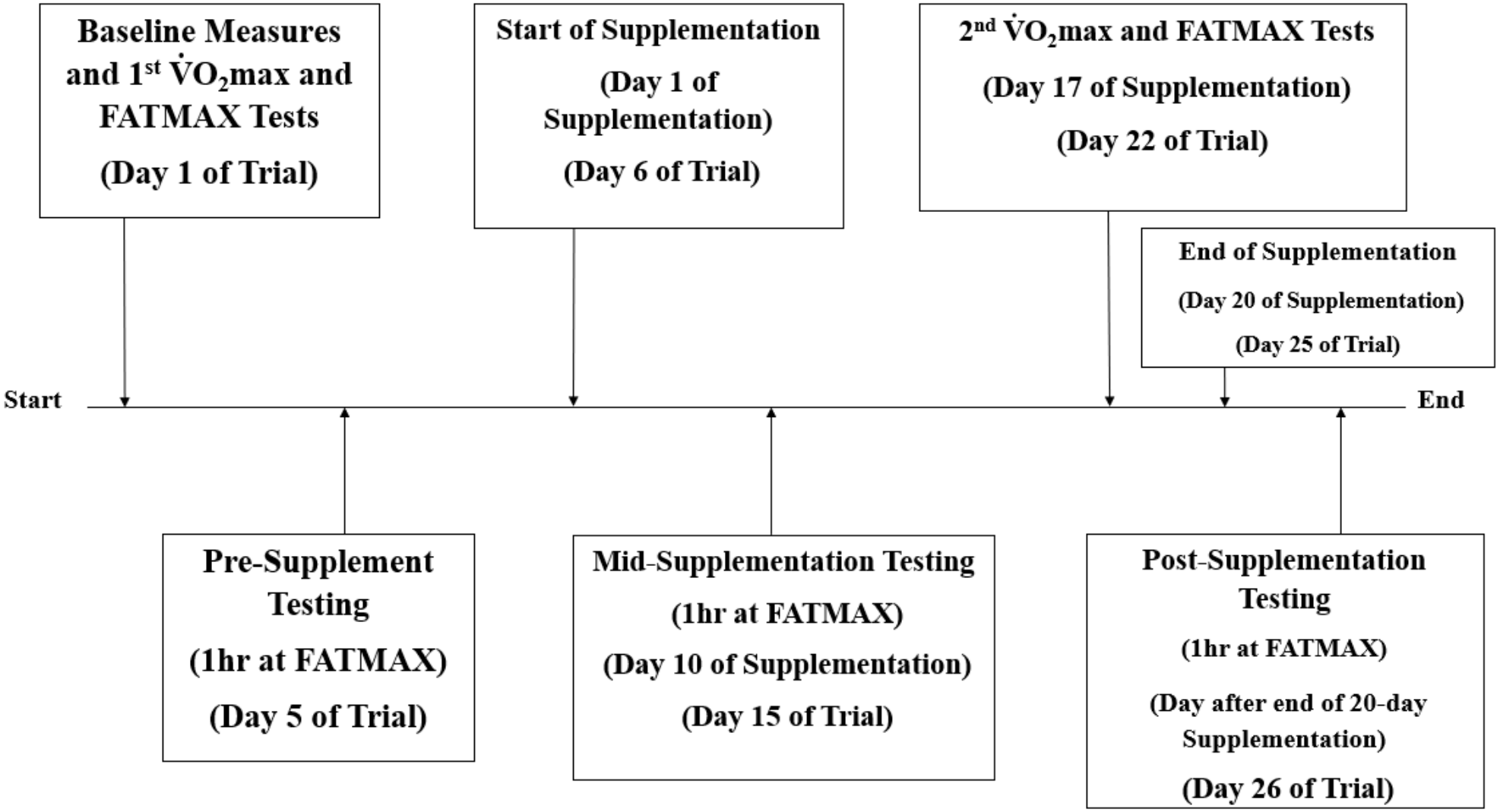


Figure 4.1. Schematic of testing protocol for each condition (MTCJ and PLA).

4.2.2.2. Dietary and Exercise Guidelines

Participants were instructed to maintain their habitual polyphenol intake, particularly anthocyanins, as opposed to complete restriction throughout the study. Refer to *section 3.2* for further details on dietary guidelines employed in this study.

Croci *et al.* (2014) recommended macronutrient dietary control greater than 48 hours before exercise to reduce day-to-day intra-individual variability in fat oxidation. Pilot testing demonstrated less variability with 72-hour dietary control (CV – 9.88%) for MFO during the one-hour sub-maximal exercise at FATMAX, compared to 48-hour dietary control (CV – 13.93%). Participants were therefore asked to replicate their 3-day food and drink consumption before each testing session (Alkhatib *et al.*, 2015; Nordby *et al.*, 2015; Roberts *et al.*, 2015). A standardised menu for the final 24-hours, of the 3-day period, was provided to each participant based on their habitual diet. This was based on findings from Roberts *et al.* (2015), who reported greater adherence to a diet standardised 24-hours prior to exercise and notably, less variance in fat oxidation compared to dietary control of only the evening meal before exercise. Standardisation of macronutrients was set at 15% protein, 55% carbohydrate and 30% fat of energy intake (Melanson *et al.*, 2002; Ben Ounis *et al.*, 2009). As subjects tend to underreport dietary consumption with food diaries, an extra 20% (Mertz *et al.*, 1991; Black *et al.*, 1993) of the 3-day average for habitual energy intake was added to the standardised menu. Standardisation was performed to ensure that all participants attended exercise testing sessions in a similar state of energy balance and fuel repletion (Horton *et al.*, 1998; Jeacocke and Burke, 2010). All participants reported 100% adherence when food diaries were assessed for percentage contributions of macronutrients to total energy intake, total polyphenols and anthocyanins. Analysis of habitual diets indicated daily consumption of 1593 mg and 57 mg for total polyphenols and anthocyanins, respectively; more than double the general population

intake for anthocyanins (23 mg) (Zamora-Ros *et al.*, 2011) but less than the total polyphenol consumption in the UK (1675 mg) (Zamora-Ros *et al.*, 2016).

4.2.2.3. Supplementation

The supplementation period lasted 20 days, similar to Roberts *et al.* (2015), where participants ceased consumption following the 20th day and during the washout period. The experimental condition included supplementation with MTCJ whilst the control involved supplementation of an energy matched placebo (Table 4.1). The MTCJ was made with 30 mL Montmorency tart cherry concentrate (Cherry Active, Active Edge Ltd, Hanworth, UK) and 100 mL water. Placebo composition consisted of 30 mL commercially available fruit-flavoured cordial (Cherries and Berries, Morrisons, Bradford, UK), with anthocyanins used only for colouring and negligible anti-oxidant content, mixed with 100 mL water. In order to match the placebo for energy, taste and visual appearance, a flavourless carbohydrate (Maltodextrin, My Protein Ltd, Northwich, UK), citric acid (100% Pure Citric Acid, VB and Sons, UK) and black food colouring (Morrisons, Bradford, UK) were added, respectively. Participants consumed two 130 mL servings per day, once in the morning immediately before breakfast, then again in the evening before dinner (Bell *et al.*, 2014b; Bell *et al.*, 2016). Due to fasting restrictions on testing days, participants delayed consumption of the morning serving until lunchtime. Refer to *section 3.3* for more information on supplementation.

In order to optimise compliance to supplementation, participants were given enough supplements to last 5 days, after which they returned to the laboratory with any unconsumed juice before being given further supplements to last another 5 days. This was repeated a total of four times to fulfil the 20-day supplementation period.

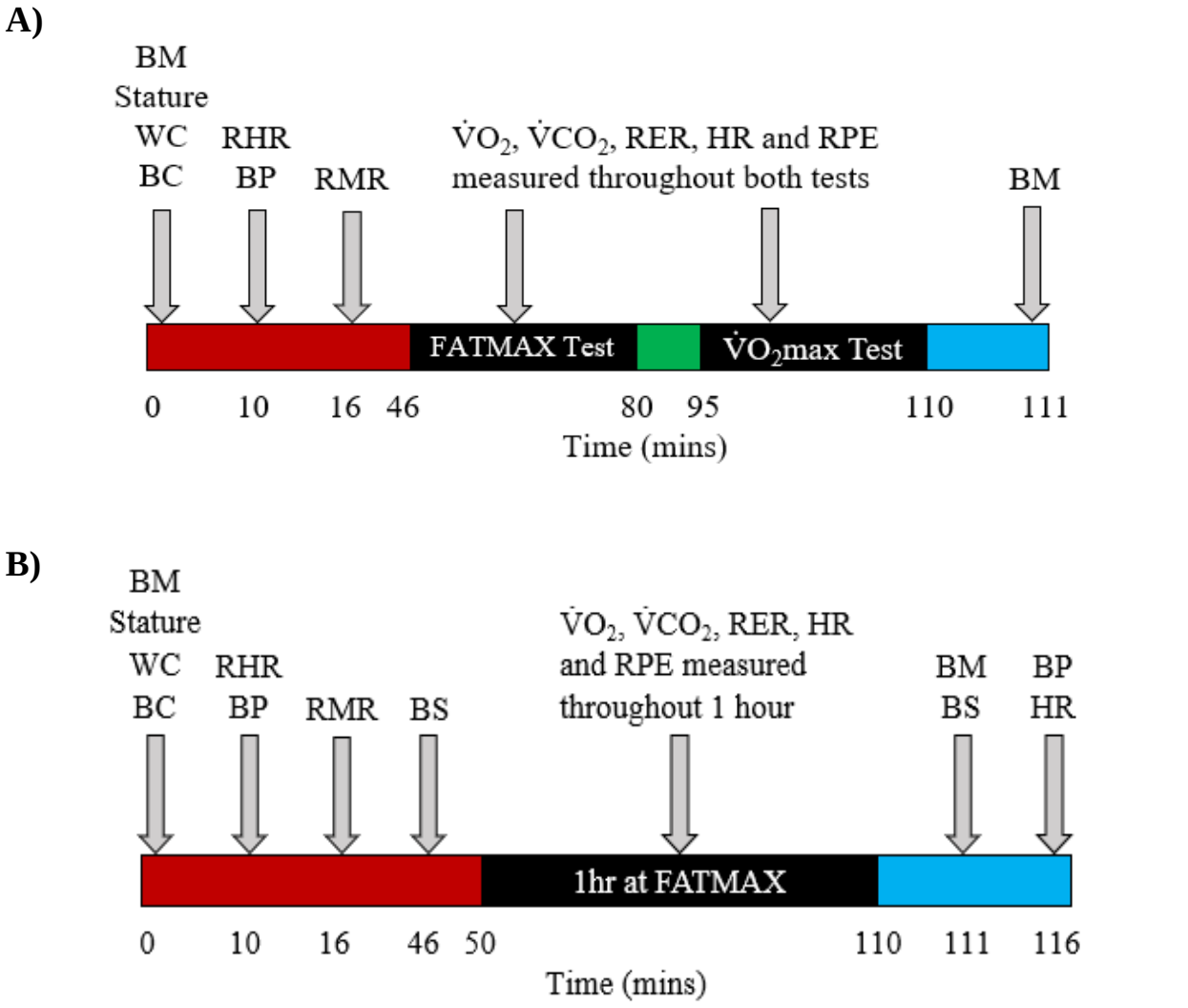
Table 4.1. Nutritional information of Montmorency tart cherry concentrate and placebo supplements.

	Montmorency tart cherry concentrate (per 30 mL)	Placebo (per 130 mL)
Energy (kcal)	102	102
Carbohydrate (g)	24.50	25.35
of which sugars (g)	17.90	25.32
Protein (g)	1.10	0.03
Fat (g)	0	0
of which saturates (g)	0	0
Fibre (g)	2.60	Trace
Total Anthocyanins (mg)	270	0

4.2.3. Testing Protocol

During all testing sessions, stature, body mass and waist circumference were measured initially, immediately followed by segmental body composition analysis (*sections 3.5.1* and *3.5.2*).

After 10 minutes rest, resting HR (Polar T31c and FT1, Polar Electro Oy, Finland) and BP (*section 3.5.3.2*) were obtained followed by RMR (*section 3.5.4*). Pre-exercise blood sampling was then performed followed by post-exercise blood sampling during the one-hour sub-maximal sessions only. HR and BP were measured after post-exercise blood sampling, approximately 5-8 minutes after the cessation of exercise (Figures 4.2A and 4.2B).



Key:
BC – Body Composition; BM – Body Mass; BP – Blood Pressure; BS – Blood Sampling; HR – Heart Rate; RHR – Resting HR; RER – Respiratory Exchange Ratio; RMR – Resting Metabolic Rate; RPE – Rating of Perceived Exertion; $\dot{V}CO_2$; Volume of Carbon Dioxide Production; $\dot{V}O_2$ – Volume of Oxygen Uptake; WC – Waist Circumference
= Pre-Exercise
= Exercise
= Post-Exercise
= Rest

Figure 4.2. Schematic of testing procedure (A) during FATMAX and $\dot{V}O_2$max testing sessions and (B) during 1-hour sub-maximal exercise at individually determined FATMAX testing sessions.

4.2.3.1. FATMAX and $\dot{V}O_2$max Protocol

During the first and fourth testing sessions of each condition, participants conducted FATMAX and $\dot{V}O_2$max tests. An incremental 3-minute step protocol was chosen to determine FATMAX. This was based on data obtained during pilot testing where two FATMAX protocols were

compared and reliability of the protocols assessed. The first protocol required participants to cycle at 70 rev.min^{-1} at an initial intensity of 30 W with increments of 15 W every 2 minutes. The second protocol increased by 10 W every 3 minutes with initial wattage and cadence the same as the first protocol. Both protocols were repeated twice separated by one week, with 72-hours dietary standardisation. Limits of agreement (LoA) and CV for MFO and FATMAX (%$\dot{V}O_{2peak}$) for both tests indicated better reliability for the latter protocol (Appendix 1).

The FATMAX test required participants to cycle on an electromagnetically braked cycle ergometer (Excalibur, Lode, Groningen, The Netherlands) at 70 rev.min^{-1}. Fore/aft position, saddle and handlebar height on the cycle ergometer were recorded for each participant, during the first exercise session and replicated for all other sessions thereafter. Participants started at an intensity of 30 W and the cycle was programmed to increase wattage by 10 W every 3 minutes. The FATMAX test was terminated once RER was greater than 1 for a continuous period of 30 seconds (Croci *et al.*, 2014). This protocol was adapted from previous studies (Achten, Gleeson and Jeukendrup, 2002; Alkhatib, 2014), in order to design an appropriate test which reflected the determination of FATMAX and MFO as accurately as possible, for the participant cohort recruited in this study. In alignment with Alkhatib *et al.* (2014), smaller 10 W increments were chosen to better determine FATMAX based on assessment of a wide range of exercise intensities (Venables, Achten and Jeukendrup, 2005; Purge, Lehismets and Jürimäe, 2015).

The $\dot{V}O_2$max test was performed 15 minutes following the FATMAX test to prevent the depletion of muscle glycogen, which occurs during the $\dot{V}O_2$max test, affecting substrate oxidation in the FATMAX test. Due to the low-intensity nature of the FATMAX test, 15 minutes provided sufficient recovery time before the $\dot{V}O_2$max test and minimised subject burden as participants did not have to attend a separate session for determination of $\dot{V}O_2$max.

The protocol for determination of $\dot{V}O_2$max consisted of cycling at 70-80 rev.min^{-1}, at an initial intensity of 100 W with wattage increasing by 20 W every minute until volitional exhaustion. The test was terminated according to BASES Sport and Exercise Physiology testing guidelines (Winter *et al.*, 2007).

HR and differentiated (overall and legs) ratings of perceived exertion (RPE) on a 6-20 scale (Borg, 1973) were recorded 15 seconds before the end of each stage for FATMAX and $\dot{V}O_2$max tests. RPE was recorded during all exercise sessions at 1, 3- and 5-minutes intervals during the $\dot{V}O_2$max, FATMAX and 1-hour sub-maximal sessions, respectively.

4.2.3.1.1. FATMAX Analysis

FATMAX was determined through visual inspection of the fat oxidation curve generated from the FATMAX sub-maximal test. The corresponding intensity at the peak of this curve was deemed to be FATMAX and was implemented during the one-hour sub-maximal sessions. This intensity was also confirmed by identifying the highest fat oxidation rate for the final minute of each stage calculated from a 15 second rolling average of each 3-minute stage. The second $\dot{V}O_2$max and FATMAX tests were used to assess changes in training status (MacRae and Mefferd, 2006) and any differences in FATMAX which may have occurred due to the previous exercise sessions and supplementation. FATMAX was adjusted to the new exercise intensity for the post-supplementation one-hour sub-maximal exercise if the change in FATMAX (expressed in terms of %$\dot{V}O_2$max) was greater than a pre-determined threshold (CV between both FATMAX determination tests >6%). The rationale behind this was to set an operational standard against which comparisons could be made to determine whether FATMAX changed after 16 days of supplementation. The threshold was established based on data collected during pilot testing, where the CV of FATMAX between two FATMAX sub-maximal determination tests (3 minutes stage protocol) were calculated. This ensured that the change in exercise

intensity represented a genuine physiological shift in FATMAX, as opposed to differences in FATMAX occurring due to equipment variance.

4.2.4. Measures and Equipment

4.2.4.1. Respiratory Gas Analysis

Real-time breath-by-breath gaseous exchange data (Metalyzer 3B, Cortex Biophysik, Leipzig, Germany) was recorded during all exercise tests. This method enabled the quantification of $\dot{V}O_2$ (L.min^{-1}), $\dot{V}CO_2$ (L.min^{-1}) and RER. Consequently, indirect calorimetry was used to calculate EE (kcal.min^{-1}) and substrate oxidation rates (g.min^{-1}) using stoichiometric equations specifically developed for exercise at intensities between 40-50% $\dot{V}O_{2peak}$, as shown below (Jeukendrup and Wallis, 2005).

$$\textit{Fat Oxidation Rate}\ (\text{g.min}^{-1}) = [(1.695 \cdot \dot{V}O_2) - (1.701 \cdot \dot{V}CO_2)] \quad (1)$$

$$\textit{CHO Oxidation Rate}\ (\text{g.min}^{-1}) = [(4.344 \cdot \dot{V}CO_2) - (3.061 \cdot \dot{V}O_2)] \quad (2)$$

$$\textit{Energy Expenditure}\ (\text{kcal.min}^{-1}) = [(0.575 \cdot \dot{V}CO_2) - (4.435 \cdot \dot{V}O_2)] \quad (3)$$

RER was determined from the ratio of CO_2 produced relative to O_2 consumed as shown in *Equation 4*.

$$RER = \dot{V}CO_2 / \dot{V}O_2 \quad (4)$$

Prior to exercise, mask (V Mask, Hand Rudolph, USA) and hairnet (Hans Rudolph, USA) sizes were determined and maintained for each participant throughout the study. The automated gas analyser was calibrated using a three-point calibration procedure as per manufacturer's instructions. First, barometric pressure was analysed followed by calibration of the analyser against a mixture of gases with known concentrations (5% CO_2, 17% O_2). Finally, the volume transducer in the analyser was calibrated with a 3-litre calibration syringe (Series 5530, Hans Rudolph, USA).

Exported data was analysed only from the final 50 minutes of all one-hour sub-maximal tests to ensure participants reached steady-state. Data was averaged for every 15 second period during the entire 50 minutes of exercise. To ensure energy and substrate oxidation rates were reliable, data during the final minute of each stage was averaged for the FATMAX test as this was the period where steady-state was most likely to be achieved (Bordenave *et al.*, 2007).

4.2.4.2. Blood Sampling and Analysis

Refer to *section 3.6.1* for details on blood sampling procedures.

Blood samples were obtained from 10 participants, as one subject was uncomfortable with the venepuncture method.

4.2.4.2.1. Plasma Volume Change

Whole blood from EDTA or lithium heparin tubes, was analysed for haemoglobin and haematocrit to calculate plasma volume change (PVC). Assay results were corrected for PVC as a result of exercise-induced changes in haemoconcentration (Allgrove *et al.*, 2011) by the equation shown below:

$$\text{Corrected Assay Results} = \%\text{PVC} * \text{Measured Post-Exercise Assay Value}$$

PVC was determined using the method and equations of Dill and Costill (1974) by means of comparing the pre- and post-exercise haemoglobin and haematocrit values.

$$BV_A = BV_B * (Hb_B / Hb_A) \quad (5)$$

$$CV_A = BV_A * Hct_A \quad (6)$$

$$PV_A = BV_A - CV_A \quad (7)$$

$$\Delta PV\ (\%) = 100 * (PV_A - PV_B) / PV_B \quad (8)$$

Where *BV* denotes blood volume, *CV* (cell volume), *Hb* (haemoglobin), *Hct* (haematocrit), *PV* (plasma volume) and ΔPV, % (percentage change in plasma volume). Subscripts of B and A denote pre-exercise and post-exercise values, respectively whilst pre-exercise BV was assumed to be 100.

Haemoglobin was analysed, via a modified azidemethemoglobin reaction, by pipetting 10µL of whole blood into a microcuvette (HemoCue Hb 201 Microcuvettes, Sweden) which was then placed into a reader (HemoCue Hb 201^{+}, Sweden). Haematocrit was analysed by pipetting 60 µL of whole blood into micro haematocrit tubes (Hawksley, UK) after which one end was sealed with Cristaseal wax (Hawksley, UK). Tubes were then placed into a centrifuge (Haematospin 1300, Hawksley, UK) and spun at 1300 $rev.min^{-1}$ for 3 minutes. Tubes were placed on a slide reader (Micro Haematocrit Tube Reader, Hawksley, UK) and percentage packed cell volume was subsequently determined.

4.2.4.2.2. Glucose

Refer to general methodology *section 3.6.2* for details on performing glucose analysis.

Overall intra-individual and inter-individual CV were 1.41% and 10.64%, respectively.

4.2.4.2.3. Total Anti-oxidant Status Assay

Total anti-oxidant capacity within serum samples, tart cherry concentrate and tart cherry juice (concentrate diluted with water), was assessed using the total anti-oxidant status colorimetric assay (Total Anti-oxidant Status NX2332, Randox Laboratories Ltd, Antrim, UK). The assay was based on the Trolox Equivalent Anti-oxidant Capacity (TEAC) method, according to the manufacturer's guidelines. Briefly, ABTS® (2,2'-Azino-di-[3-ethylbenzthiazoline sulphonate]) was incubated with metmyoglobin (chromogen) and hydrogen peroxide to generate the radical cation $ABTS^{®+}$ and the appearance of a stable blue-green colour. Repression of the colour change (Figure 4.3), due to inhibition of $ABTS^{®+}$ by anti-oxidants, was proportional to the concentration of anti-oxidants within the sample, indicating a negative linear correlation (Figure 4.4). The assay principle is shown below.

$HX\text{-}Fe^{III} + H_2O_2 \longrightarrow X\text{–}[Fe^{IV} = 0] + H_2O$

$ABTS^{®} + X\text{–}[Fe^{IV} = 0] \longrightarrow ABTS^{®+} + HX\text{-}Fe^{III}$

$HX\text{-}Fe^{III}$: Metmyoglobin

$X\text{–}[Fe^{IV} = 0]$: Ferrylmyoglobin

H_2O_2 : Hydrogen Peroxide

H_2O : Water

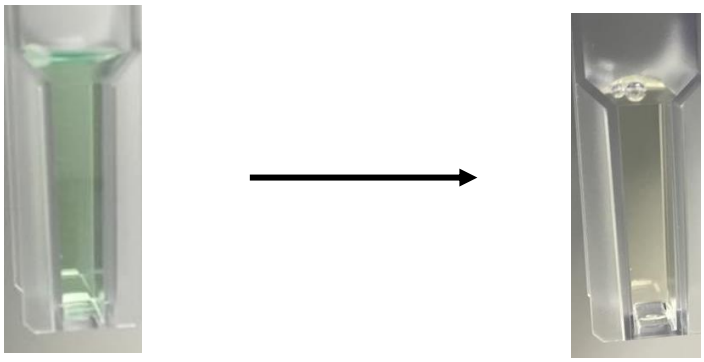


Figure 4.3. Example of colour change from stable blue-green colour (left) to clear (right), due to inhibition of $ABTS^{®+}$ by anti-oxidants.

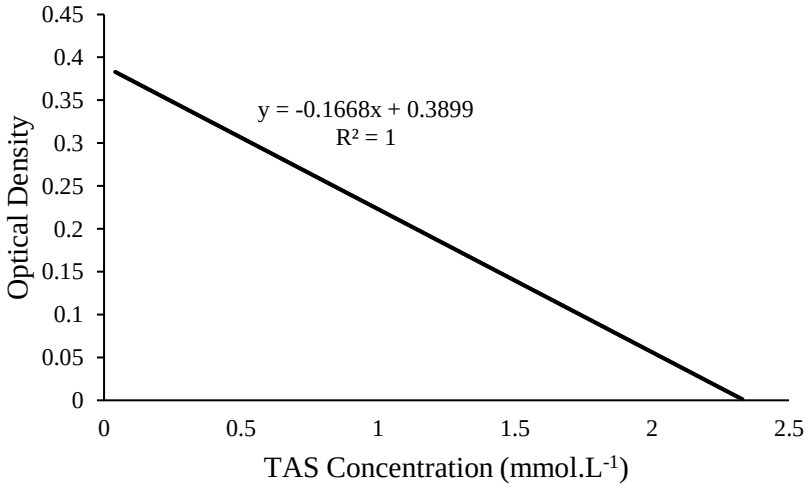


Figure 4.4. Representative two-point standard curve used for determining TAS concentrations in serum samples.

Absorbency of the samples was measured at 37ºC and a wavelength of 600 nm. According to the manufacturer's guidelines, all samples were assessed within 14 days of blood sampling.

Analytical variance was determined by analysing quality control (Total Anti-oxidant Status Control NX2331, Randox Laboratories Ltd, Antrim, UK) samples ten times. All quality control values were within the quality control range for TAS (1.26 – 1.88 mmol.L^{-1}), suggesting the spectrophotometer and the assay were valid and reliable; analytical variance was found to be 6.71%. The accuracy of the assay was 94.27% when comparing the ten quality control samples to the expected quality control concentration. Assay linearity was reported to be 2.5 mmol.L^{-1}, thus all experimental samples were within the upper detection limit of the assay.

Intra-assay variation for serum samples and MTCJ was determined by repeatedly analysing the same samples ten times. Overall intra-assay and MTCJ CV were 3.49% and 1.26%, respectively. Intra-individual variability for pre- and post-exercise serum samples were found to be 2.94% and 5.12%, respectively for this phase. Pre- and post-exercise inter-individual CV's were 4.72% and 5.56%, respectively.

4.2.4.2.4. Triglycerides Assay

Refer to general methodology *section 3.6.4* for details on performing triglyceride analysis. Overall intra-assay CV was 4.96% and serum pre- and post-exercise inter-individual CV were 7.91% and 11.50%, respectively. Pre- and post-exercise intra-individual CV's were 2.21% and 5.47%, respectively.

4.2.4.2.5. Total Cholesterol Assay

Refer to general methodology *section 3.6.5* for details on performing total cholesterol analysis. Intra-individual CV was 1.77% for pre-exercise serum samples, 3.66% for post-exercise samples and 1.99% for overall intra-assay CV. Pre- and post-exercise inter-individual CV were 4.96% and 6.16%, respectively.

4.2.4.2.6. HDL Assay

Refer to general methodology *section 3.6.6* for details on performing HDL analysis.

Overall intra-assay CV was 4.66% and serum pre- and post-exercise intra-individual CV were 1.17% and 1.76%, respectively. Pre- and post-exercise inter-individual CV were 4.03% and 4.15%, respectively.

4.2.4.2.7. Total Cholesterol:HDL Ratio

Refer to general methodology *section 3.6.7* for details on calculating TC:HDL ratio.

4.2.4.2.8. LDL

Indirect calculation of LDL was conducted using the formula outlined in *Equation 9* (Ahmadi *et al.*, 2008):

$$\text{LDL (mmol.L}^{-1}) = \left(\frac{\text{Total Cholesterol}}{1.19}\right) + \left(\frac{\text{Triglycerides}}{0.81}\right) - \left(\frac{\text{HDL}}{1.1}\right) - 0.98 \qquad (9)$$

4.2.5. Data Analysis

Statistical analysis was performed using SPSS v22.0 (IBM, Chicago, USA) where data are reported as mean ± standard deviation (±SD). Data normality was checked using a Shapiro-Wilk test, upon violation of the normality assumption, analysis of variance (ANOVA) was still conducted as the model is sufficiently robust to detect statistically significant differences between means, in terms of type 1 error (Blanca *et al.*, 2017). Greenhouse-Geisser correction was applied upon violation of Mauchly's test of sphericity for ANOVAs ($P < 0.05$). Statistical significance was set at $P < 0.05$. A within-group 3-way, 2 x 3 x 2 (condition x supplementation x exercise), repeated-measures ANOVA with *post-hoc* Bonferroni's adjustment, measured differences of body mass, BMI, HR, BP, glucose, TAS, triglycerides, total cholesterol, HDL and TC:HDL. Waist circumference, body composition and resting EE, RER, fat and carbohydrate (CHO) oxidation were analysed using a 2-way, 2 x 3 (condition x

supplementation) repeated-measures ANOVA design with *post-hoc* Bonferroni's adjustment. A paired-samples *t*-test was used to identify differences between individual data points within conditions and between conditions for the corresponding time point.

Parameters measured during the FATMAX and $\dot{V}O_2$max tests, including MFO and power output at FATMAX, FATMAX (%$\dot{V}O_{2peak}$), RER at FATMAX, percentage of maximal HR (%HR_{max}) at FATMAX and $\dot{V}O_{2peak}$, were analysed using a 2 x 2 (condition x time) repeated-measures ANOVA. A paired samples *t*-test was used to identify differences between conditions for the first and second FATMAX tests.

Variables measured during the one-hour sub-maximal exercise at FATMAX including, RER, HR, RPE (overall), RPE (legs), EE, CHO oxidation, fat oxidation and percentage contribution of fat and CHO to total EE, were analysed using a 2 x 3 (condition x time) repeated-measures ANOVA, when averaged for the final 50 minutes.

Partial Eta-Squared ($\eta^2_{partial}$) was used to report effect sizes for ANOVA where effects were classified as small (0.01-0.08), moderate (0.09-0.25) and large (>0.25) (Cohen, 1988). Cohen's *d* effect size was used for paired-samples *t*-test where effects were classified as no effect (0-0.1), small (0.2-0.4), moderate (0.5-0.7) and high (≥0.8) (Cohen, 1988).

Test-retest reliability was measured using Bland-Altman plots (Bland and Altman, 1986) for MFO and %$\dot{V}O_{2peak}$, during FATMAX tests and one-hour sub-maximal tests conducted in pilot testing. Reliability of MFO and %$\dot{V}O_{2peak}$ measured during FATMAX tests and one-hour sub-maximal tests was performed in the same 11 participants recruited in this study. LoA between the tests were calculated by plotting differences between corresponding measurements against the mean of the measurements. Reference lines for the mean difference ±1.96 SD are shown on the plots. LoA for the FATMAX test represented by MFO and %$\dot{V}O_{2peak}$ were

0.03 [-0.13 – 0.20] g.min^{-1} and -0.49 [-5.91 – 4.93] %$\dot{V}O_{2peak}$, respectively, indicating high reliability of the test (Appendix 2). During the one-hour sub-maximal exercise, LoA for MFO and %$\dot{V}O_{2peak}$ were -0.01 [-0.11 – 0.09] g.min^{-1} and 0.45 [-2.93 – 3.83] %$\dot{V}O_{2peak}$, respectively, suggesting high agreement and reliability for the FATMAX intensity during prolonged exercise (Appendix 2). Additionally, agreement of fat oxidation rates during the one-hour sub-maximal test and the FATMAX determination protocol was assessed. Fat oxidation rates averaged for the final 50 minutes of the one-hour sub-maximal exercise test and mean fat oxidation rates obtained during the final minute of the stage eliciting MFO in the FATMAX determination test were compared (Appendix 2). The narrow limits of agreement (0.00 [-0.09 – 0.09] g.min^{-1}) indicated a low degree of variation, therefore suggesting that the one-hour sub-maximal exercise at FATMAX demonstrated good agreement with fat oxidation rates for the final minute of the intensity eliciting FATMAX (Appendix 3).

In order to determine whether independent variables could explain the variance observed for MFO, bivariate correlations were conducted between MFO values obtained during the final minute of the stage corresponding to FATMAX at baseline and independent variables affecting FATMAX, including age, anthropometrics, body composition, dietary intake and $\dot{V}O_{2peak}$. No significant correlations ($P > 0.05$) were found for any independent variables, thus regression analysis was not performed.

4.3. Results

4.3.1. Exercise Results

4.3.1.1. One-hour Sub-Maximal Cycling Tests

No significant interactions or main effects for condition and time ($P > 0.05$) were observed for mean EE, percentage contributions of fat and CHO to EE, $\dot{V}O_2$, $\dot{V}CO_2$, HR, overall RPE, legs RPE and serum glucose during one-hour cycling at individual FATMAX; suggesting exercise

intensities and physiological responses were similar between conditions over the supplementation period (Table 4.2).

Table 4.2. Mean ± SD values of final 50 min for variables measured during one-hour sub-maximal exercise at individual FATMAX throughout the study duration for PLA and MTCJ.

		Pre-Supplementation	Mid-Supplementation	Post-Supplementation
CHO Oxidation ($g.min^{-1}$)	PLA	0.85 ± 0.33	0.84 ± 0.27	0.89 ± 0.25
	MTCJ	0.98 ± 0.25	0.88 ± 0.29	0.99 ± 0.19
EE ($kcal.min^{-1}$)	PLA	1.09 ± 0.24	1.10 ± 0.24	1.19 ± 0.29
	MTCJ	1.19 ± 0.22	1.18 ± 0.26	1.20 ± 0.20
Contribution of Fat to EE (%)	PLA	22.49 ± 17.11	23.33 ± 8.03	21.67 ± 10.34
	MTCJ	19.88 ± 8.03	24.06 ± 10.96	20.51 ± 7.37
Contribution of CHO to EE (%)	PLA	77.51 ± 17.11	76.67 ± 8.03	78.33 ± 10.34
	MTCJ	80.12 ± 8.03	75.94 ± 10.96	79.49 ± 7.37
$\dot{V}O_2$ ($L.min^{-1}$)	PLA	1.16 ± 0.26	1.16 ± 0.25	1.18 ± 0.31
	MTCJ	1.25 ± 0.23	1.25 ± 0.28	1.26 ± 0.21
$\dot{V}CO_2$ ($L.min^{-1}$)	PLA	1.00 ± 0.25	1.00 ± 0.24	1.03 ± 0.25
	MTCJ	1.10 ± 0.21	1.08 ± 0.24	1.12 ± 0.18
RER (AU)	PLA	0.87 ± 0.05	0.87 ± 0.03	0.88 ± 0.04
	MTCJ	0.88 ± 0.03	0.87 ± 0.04	0.89 ± 0.03
HR ($beats.min^{-1}$)	PLA	114 ± 17	112 ± 18	113 ± 17
	MTCJ	116 ± 9	116 ± 14	115 ± 15
RPE (Overall) (AU)	PLA	10 ± 2	10 ± 1	10 ± 2
	MTCJ	11 ± 2	10 ± 2	11 ± 1
RPE (Legs) (AU)	PLA	10 ± 2	10 ± 2	11 ± 1
	MTCJ	11 ± 2	11 ± 2	11 ± 1

AU (Arbitrary Units); CHO (Carbohydrate); EE (Energy Expenditure); HR (Heart Rate); MTCJ (Montmorency Tart Cherry Juice); PLA (Placebo); RER (Respiratory Exchange Ratio); RPE (Ratings of Perceived Exertion); $\dot{V}CO_2$ (Volume of Carbon Dioxide Production); $\dot{V}O_2$ (Volume of Oxygen Uptake).

Mean fat oxidation rates during the final 50 minutes of the one-hour cycling exercise at individual FATMAX were not significantly different between conditions (PLA: 0.25 ± 0.10 $g.min^{-1}$ and MTCJ: 0.26 ± 0.09 $g.min^{-1}$; $F_{(1,\ 10)}$ = 0.35; P = 0.567, $\eta^2_{partial}$ = 0.034), time (Pre-Supplementation: 0.25 ± 0.09 $g.min^{-1}$, Mid-Supplementation:

0.27 ± 0.08 g.min^{-1}, Post-Supplementation: 0.25 ± 0.11 g.min^{-1}; $F_{(2, 20)}$ = 1.22; P = 0.318, $\eta^2_{partial}$ = 0.108) or interaction between condition and time ($F_{(2, 20)}$ = 0.273; P = 0.764, $\eta^2_{partial}$ = 0.027) (Figure 4.5).

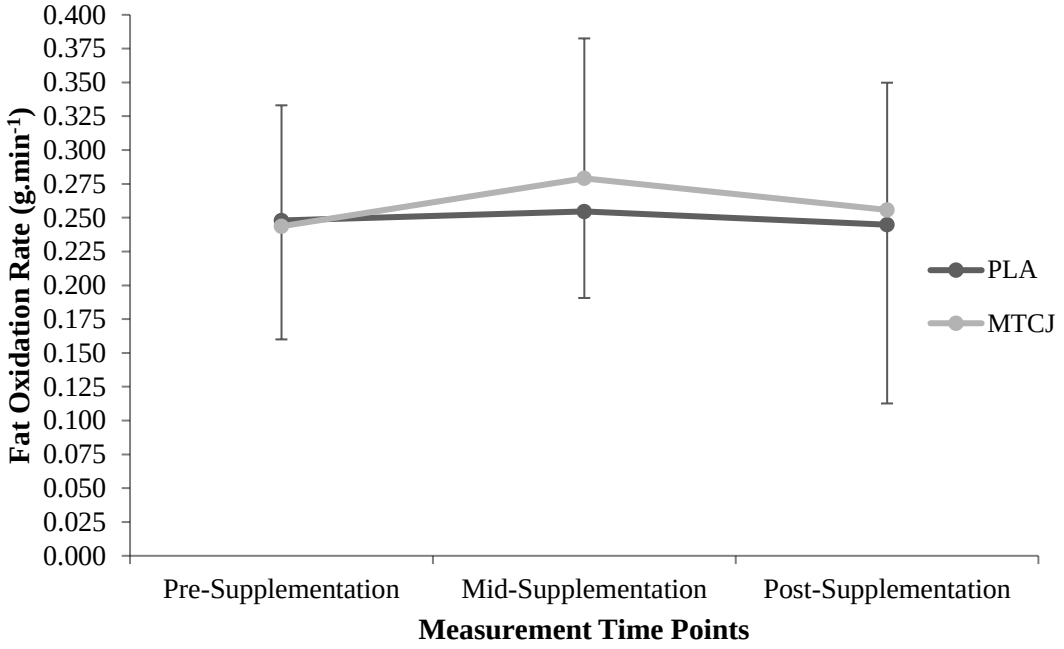


Figure 4.5. Mean (±SD) fat oxidation rates measured during final 50 minutes exercise at individual FATMAX for PLA and MTCJ.

There were also no main effects (P > 0.05) for condition, time or interaction for mean CHO oxidation rates. A significant main effect for time (Pre-Supplementation: 0.88 ± 0.04, Mid-Supplementation: 0.87 ± 0.04, Post-Supplementation: 0.89 ± 0.04; $F_{(2, 20)}$ = 4.14; P = 0.031, $\eta^2_{partial}$ = 0.293) was detected for mean RER but not between conditions or the interaction (P > 0.05). *Post-hoc* analysis identified a trend towards significance between mid-supplementation and post-supplementation (P = 0.070).

4.3.1.2. FATMAX and $\dot{V}O_2$max Determination Tests

No significant differences between conditions (PLA: 0.26 ± 0.05 g.min^{-1} and MTCJ: 0.23 ± 0.04 g.min^{-1}; $F_{(1, 10)}$ = 2.79; P = 0.126, $\eta^2_{partial}$ = 0.22) or time

(Test 1: 0.25 ± 0.08 g.min^{-1} and Test 2: 0.25 ± 0.10 g.min^{-1}; $F_{(1,10)}$ = 0.06; P = 0.807, $\eta^2_{partial}$ = 0.01) were found for MFO during the FATMAX determination tests. A tendency ($t_{(10)}$ = 2.210; P = 0.052, d = 0.59) towards significance was observed between conditions for MFO during the second FATMAX test (PLA: 0.28 ± 0.12 g.min^{-1} and MTCJ: 0.22 ± 0.08 g.min^{-1}).

No main effects (P > 0.05) for condition, time or interaction were detected for FATMAX (%$\dot{V}O_{2peak}$) (Test 1 – PLA: 42.77 ± 8.69% and MTCJ: 45.94 ± 9.28%, Test 2 – PLA: 45.28 ± 12.07% and MTCJ: 47.91 ± 11.52%), MFO (Test 1 – PLA: 0.25 ± 0.08 g.min^{-1} and MTCJ: 0.24 ± 0.08 g.min^{-1}, Test 2 – PLA: 0.28 ± 0.12 g.min^{-1} and MTCJ: 0.22 ± 0.08 g.min^{-1}), RER at FATMAX (Test 1 – PLA: 0.87 ± 0.04 and MTCJ: 0.88 ± 0.04, Test 2 – PLA: 0.87 ± 0.03 and MTCJ: 0.90 ± 0.03), %HR_{max} at FATMAX (Test 1 – PLA: 60 ± 10% and MTCJ: 63 ± 7%, Test 2 – PLA: 60 ± 10% and MTCJ: 63 ± 8%), power output at FATMAX (Test 1 – PLA: 66 ± 20 W and MTCJ: 72 ± 18 W, Test 2 – PLA: 70 ± 19 W and MTCJ: 78 ± 18 W) and $\dot{V}O_{2peak}$ (Test 1 – PLA: 36.10 ± 5.17 mL.kg^{-1}.min^{-1} and MTCJ: 36.33 ± 5.34 mL.kg^{-1}.min^{-1}, Test 2 – PLA: 37.07 ± 5.77 mL.kg^{-1}.min^{-1} and MTCJ: 36.94 ± 5.89 mL.kg^{-1}.min^{-1}). Values tended to be greater with MTCJ ($F_{(1,10)}$ = 3.65; P = 0.085, $\eta^2_{partial}$ = 0.27) for RER at FATMAX, particularly during the second test (0.90 ± 0.03) compared to the first test (0.88 ± 0.04) ($t_{(10)}$ = -2.040; P = 0.068, d = 0.63). RER at FATMAX was significantly greater ($t_{(10)}$ = -2.770; P = 0.020, d = 1.10) during the second test with MTCJ (0.90 ± 0.03) compared to PLA (0.87 ± 0.03).

Paired-samples t-test between conditions did not outline significant differences ($t_{(10)}$ = -2.040; P = 0.256, d = 0.63) for the shift in wattage for FATMAX; 3 participants increased wattage with PLA whilst 4 participants increased with MTCJ. Thus, the shift in wattage between conditions was considered negligible and not a limitation of the research.

4.3.2. Anthropometric and Functional Variables

No interactions or main effects for condition and time were detected for anthropometric measurements in Table 4.3 ($P > 0.05$). However, there was a tendency towards significantly lower percentage body fat ($t_{(10)} = 1.887$; $P = 0.080$, $d = 0.05$) and fat mass ($t_{(10)} = 1.903$; $P = 0.081$, $d = 0.05$) values pre- to post-supplementation with PLA compared to no difference with MTCJ for percentage body fat ($t_{(10)} = -0.841$; $P = 0.420$, $d = 0.02$) and fat mass ($t_{(10)} = -1.386$; $P = 0.196$, $d = 0.04$).

No interactions or main effects for condition, time and exercise ($P > 0.05$) were obtained for functional variables presented in Table 4.3, apart from a main effect of exercise for HR ($F_{(1,10)} = 25.493$; $P < 0.001$, $\eta^2_{partial} = 0.718$), as expected.

The change in resting EE from the mid- to post-supplementation trial was significantly different between conditions ($t_{(10)} = -2.602$; $P = 0.026$, $d = 0.86$) suggesting different responses during days 10-20 of supplementation. A tendency towards significance was detected with MTCJ where resting CHO oxidation increased from mid- to post-supplementation but not with PLA ($t_{(10)} = -2.213$; $P = 0.051$, $d = 0.77$).

Table 4.3. Mean ± SD for anthropometric, body composition and resting functional variables obtained pre- and/or post-exercise in both conditions at baseline and during pre-, mid- and post-supplementation trials.

		Pre-Supplementation		Mid-Supplementation		Post-Supplementation	
		Pre-Exercise	Post-Exercise	Pre-Exercise	Post-Exercise	Pre-Exercise	Post-Exercise
Body Mass (kg)	PLA	75.75 ± 13.38	75.37 ± 13.30	75.84 ± 13.33	75.44 ± 13.24	75.48 ± 13.60	75.08 ± 13.70
	MTCJ	75.83 ± 13.36	75.47 ± 13.42	75.94 ± 14.15	75.55 ± 14.25	76.05 ± 13.81	75.70 ± 13.85
BMI ($kg.m^2$)	PLA	24.48 ± 3.30	24.39 ± 3.30	24.54 ± 3.29	24.41 ± 3.29	24.39 ± 3.33	24.28 ± 3.38
	MTCJ	24.53 ± 3.29	24.41 ± 3.29	24.57 ± 3.61	24.41 ± 3.61	24.60 ± 3.48	24.51 ± 3.49
Waist Circumference (cm)	PLA	77.50 ± 11.30		77.30 ± 11.90		78.00 ± 11.30	
	MTCJ	75.50 ± 10.80		76.10 ± 11.50		76.60 ± 12.30	
Fat Mass (kg)	PLA	16.43 ± 8.21		15.96 ± 8.46		16.05 ± 7.97	
	MTCJ	15.95 ± 7.91		16.04 ± 8.60		16.24 ± 8.34	
Fat Free Mass (kg)	PLA	58.75 ± 13.01		59.57 ± 13.00		59.15 ± 13.08	
	MTCJ	55.76 ± 20.27		59.64 ± 13.45		54.05 ± 18.72	
Whole-body Fat (%)	PLA	21.91 ± 10.48		21.14 ± 10.45		21.37 ± 10.05	
	MTCJ	20.41 ± 10.05		21.23 ± 10.50		21.47 ± 10.43	
Trunk Fat (%)	PLA	22.58 ± 9.66		21.53 ± 9.59		21.98 ± 9.37	
	MTCJ	21.85 ± 9.29		21.65 ± 9.58		21.95 ± 9.70	
Resting HR ($beats.min^{-1}$)	PLA	65 ± 8	74 ± 12	62 ± 6	79 ± 16	65 ± 6	80 ± 10^
	MTCJ	65 ± 11	73 ± 8	65 ± 9	72 ± 11	64 ± 7	76 ± 7
SBP (mmHg)	PLA	117 ± 16	115 ± 10	115 ± 13	121 ± 18	116 ± 11	114 ± 12
	MTCJ	118 ± 12	121 ± 13	119 ± 11	117 ± 12	117 ± 12	116 ± 12
DBP (mmHg)	PLA	73 ± 10	73 ± 7	72 ± 10	75 ± 9	72 ± 8	72 ± 10
	MTCJ	74 ± 10	75 ± 10	72 ± 9	75 ± 8	72 ± 10	74 ± 7

Resting RER (AU)	PLA	0.83 ± 0.03	0.84 ± 0.03	0.84 ± 0.04
	MTCJ	0.84 ± 0.03	0.83 ± 0.03	0.84 ± 0.04
Resting EE ($kcal.day^{-1}$)	PLA	1718 ± 274	1865 ± 291	1745 ± 279
	MTCJ	1823 ± 233	1825 ± 294	1924 ± 375
Resting Fat Oxidation ($g.min^{-1}$)	PLA	0.08 ± 0.03	0.07 ± 0.02	0.07 ± 0.03
	MTCJ	0.07 ± 0.02	0.08 ± 0.03	0.08 ± 0.02
Resting CHO Oxidation ($g.min^{-1}$)	PLA	0.14 ± 0.03	0.15 ± 0.05	0.15 ± 0.06
	MTCJ	0.16 ± 0.05	0.14 ± 0.05	0.17 ± 0.06

AU (Arbitrary Units); BMI (Body Mass Index); CHO (Carbohydrate); DBP (Diastolic Blood Pressure); EE (Energy Expenditure); HR (Heart Rate); MTCJ (Montmorency Tart Cherry Juice); PLA (Placebo); RER (Respiratory Exchange Ratio); SBP (Systolic Blood Pressure).

4.3.3. Glucose and TAS Biomarkers

No significant interactions or main effects for condition, time and exercise were detected for glucose ($P > 0.05$).

A main effect for time ($F_{(2, 18)} = 11.137$; $P = 0.001$, $\eta^2_{partial} = 0.55$) and a significant interaction ($F_{(1, 9)} = 19.122$; $P = 0.002$, $\eta^2_{partial} = 0.68$) between condition and exercise were detected for TAS (Figure 4.6). *Post-hoc* analysis revealed significantly lower concentrations post-supplementation compared to mid-supplementation. There was also a tendency towards significance for the interaction between time and exercise ($F_{(2, 18)} = 3.466$; $P = 0.053$, $\eta^2_{partial} = 0.28$).

Pre-exercise TAS with MTCJ was significantly lower post-supplementation (1.18 ± 0.02 mmol.L^{-1}; 98.92% of baseline) compared to mid-supplementation (1.33 ± 0.05 mmol.L^{-1}; 111.93% of baseline) by 11.45%. In comparison, values were statistically similar with PLA ($t_{(9)} = 1.464$; $P = 0.177$, $d = 0.47$) (Table 4.4). There was a tendency towards significance ($t_{(9)} = -1.998$; $P = 0.077$, $d = 0.71$) for the difference between pre-supplementation (1.17 ± 0.03 mmol.L^{-1}; 101.26% of baseline) and mid-supplementation (1.33 ± 0.05 mmol.L^{-1}; 111.93% of baseline) values prior to exercise with MTCJ, but not for PLA ($t_{(9)} = -0.556$; $P = 0.585$, $d = 0.24$). After the onset of supplementation, the change in TAS during one-hour FATMAX exercise was significantly different ($t_{(19)} = 2.291$; $P = 0.034$, $d = 0.64$), where an average increase of 1.75% was seen with MTCJ and a decrease of 5.17% with PLA.

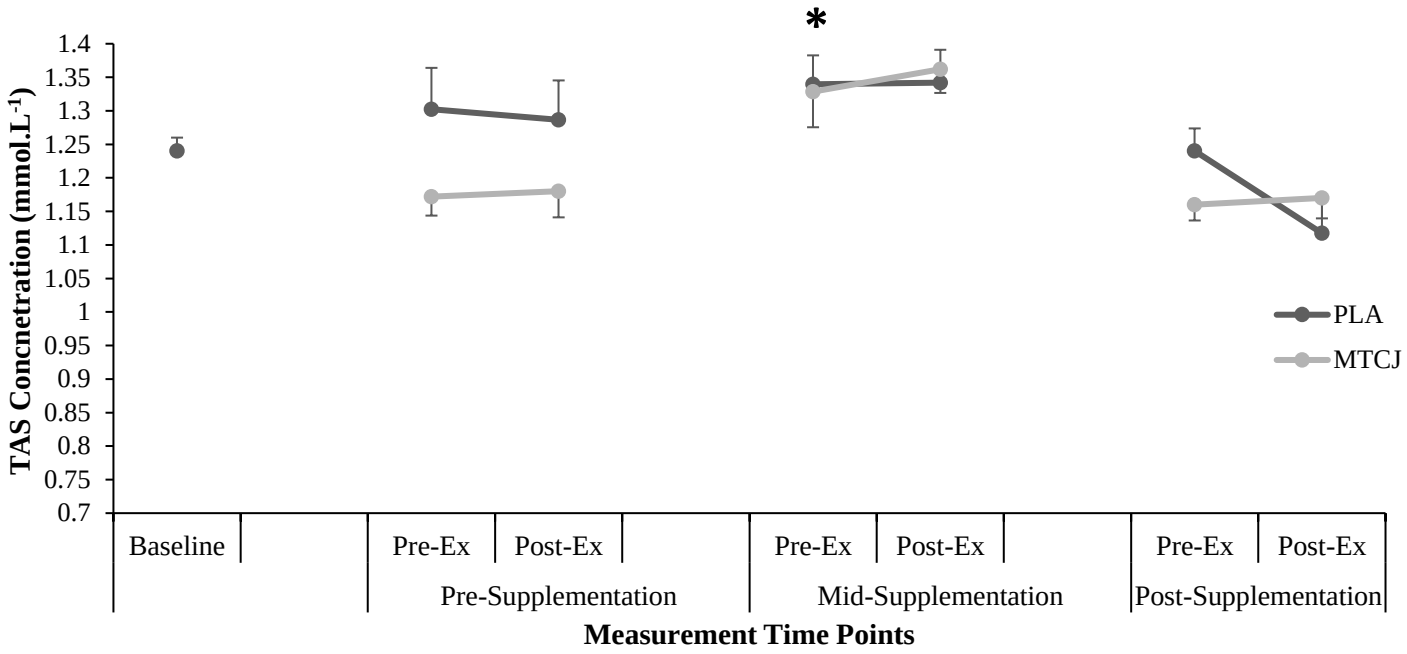


Figure 4.6. Mean (±SD) TAS concentrations presented at all measured time points for PLA and MTCJ. *Denotes significant difference between corresponding time point during post-supplementation for MTCJ.

Table 4.4. Mean ± SD for all blood-based biomarkers measured for PLA and MTCJ during pre-, mid-, post-supplementation, before and after 1-hour FATMAX exercise.

			Pre-Supplementation		Mid-Supplementation		Post-Supplementation	
		Baseline	Pre-Exercise	Post-Exercise	Pre-Exercise	Post-Exercise	Pre-Exercise	Post-Exercise
Glucose ($mmol.L^{-1}$)	PLA	5.19 ± 0.71	5.15 ± 0.56	5.47 ± 0.77	5.01 ± 0.57	5.33 ± 0.70	5.13 ± 0.86	5.52 ± 0.65
	MTCJ		5.22 ± 0.86	5.33 ± 0.52	5.21 ± 0.73	5.39 ± 0.56	5.07 ± 0.68	5.24 ± 0.82
Triglycerides ($mmol.L^{-1}$)	PLA	0.5 ± 0.3	0.5 ± 0.0	0.5 ± 0.0	0.7 ± 0.1	0.7 ± 0.1	0.5 ± 0.0	0.6 ± 0.0
	MTCJ		0.6 ± 0.0	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.0	0.6 ± 0.0
Total Cholesterol ($mmol.L^{-1}$)	PLA	3.77 ± 0.67	3.25 ± 0.03	3.20 ± 0.10	2.96 ± 0.11	2.74 ± 0.16	3.29 ± 0.05	3.17 ± 0.06
	MTCJ		3.11 ± 0.06	3.01 ± 0.05	2.94 ± 0.20	2.75 ± 0.21	3.40 ± 0.07	2.94 ± 0.20
LDL ($mmol.L^{-1}$)	PLA	1.48 ± 0.83	1.30 ± 0.08	1.23 ± 0.13	0.92 ± 0.13	0.63 ± 0.20	1.35 ± 0.07	1.31 ± 0.16
	MTCJ		1.09 ± 0.12	1.00 ± 0.13	0.79 ± 0.32	0.66 ± 0.23	1.43 ± 0.12	1.28 ± 0.14
TC:HDL (AU)	PLA	2.28 ± 0.16	1.98 ± 0.08	1.93 ± 0.09	1.83 ± 0.06	1.61 ± 0.18	2.06 ± 0.06	1.98 ± 0.15
	MTCJ		2.00 ± 0.14	1.85 ± 0.12	1.69 ± 0.21	1.54 ± 0.12	2.21 ± 0.09	2.05 ± 0.13
Haemoglobin ($g.L^{-1}$)	PLA	143 ± 15	142 ± 14	142 ± 15	143 ± 17	143 ± 14	139 ± 16	145 ± 15
	MTCJ		144 ± 16	146 ± 17	146 ± 17	148 ± 18	142 ± 16	144 ± 13
Haematocrit (%)	PLA	44 ± 1	44 ± 1	44 ± 1	45 ± 1	45 ± 1	44 ± 1	45 ± 1
	MTCJ		45 ± 1	45 ± 1	46 ± 1	46 ± 1	44 ± 1	45 ± 1

AU (Arbitrary Units); LDL (Low-density Lipoprotein); MTCJ (Montmorency Tart Cherry Juice); PLA (Placebo); TAS (Total Anti-oxidant Status); TC:HDL (Total Cholesterol to HDL Ratio)

4.3.4. Lipid Biomarkers

No significant interactions or main effects for condition, time and exercise were detected for triglycerides and LDL ($P > 0.05$).

A main effect for time only was found for HDL ($F_{(1.3, 11.703)} = 7.098$; $P = 0.016$, $\eta^2_{partial} = 0.441$). *Post-hoc* analysis showed post-supplementation concentrations were significantly lower than mid-supplementation ($P < 0.001$) (Figure 4.7). As with TAS, a significant ($t_{(9)} = 3.123$; $P = 0.012$, $d = 0.68$) reduction in pre-exercise HDL concentrations was observed from mid-supplementation (1.84 ± 0.47 mmol.L^{-1}; 114.48% of baseline) to post-supplementation (1.56 ± 0.34 mmol.L^{-1}; 98.62% of baseline) with MTCJ whereas no difference was found for PLA ($t_{(9)} = 0.719$; $P = 0.490$, $d = 0.15$) (Table 4.4).

Mean post-exercise values for total cholesterol (main effect for exercise: $F_{(1, 9)} = 8.951$; $P = 0.015$, $\eta^2_{partial} = 0.5$) and TC:HDL ratio (main effect for exercise: $F_{(1, 9)} = 8.951$; $P = 0.015$, $\eta^2_{partial} = 0.5$) were significantly lower than pre-exercise values. In addition, both total cholesterol ($F_{(1.23, 11.154)} = 6.092$; $P = 0.026$, $\eta^2_{partial} = 0.404$) and TC:HDL ($F_{(2, 18)} = 10.995$; $P = 0.001$, $\eta^2_{partial} = 0.55$) demonstrated main effects for time, however no main effect for condition, or significant interactions were detected ($P > 0.05$).

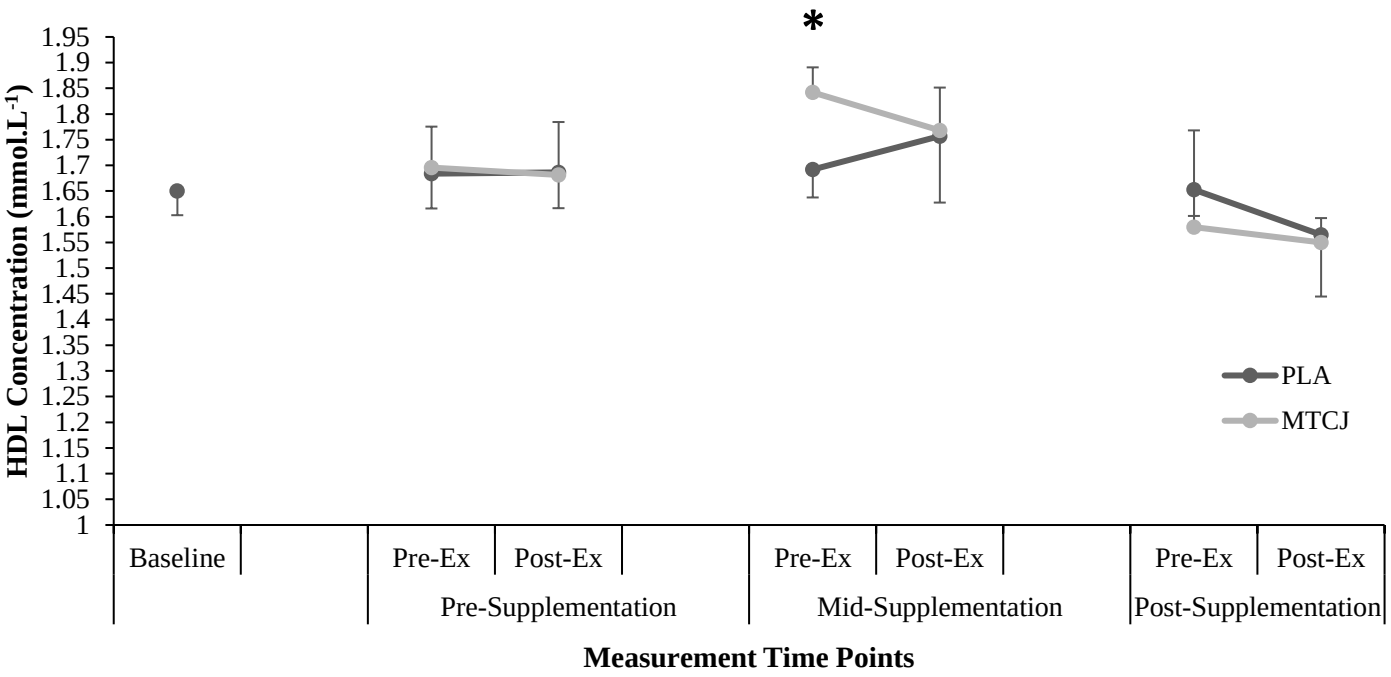


Figure 4.7. Mean (±SD) HDL concentrations presented at all measured time points for PLA and MTCJ. *Denotes significant difference between corresponding time point during post-supplementation for MTCJ.

4.4. Discussion

This was the first study in any population to combine exercise and tart cherry supplementation whilst examining cardio-metabolic biomarkers. The main findings of this study were that 20 days of MTCJ supplementation did not significantly increase fat oxidation rates at rest or during FATMAX exercise, nor did it alter waist circumference, body composition or cardio-metabolic biomarkers compared to the placebo condition.

4.4.1. Metabolic Responses

The present study did not find statistically significant differences between conditions for RER, EE, fat and CHO oxidation rates, contrasting previously reported results regarding the effect of dietary anthocyanin supplementation on substrate metabolism with exercise. Cook *et al.* (2015) observed a 16% increase in fat oxidation with 7 days encapsulated, anthocyanin-rich, New Zealand blackcurrant extract (NZBE) supplementation compared to placebo during cycling exercise at 65% $\dot{V}O_2$max. Crucially, Cook *et al.* (2015) instructed participants to

consume one NZBE capsule, containing 105 mg anthocyanins, 2 hours prior to commencing exercise thus coinciding peak bioavailability and concentrations of anthocyanins, secondary metabolites and phase II conjugates in plasma and target tissues with the time of measurement of substrate oxidation rates during exercise. Muscle oxygenation responses to Montmorency tart cherry capsule consumption were improved with similar time-dependent supplementation strategies, whereby short-term (7 days) and acute (60 minutes) loading was provided to coincide anthocyanin pharmacokinetics with measurement of physiological responses (Morgan, Barton and Bowtell, 2019). RER during 10 minutes of steady-state exercise at ~65% $\dot{V}O_{2peak}$ was not different after consuming Montmorency tart cherry capsules (Morgan, Barton and Bowtell, 2019), however the short exercise timeframe likely negated the ability to observe significantly lower RER. This provides evidence that fat oxidation and other physiological parameters can be improved with short-term (7 days) and acute (2 hours before exercise) supplementation of dietary anthocyanins. Highlighting the importance of timing of consumption to maximise bioavailability and concentrations in plasma and target tissues. Subsequently, the inclusion of an overnight fast may explain why Montmorency tart cherry anthocyanins and its metabolites were not able to significantly augment fat oxidation rates.

Differences in fat oxidation responses to NZBE anthocyanin supplementation may be explained by the different types of anthocyanins present compared to MTCJ. The main anthocyanins in NZBE (35-50% delphinidin-3-rutinoside and 5-20% delphinidin-3-glucoside) are derived from the anthocyanidin, delphinidin (Cook *et al.*, 2017b), whereas cyanidin based anthocyanins (cyanidin-3-glucosylrutinoside and cyanidin-3-rutinoside) comprise 93% of the total anthocyanin content in Montmorency tart cherries (Kirakosyan *et al*., 2009). It has been previously reported that delphinidin based anthocyanins provide more effective cardio-metabolic protection than cyanidin based anthocyanins (Overall *et al.*, 2017). Additionally, cyanidin is less stable in the gastrointestinal environment, although it is not dependent on gut

bacteria for metabolism or absorption whereas delphinidin is dependent on gut bacteria (Overall *et al*., 2017). Healthy individuals are expected to have greater diversity and concentrations of 'good' gut bacteria, acting to increase the bioavailability of anthocyanins and their metabolites (Hidalgo *et al.*, 2012; Fernandes *et al.*, 2014). Since gut bacteria do not modulate cyanidin based anthocyanin metabolism and absorption, it is not surprising that cardio-metabolic markers or fat oxidation rates were not improved in the healthy participants from this study.

It is feasible that consumption of a fruit extract (NZBE) in capsule form would not influence substrate oxidation rates as much as supplements in juice form (MTCJ) when ingested immediately before exercise, given the likely differences in macronutrient content and metabolism such as anthocyanin degradation and absorption rates. Perhaps this explains why Cook *et al.* (2015) were able to observe greater fat oxidation with NZBE. Cook *et al.* (2015) reported NZBE anthocyanins contributed 30.70% of fat to total EE during exercise, whilst MTCJ peaked during the mid-supplementation test at 24.06% and averaged 21.48% throughout the present study. Similar results were reported with the placebo, with the most likely explanation being the high carbohydrate content of the MTCJ and placebo drinks, compared to the capsules provided in the NZBE study which were completely devoid of carbohydrate. The significant increase in resting EE was facilitated by upregulation of CHO oxidation, signifying that the higher CHO content of MTCJ did induce greater glycolytic flux from days 10-20 of supplementation. This provides an explanation for the lower MFO rates and percentage contributions of fat to total EE in the present study compared to Cook *et al.* (2015). MTCJ contained fructose and glucose, thus the significant elevation in CHO oxidation at rest with MTCJ during the final 10 days of supplementation may be due to fructose accumulation. In support of these findings, co-ingestion of glucose and fructose, as opposed to either alone, has

been shown to significantly increase CHO oxidation rates in healthy participants (Jentjens and Jeukendrup, 2005).

4.4.2. Anti-oxidant Responses

The tendency towards significance for the 13.35% increase in TAS from pre-supplementation to mid-supplementation with MTCJ, may explain the tendency for greater fat oxidation with MTCJ during one-hour FATMAX exercise between these time points. The increase in oxygen flux and therefore generation of ROS during exercise (Radak *et al.*, 2013), as a by-product of fatty acid oxidation at FATMAX, may have been mitigated by MTCJ anti-oxidants. These anti-oxidants may have retarded mitophagy and preserved mitochondrial function, thus enabling the continuation of fat oxidation (Montgomery and Turner, 2014). The 12.76% decrease in TAS from mid-supplementation to post-supplementation prior to exercise suggests TAS does not increase linearly with further ingestion of MTCJ after 10 days but returns to baseline. As far as the author is aware, this is the first study to demonstrate such an effect. Consequently, this may have contributed to the different responses observed between conditions for HDL and resting EE during days 10-20 of supplementation. Endogenously derived anti-oxidants are the primary contributors to the anti-oxidant balance; thus supplementation of exogenous anti-oxidants may inhibit synthesis of endogenous anti-oxidants in order to maintain a homeostatic balance (Poljsak, Šuput and Milisav, 2013). Thus, it is plausible that the synthesis of endogenous anti-oxidants was reduced during the final 10 days of exogenous anti-oxidant supplementation with MTCJ resulting in a net reduction of TAS. A potential increase in oxidative stress from mid- to post-supplementation may have contributed to inefficient oxidative metabolism and thus greater resting EE (Frisard and Ravussin, 2006). These findings are supported by Timmers *et al.* (2011) who reported significantly lower basal EE due to improved mitochondrial function and therefore metabolic efficiency after 30 days resveratrol supplementation, a potent anti-oxidant, in humans that were obese but otherwise healthy.

Furthermore, the reduced synthesis/uptake of anti-oxidants may be a defensive mechanism against an excessively elevated anti-oxidant balance which may affect hormetic responses. Such is the complexity of the interactions of free radicals, pro-oxidant species, anti-oxidants and the cellular mechanisms involved, that no one reason is liable to be responsible for this potential anti-oxidant effect.

4.4.3. Lipid Responses

In relation to lipid responses, it is reasoned a lack of cardio-metabolic dysregulation does not provide sufficient scope for a cherry intervention to further regulate cardio-metabolic function at rest (Kelley *et al.*, 2006, 2013; Ataie-Jafari *et al.*, 2008; Martin *et al.*, 2010). Despite the addition of an exercise intervention, a similar reasoning can be applied to the present study, given the healthy baseline lipid concentrations presented by the participants in this study. This observation is further supported by rodents susceptible to dyslipidaemia responding significantly better to cherry consumption than those without dyslipidaemia (Seymour *et al.*, 2008, 2009; Wu *et al.*, 2014). The initial physiological status of participants seems to influence the efficacy of cherry interventions.

It is apparent accumulation of anthocyanins and their metabolites with long-term supplementation likely did not occur in the present study. This is likely due to the poor bioavailability, intestinal absorption and rapid elimination rates of anthocyanins (Manach *et al.*, 2004). Alternatively, it may be that the anthocyanin and secondary metabolite concentrations reached a ceiling level below a physiologically relevant threshold (Krga *et al.*, 2016), to affect serum lipids, thus explaining pre-exercise HDL responses to MTCJ consumption. A biphasic response was observed where a non-significant, yet clinically relevant, increase in HDL from pre- to mid-supplementation was followed by a significant decline from mid- to post-supplementation with MTCJ but not PLA. Based on data from

epidemiological studies with middle-age and high-risk subjects, suggesting an increase of 0.026 mmol.L^{-1} could reduce the risk of coronary heart disease by 2% in males (Gordon *et al.*, 1989), the change in HDL from pre- to mid-supplementation may result in a risk reduction of 12% with MTCJ. However, this finding should be interpreted with caution as the translation across to healthy subjects of both sexes and the lack of statistical and clinically significant improvements for other lipid profile markers with MTCJ, renders the increase in HDL less important. The decline from mid- to post-supplementation suggests that administering cherry interventions longer than 10 days does not maintain elevated HDL concentrations, but rather a return to baseline as supported by findings from Kelley *et al.* (2006), Ataie-Jafari *et al.* (2008), Martin *et al.* (2010) and Lynn *et al.* (2014).

Finally, limitations associated with this study include the overnight fast dietary restriction put in place to reduce intra- and inter-individual variability, but at the expense of upholding ecological validity. Secondly, previous research which demonstrated improvements in body mass, fat mass and metabolic function required participants to exercise at FATMAX 2-4 times per week for 2-12 months (Brun, Romain and Mercier, 2011). Thus, MTC phytochemicals were not able to compound upon any cellular and molecular adaptations promoting fat oxidation which may have occurred with FATMAX training. Thirdly, the small sample size did not assist the ability to find significant differences with MTCJ as *post-hoc* power analysis suggested the study was underpowered ($1-\beta = 0.18$; $\alpha = 0.05$; $n = 11$) for detecting a significant interaction between condition and time (pre-, mid-, post-supplementation) for fat oxidation during one-hour sub-maximal exercise. Additionally, due to the differences in viscosity of the placebo and MTCJ, 9/11 participants correctly identified which supplement they were provided when asked at the end of the study. Consequently, this may have contributed to the lack of significance between conditions as participants could manipulate their activity during the study. This highlights a limitation of juice concentrate as a form of supplementation and

perhaps alternative forms such as capsules are preferable to uphold anonymity between conditions.

Future work should attempt to elucidate the cellular and molecular mechanisms, in humans to provide a basis upon which theories explaining the obtained responses can be either accepted or refuted. This would provide crucial information by which supplementation strategies can be altered to maximise the efficacy of MTCJ. Based on Cook's *et al.* (2015) findings, it would be appropriate to suggest that any effect of anthocyanin supplementation on fat oxidation is short-term and provides a rationale to acutely supplement MTC in future studies. The augmentation of fat oxidation with acute supplementation may then contribute to mitigating the development of cardio-metabolic symptoms in clinical populations such as those with MetS.

4.5. Conclusion

This was the first study to examine the effect of cherry supplementation on fat oxidation rates at rest and during exercise. Findings showed that MTCJ did not significantly increase fat oxidation rates at rest or during FATMAX exercise. Additionally, secondary cardio-metabolic markers were also not significantly different with MTCJ supplementation, indicating this intervention does not act as a CRM in healthy participants. Consequently, it is unnecessary for healthy participants to supplement MTCJ to improve cardio-metabolic biomarkers. Previous studies to report a significant response to cherry supplementation in animal (Seymour *et al.*, 2008, 2009; Wu *et al.*, 2014) and human (Ataie-Jafari *et al.*, 2008; Martin *et al.*, 2010; Keane *et al.*, 2016bc) studies occurred when initial values were abnormal, thus further research is warranted in clinical populations, including MetS.

Chapter 5

Effects of Montmorency Tart Cherry Supplementation on Cardio-Metabolic Markers in Participants with Metabolic Syndrome: a pilot study

Publication arising from this Chapter (Appendix 11): Desai, T., Roberts, M. and Bottoms, L. (2019). Effects of Montmorency tart cherry supplementation on cardio-metabolic markers in metabolic syndrome participants: A pilot study. *Journal of Functional Foods, 57*, pp. 286-298.

5.1. Introduction

Substantial literature exists (Basu *et al.*, 2009, 2011; Basu *et al.*, 2010; Basu and Lyons, 2012; Stull *et al.*, 2015; Amiot, Riva and Vinet, 2016; Vendrame *et al.*, 2016), as shown in *section 2.2.5.2.2.1*, that highlights consumption of anthocyanin-rich dietary interventions are beneficial for mitigating symptoms associated with MetS, despite findings from **chapter 4**. Montmorency tart cherries (MTC) may be one such intervention not only rich in anthocyanins, but also other phenolics resulting in being one of the most potent dietary anti-oxidant interventions when consumed in habitual portion sizes (Ou *et al.*, 2012). The anti-oxidative properties of MTC may only be beneficial in the presence of a pro-oxidant balance, therefore as oxidative stress is typical in humans with MetS (Chiva-Blanch and Badimon, 2017), the anti-oxidant hypothesis may be a potential mechanism by which MTC may improve MetS symptoms. This study proceeded to explore the potential beneficial effects of MTC on cardio-metabolic function by addressing some of the issues encountered in **chapter 4**. Specifically, recruitment of participants with MetS possessing some cardio-metabolic dysfunction rather than healthy participants. Secondly, acute administration of supplements to coincide with the pharmacokinetics of MTC phytochemicals as previously conducted by Keane *et al.* (2016ab). Based on findings from Keane *et al.* (2016b) this study acutely administered Montmorency Tart Cherry Capsules (MTCC), for the first time in a clinical health study, and Montmorency Tart Cherry Juice (MTCJ) and assessed responses on blood-based and functional cardio-metabolic markers. Lastly, to determine whether the high carbohydrate content present in MTCJ affects glycaemic, insulinaemic and lipaemic metabolism, by comparing responses to the lower carbohydrate MTCC supplements for the first time.

As mentioned in *section 2.2.4*, MetS encompasses a cluster of cardio-metabolic conditions including insulin resistance, central adiposity, dyslipidaemia and pre-hypertension (Kaur, 2014). The presence of three or more of these symptoms combined, augments the incidence of

chronic diseases such as CVD by 2-fold and T2D by 5-fold (Falkner and Cossrow, 2014). Ultimately, this highlights the strong association between MetS and higher rates of both cardiovascular and total mortality (Falkner and Cossrow, 2014).

Despite the difficulty in measuring MetS prevalence rates, it is accepted that global rates are on the rise (O'Neill and O'Driscoll, 2015). In the UK, current indications suggest approximately 25% of the population >20 years of age have MetS (Scuteri *et al.*, 2015), equating to ~12 million individuals. In the United States [~35% MetS prevalence rate (Aguilar *et al.*, 2015)], low fruit and vegetable intake accounted for 15.1% of all diet-related cardio-metabolic deaths (Micha *et al.*, 2017). Therefore, fruit and vegetables rich in polyphenols, especially anthocyanins, are attractive interventions against cardio-metabolic disease risk factors (Vendrame *et al.*, 2016) such as insulin resistance (Muraki *et al.*, 2013), dyslipidaemia (Liu *et al.*, 2016), hypertension (Del Rio *et al.*, 2013) and central adiposity (Bertoia *et al.*, 2016); as supported by epidemiological evidence (Arts and Hollman, 2005; Wallace, 2011; Cassidy *et al.*, 2013).

Given the promising evidence provided above, the present study set out to determine whether acute administration of MTC could improve cardio-metabolic function in individuals with MetS. To the authors knowledge, no study has been conducted assessing the responses of humans with MetS to MTC supplementation, however individual aspects of the MetS cluster have been explored in pathological populations. Keane *et al.* (2016b) showed significant reductions in systolic blood pressure (SBP) after acute ingestion of MTCJ in early-hypertensive males. Additionally, Martin *et al.* (2010, 2011) demonstrated significant reductions in serum triglycerides, triglyceride/HDL ratio and VLDL concentrations after 4 weeks tart cherry juice supplementation (230 $mL.day^{-1}$) in participants with central adiposity and dyslipidaemia. Moreover, Ataie-Jafari *et al.* (2008) reported 6 weeks concentrated sour cherry juice

supplementation improved $HbA1_c$, in females with T2D, along with total cholesterol and LDL in those participants with hyperlipidaemia.

As previously eluded to in *section 2.1.6.2*, the anthocyanin pharmacokinetics after consuming whole Montmorency tart cherries (Seymour *et al.*, 2014) followed the general pattern and time-course of anthocyanin metabolism (Kay, Mazza and Holub, 2005); parent anthocyanin concentrations in plasma peaked 2-4 hours post-ingestion and excretion rates in urine peaked 6-8 hours post-ingestion. The pharmacokinetics of anthocyanins and metabolites derived from powdered freeze-dried MTC are currently unknown when administered in encapsulated form, but it is anticipated that their bioavailability may be enhanced. Hydroxypropyl methylcellulose (HPMC) is a material used to form the shell of capsules, and both *in vitro* (Glube, Moos and Duchateau, 2013) and *in vivo* (Lown *et al.*, 2017) studies have demonstrated disintegration of HPMC 30 minutes post-ingestion in the presence of intestinal fluid. This material has previously been shown to delay the release of green tea catechins (Glube, Moos and Duchateau, 2013) and mulberry extract anthocyanins (Lown *et al.*, 2017) which consequently delayed glucose and insulin responses until 30 minutes after ingestion, coinciding with the disintegration and dissolution of the capsule shell (Lown *et al.*, 2017). Capsules as a delivery vehicle of phytochemicals to target tissues via systemic circulation are useful as they are programmed to selectively degrade once in the gastrointestinal tract, therefore potentially maximising bioavailability (Oidtmann *et al.*, 2012). This is achieved due to protection of phytochemicals by the exterior capsule shell against factors known to influence their stability such as pH, temperature, light, enzymes (particularly in saliva) and sugars (Robert and Fredes, 2015). It has been previously eluded to in **chapter 2**, that phytochemical, particularly anthocyanin bioavailability is notoriously poor (Kay, 2006). Subsequently, MTCC was provided to assess whether encapsulation, and thus protection of MTC phytochemicals from premature degradation, potentially maximises bioavailability and therefore their

beneficial effects on cardio-metabolic function. Finally, anthocyanin research on their anti-oxidant effect suggests that lower concentrations, based on the concept of hormesis, are more effective at inducing cellular, molecular and thus functional changes to improve health (Wallace, 2011). The delivery of a lower dose in this study (30 mL MTCJ) also has practical and financial benefits to consumers.

Based around the pharmacokinetics of MTC phytochemicals, this study aimed to explore the short-term responses to acute supplementation of MTCJ and MTCC on cardiac haemodynamics, arterial stiffness by pulse wave analysis (PWA), substrate metabolism and various blood-based cardio-metabolic biomarkers in humans with MetS. Given that these subjects present at least three or more risk factors resulting in a holistic state of cardio-metabolic dysregulation, it was hypothesised that the MTC interventions would have positive responses on various diagnostic cardio-metabolic markers, in particular glucose, lipids and SBP based on previous human studies with tart cherry supplementation (Keane *et al.* 2016b; Martin *et al.,* 2010; Ataie-Jafari *et al.,* 2008). Furthermore, it was hypothesised that MTCC would be more effective than MTCJ or placebo in mediating these benefits, as MTCC would induce less glycaemic, insulinaemic and lipaemic stress, and bioavailability of phytochemicals was anticipated to be superior.

Pharmacokinetic studies comparing 30 and 60 mL MTC concentrate consumption in healthy humans (Bell *et al.,* 2014b; Keane *et al.,* 2016a), suggested a physiological dose-response effect of circulating parent anthocyanins and secondary phenolic acid metabolites. Hence, Part B of this study aimed to compare whether a dose-response effect would be observed on cardio-metabolic biomarkers in individuals with MetS. The rationale for this arm of the study was based on previous literature reporting beneficial cardiovascular responses to an acute 60 mL dose of MTC concentrate (Keane *et al.,* 2016bc). It was hypothesised that a dose-response effect would be observed for cardio-metabolic biomarkers in individuals with MetS.

5.2. Methods

5.2.1. Part A

5.2.1.1. Participants

Eleven (6 males and 5 females) participants with Metabolic Syndrome (MetS) (Tables 5.1 and 5.2) volunteered for this pilot study. All participants were screened for MetS prior to formal inclusion onto the study according to the harmonised criteria outlined by Alberti *et al.* (2009), where 3 of the 5 qualifying criteria [Waist Circumference: ethnicity and sex specific criteria; Fasting Triglycerides: ≥1.69 mmol.L^{-1}; Fasting High-Density Lipoprotein: <1.03 mmol.L^{-1} (men), <1.29 mmol.L^{-1} (women); Blood Pressure: ≥130 mmHg SBP or ≥85 mmHg DBP; Fasting Glucose: ≥5.6 mmol.L^{-1}] had to be met. Recruitment (Figure 5.1) was conducted via word of mouth, flyers and email advertisements. Participants were excluded from the study if they did not meet the criteria for MetS at screening, were smokers, pregnant, heavy alcohol consumers (>14 units per week), current or previous diagnosis of chronic disease (gastrointestinal, cardiovascular, hepatic or renal), or were on statins, hyperlipidaemic, anti-hypertensive, anti-diabetic, anti-inflammatory or steroidal medication. Verbal and written information was provided to all participants regarding the study purpose and procedures. Ethical approval was obtained from the University of Hertfordshire Health, Science, Engineering and Technology Ethics Committee and informed consent was provided by all participants prior to enrolment. This study was registered as a clinical trial on clinicaltrials.gov (NCT03615885).

Table 5.1. Selected baseline characteristics obtained during screening (n = 11).

Characteristics	Mean ± SD
Age (years)	49 ± 12
Stature (m)	1.72 ± 0.11
Body Mass (kg)	99.5 ± 20.5
BMI ($kg.m^{-2}$)	33 ± 5
Whole-body Fat (%)	38 ± 10
Trunk Fat (%)	34 ± 13
Fat Mass (kg)	41 ± 17
Fat Free Mass (kg)	58 ± 15
Fasting Total Cholesterol ($mmol.L^{-1}$)	3.83 ± 0.91
Fasting Insulin ($pmol.L^{-1}$)	115.67 ± 73.75
HOMA2-IR (AU)	2.2 ± 1.4
HOMA2-β (%)	132.5 ± 52.9
HOMA2-%S (%)	60.4 ± 27.3

AU (Arbitrary Units); BMI (Body Mass Index); HOMA2-IR (Homeostatic Model Assessment of Insulin Resistance); HOMA2-β (Homeostatic Model Assessment of pancreatic β-cell function); HOMA2-%S (Homeostatic Model Assessment of Insulin Sensitivity).

Table 5.2. Individual baseline characteristics of MetS criteria obtained during screening (n = 11).

Characteristics	Mean ± SD	Participant										
		1	2	3	4	5	6	7	8	9	10	11
Waist Circumference (cm)	106.0 ± 13.4	102.2*	133*	93	90.8	119*	119*	95	104.1*	94	112*	104*
Fasting Glucose ($mmol.L^{-1}$)	5.63 ± 0.76	6.20*	5.33	6.72*	4.48	5.93	6.50*	6.21*	5.12	5.78	4.85	4.79
Fasting Triglycerides ($mmol.L^{-1}$)	1.9 ± 0.5	1.9*	2.0*	2.6*	1.7*	0.9	1.5	2.6*	2.1*	2.0*	2.1*	1.5
Fasting HDL ($mmol.L^{-1}$)	1.23 ± 0.36	2.23	1.25	1.22*	0.94*	1.23*	0.83*	1.19*	1.30	1.06*	1.28*	1.01*
SBP (mmHg)	132 ± 9	122	146*	130*	130*	136*	143*	129	132*	142*	120	126
DBP (mmHg)	80 ± 9	67	90*	86*	87*	79	91*	72	72	88*	67	85*

DBP (Diastolic Blood Pressure); HDL (High-density Lipoprotein); SBP (Systolic Blood Pressure).

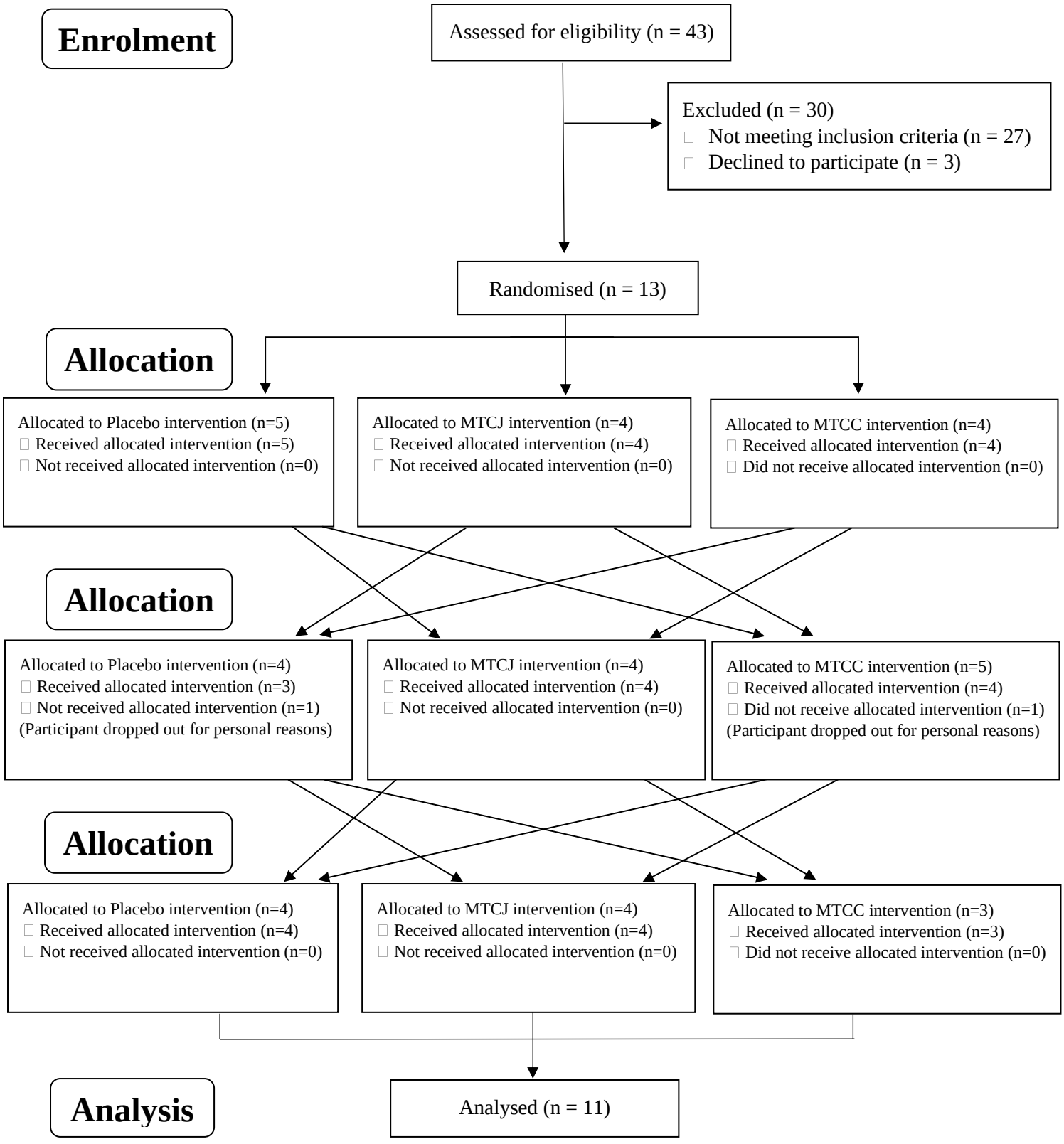


Figure 5.1. CONSORT flow diagram of the participants recruited, screened, tested, analysed and excluded during the course of the study.

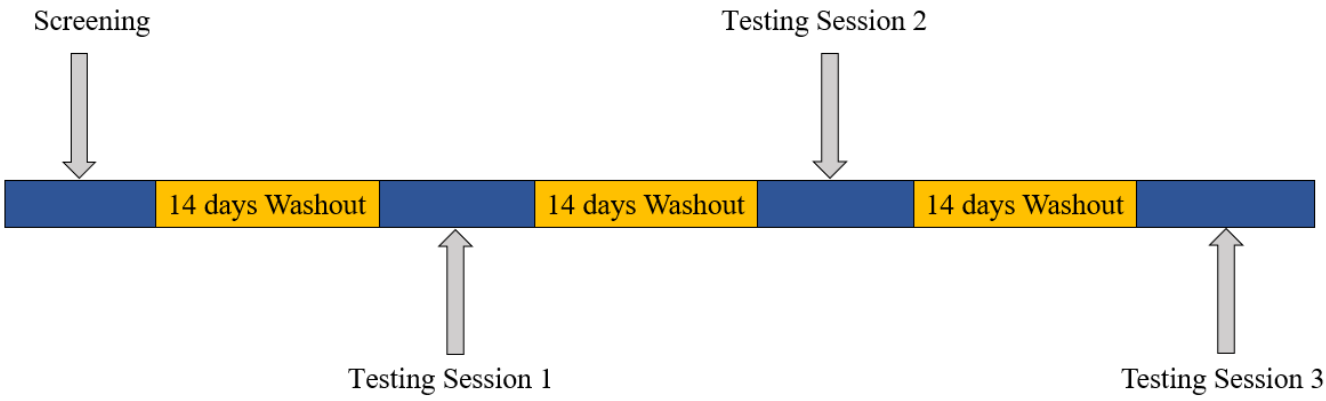
5.2.1.2. Procedures

5.2.1.2.1. Research Design

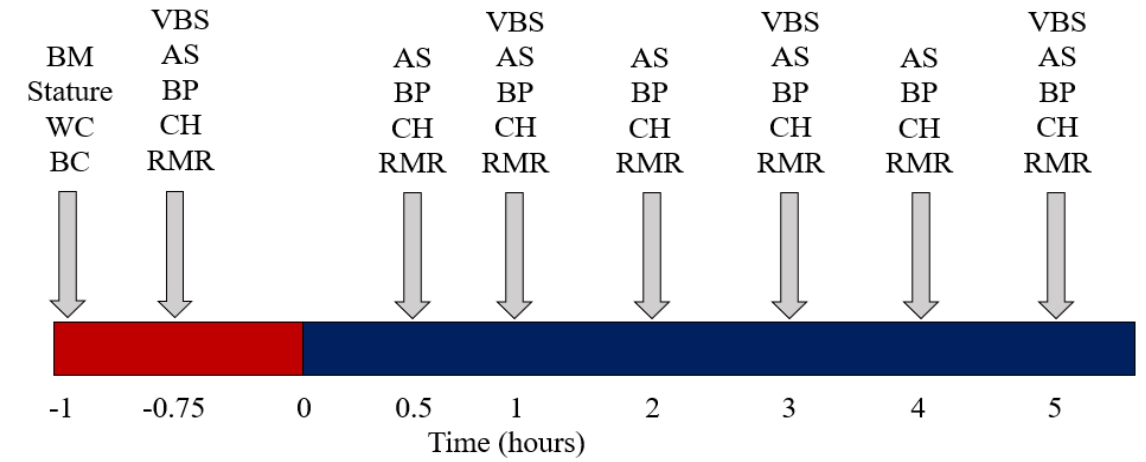
A single-blind (blinded to participant), placebo-controlled, randomised, crossover design was utilised; each participant acted as their own control. During the 6-week study duration, all participants completed 3 testing sessions during which one of three [placebo (PLA) or MTCJ or MTCC] different supplements were provided each time. As in **chapter 4**, a 14-day washout period (Keane *et al.*, 2016b; Cook *et al.*, 2015; Howatson *et al.*, 2012) was incorporated between testing sessions and between the screening and first testing sessions.

Testing sessions lasted 6 hours and were identical in terms of design and testing procedures. Schematics of the overall study design and specific procedures during a testing session are depicted in Figure 5.2. Baseline anthropometric (stature, body mass, waist circumference, body fat percentage) and functional (PWA, cardiac haemodynamics, RMR) measurements were obtained prior to consumption of the supplement. Functional measurements were recorded at 30 minutes, 1, 2, 3, 4- and 5-hours post-bolus. Blood sampling was performed at baseline, 1, 3- and 5-hours post-bolus only.

A)



B)



Key:
AS – Arterial Stiffness; BC – Body Composition; BM – Body Mass; BP – Blood Pressure; CH – Cardiac Haemodynamics; RMR – Resting Metabolic Rate; VBS – Venous Blood Sampling; WC – Waist Circumference

= Pre-Bolus
= Post-Bolus

Figure 5.2. (A) Schematic representation of the overall study design. (B) Schematic representation of the specific procedures during each testing session.

5.2.1.2.2. Dietary Guidelines

Refer to general methodology *section 3.2* for further details on dietary guidelines employed in this study.

All participants complied with dietary guidelines upon analysis for percentage contributions of macronutrients to total energy intake [(protein 16 ± 23%), (CHO 45 ± 56%), (fat 39 ± 38%)], total polyphenols (70 ± 83 mg) and anthocyanins (26 ± 22 mg).

5.2.1.2.3. Supplementation

This study acutely administered three different supplements including a placebo which acted as the control condition and two experimental conditions, MTCJ and MTCC. The placebo was composed of 30 mL commercially available fruit-flavoured cordial (Cherries and Berries, Morrisons, Bradford, UK) mixed with 100 mL water. The placebo drink was also matched against MTCJ for energy content, taste and visual appearance by adding a flavourless carbohydrate (Maltodextrin, My Protein Ltd, Northwich, UK), citric acid (100% Pure Citric Acid, VB and Sons, UK), red and black food colouring (Morrisons, Bradford, UK), respectively. MTCJ consisted of 30 mL Montmorency tart cherry concentrate (Cherry Active, Active Edge Ltd, Hanworth, UK) and 100 mL water. To match the anthocyanin content of MTCJ, participants consumed 10 Montmorency tart cherry capsules (Cherry Active Capsules, Active Edge, Ltd, Hanworth, UK) with 130 mL water. Each capsule contained 435 mg of freeze-dried Montmorency tart cherry skin powder, with Table 5.3 detailing nutritional analysis. Based on the ease of distinguishing between PLA and MTCJ in **chapter 4**, further anonymity of the supplementation was ensured in **chapter 5** by blinding the participants to the source of anthocyanins. This was achieved by explaining that an ‘anthocyanin-rich supplement’ would be provided rather than disclosing Montmorency tart cherries as the specific source. Further anonymity was placed by indicating to participants placebo capsules may be provided. Only 2/11 participants correctly identified the interventions provided

(intervention or placebo) and none identified the intervention as 'cherry' or 'containing cherries' (either sweet or tart). Refer to *section 3.3* for more information on supplementation.

Table 5.3. Nutritional information of each supplement provided.

	Montmorency tart cherry concentrate (per 30 mL)	Montmorency tart cherry capsules (per capsule – 435 mg)	Placebo (per 130 mL)
Energy (kcal)	102	1.3	102
Carbohydrate (g)	24.5	0.3	25.35
of which sugars (g)	17.9	0.1	25.32
Protein (g)	1.1	Trace	0.03
Fat (g)	0	Trace	0
of which saturates (g)	0	0	0
Fibre (g)	2.6	Trace	Trace
Salt (g)	0	Trace	0
Total Anthocyanins (mg)	270	27	0

5.2.1.2.4. Measures and Equipment

5.2.1.2.4.1. Anthropometrics

Refer to general methodology *section 3.5.1* for details on procedures of measuring stature, body mass and waist circumference.

Refer to general methodology *section 3.5.2* for details on procedures of measuring segmental body composition.

5.2.1.2.4.2. Cardiac Haemodynamics

Refer to general methodology *section 3.5.3.1* for details on procedures for obtaining cardiac haemodynamic measurements.

5.2.1.2.4.3. Blood Pressure

Refer to general methodology *section 3.5.3.2* for details on procedures for obtaining blood pressure measurements.

5.2.1.2.4.4. Pulse Wave Analysis

Refer to general methodology *section 3.5.3.3* for details on procedures for obtaining PWA measurements.

5.2.1.2.4.5. Resting Metabolic Rate

Refer to general methodology *section 3.5.4* for details on procedures for obtaining RMR measurements.

In addition to values for RER and EE ($kcal.day^{-1}$), resting fat and carbohydrate oxidations rates were also calculated at all time points.

5.2.1.2.5. Blood Sampling and Analysis

5.2.1.2.5.1. Blood Sampling

Refer to general methodology *section 3.6.1* for details on blood sampling procedures.

Due to logistical difficulties, blood sampling through cannulation was not possible, therefore venous blood was sampled through 4 individual venepunctures (one at each time point: pre-bolus and 1, 3, 5-hours post-bolus). A total of 5 mL venous blood was collected into serum-separation tubes for each sample.

Pilot work was initially conducted to calculate PVC from haemoglobin and haematocrit obtained from whole blood sampled into EDTA tubes. Due to the resting nature of the study and replicating total water consumption for each session, there was no change ($P > 0.05$) in plasma volume between time points (pre-bolus and 1, 3, 5-hours post-bolus) and sessions, thus biomarker concentrations in assays were not corrected for PVC. In support of this, Keane *et al.*

(2016b) sufficiently controlled PVC through replicating water consumption and thus did not correct biomarker concentrations for PVC during their 8-hour protocol.

5.2.1.2.5.2. Glucose

Refer to general methodology *section 3.6.2* for details on performing glucose analysis.

Overall intra-individual and inter-individual CV were 1.93% and 13.43%, respectively.

5.2.1.2.5.3. Insulin and Insulin Resistance and Sensitivity Indexes

Refer to general methodology *section 3.6.8* for details on performing insulin analysis.

Inter- and intra-plate CV were 9.1% and 7.8%, respectively.

Refer to general methodology *section 3.6.8.1* for details on insulin resistance and sensitivity analysis.

5.2.1.2.5.4. Triglycerides

Refer to general methodology *section 3.6.4* for details on performing triglyceride analysis.

Overall intra-individual and inter-individual CV were 2.33% and 5.94%, respectively.

5.2.1.2.5.5. Total Cholesterol

Refer to general methodology *section 3.6.5* for details on performing total cholesterol analysis.

Overall intra-individual and inter-individual CV were 4.45% and 5.63%, respectively.

5.2.1.2.5.6. HDL

Refer to general methodology *section 3.6.6* for details on performing HDL analysis.

Overall intra-individual and inter-individual CV were 3.63% and 6.12%, respectively.

5.2.1.2.5.7. Total Cholesterol:HDL Ratio

Refer to general methodology *section 3.6.7* for details on performing TC:HDL analysis.

5.2.1.2.5.8. LDL

LDL was determined indirectly using the Friedewald formula (Friedewald, Levy and Fredrickson, 1972).

5.2.1.3. Data Analysis

Statistical analysis was performed using SPSS v22.0 (IBM, Chicago, USA) where data are reported as mean ± standard deviation (±SD). Data normality was checked using a Shapiro-Wilk test. Greenhouse-Geisser correction was applied upon violation of Mauchly's test of sphericity for ANOVAs ($P < 0.05$). Statistical significance was set at $P < 0.05$. Due to this being a pilot study and the lack of prior data on the effects of MTC products in humans with MetS from which to conduct power calculations, a minimum sample size of 10 was established (Udani *et al.*, 2011).

To account for day-to-day physiological variances at pre-bolus between conditions for each variable, data was analysed as change from pre-bolus for each time point measured post-bolus. This enabled a fair assessment of the post-bolus responses to each condition from pre-bolus across all variables. The pre-bolus time point was not included as a covariate, as one-way ANOVA analysis indicated no significant differences between conditions ($P > 0.05$) for all variables at the pre-bolus time point, hence two-way repeated-measures ANOVA was performed.

A within-group two-way, 3 x 6, condition (PLA vs MTCC vs MTCJ) x time (30 minutes, 1, 2, 3, 4- and 5-hours post-bolus), repeated-measures ANOVA design with *post-hoc* Bonferroni adjustment, measured differences for SBP, DBP, RMR, cardiac haemodynamic and PWA parameters on change from pre-bolus values for each condition.

Blood-based biomarkers were analysed using the same model but with a 3 x 3, condition (PLA vs MTCJ vs MTCC) by time (1, 3- and 5-hours post-bolus) design on change from pre-bolus

values for each condition. A paired-samples *t*-test was used to identify differences between individual data points within conditions and between conditions for the corresponding time point.

Partial Eta-Squared ($\eta^2_{partial}$) was used to report effect sizes for ANOVA where effects were classified as small (0.01-0.08), moderate (0.09-0.25) and large (>0.25) (Cohen, 1998). Cohen's *d* effect size was used for paired-samples *t*-test and *post-hoc* interaction comparisons where effects were classified as no effect (0-0.1), small (0.2-0.4), moderate (0.5-0.7) and high (≥0.8) (Cohen, 1998).

Bivariate correlations were performed between SBP and PWA parameters on change from pre-bolus data for each time point measured post-bolus. Coefficients were classified as weak (0.1-0.2), moderate (0.3-0.5), strong (>0.5) (Cohen, 1988).

5.2.2. Part B

5.2.2.1. Participants

Six (4 male and 2 female) participants with MetS (Tables 5.4 and 5.5) from the original cohort in Part A volunteered for this additional arm of the study.

Table 5.4. Baseline characteristics obtained during screening of all participants (n = 6).

Characteristics	Mean ± SD
Age (years)	53 ± 13
Stature (m)	1.76 ± 0.13
Body Mass (kg)	106.4 ± 25.0
BMI ($kg.m^{-2}$)	34 ± 6
Whole-body Fat (%)	33 ± 7
Trunk Fat (%)	31 ± 12
Fat Mass (kg)	40 ± 14
Fat Free Mass (kg)	57 ± 16
Fasting Total Cholesterol ($mmol.L^{-1}$)	3.86 ± 0.33
Fasting Insulin ($pmol.L^{-1}$)	104.50 ± 20.04
HOMA2-IR (AU)	2.0 ± 1.1
HOMA2-β (%)	106.1 ± 41.1
HOMA2-%S (%)	64.6 ± 35.2

AU (Arbitrary Units); BMI (Body Mass Index); HOMA2-IR (Homeostatic Model Assessment of Insulin Resistance); HOMA-β (Homeostatic Model Assessment of pancreatic β-cell function); HOMA2-%S (Homeostatic Model Assessment of Insulin Sensitivity).

Table 5.5. Individual baseline characteristics of MetS criteria obtained during screening (n = 6).

Characteristics	Mean ± SD	Participant					
		1	2	3	4	5	6
Waist Circumference (cm)	109.6 ± 13.9	102.2*	133*	119*	95	104.1*	104*
Fasting Glucose ($mmol.L^{-1}$)	5.69 ± 0.70	6.20*	5.33	6.50*	6.21*	5.12	4.79
Fasting Triglycerides ($mmol.L^{-1}$)	1.9 ± 0.4	1.9*	2.0*	1.5	2.6*	2.1*	1.5
Fasting HDL ($mmol.L^{-1}$)	1.30 ± 0.49	2.23	1.25	0.83*	1.19*	1.30	1.01*
SBP (mmHg)	133 ± 10	122	146*	143*	129	132*	126
DBP (mmHg)	80 ± 10	67	90*	91*	72	72	85*

DBP (Diastolic Blood Pressure); HDL (High-density Lipoprotein); SBP (Systolic Blood Pressure).

5.2.2.2. Procedures

The same procedures as in Part A were used during this arm, except for the supplement provided. Additionally, due to an equipment malfunction, RMR was not measured in this arm.

5.2.2.2.1. Supplementation

This arm acutely administered MTCJ composed of 60 mL MTC concentrate (Cherry Active, Active Edge Ltd, Hanworth, UK) and 100 mL water (MTCJ60) (refer to Table 5.6 for nutritional information comparing MTCJ60 to PLA, MTCC and MTCJ). As in Part A, anonymity was achieved by explaining to participants that a placebo or 'anthocyanin-rich supplement' would be provided; Montmorency tart cherries as the specific source was not disclosed. Further anonymity was placed by indicating to participants placebo capsules may be provided. Only 1/6 participants correctly distinguished between placebo and the interventions provided.

Table 5.6. Nutritional information of 60 mL Montmorency tart cherry concentrate against other supplements previously provided.

	MTC concentrate (per 60 mL)	MTC concentrate (per 30 mL)	MTC capsules (per capsule – 435 mg)	Placebo (per 130 mL)
Energy (kcal)	204	102	1.3	102
Carbohydrate (g)	49	24.5	0.3	25.35
of which sugars (g)	35.8	17.9	0.1	25.32
Protein (g)	2.2	1.1	Trace	0.03
Fat (g)	0	0	Trace	0
of which saturates (g)	0	0	0	0
Fibre (g)	5.2	2.6	Trace	Trace
Salt (g)	0	0	Trace	0
Total Anthocyanins (mg)	540	270	27	0

5.2.2.3. Data Analysis

The same statistical tests were performed as in *section 5.2.1.3,* with slight differences due to study design.

As in Part A, to account for day-to-day physiological variances at pre-bolus between conditions for each variable, data was analysed as change from pre-bolus for each time point measured post-bolus.

A within-group two-way, 4 x 6, condition (PLA vs MTCC vs MTCJ vs MTCJ60) x time (30 minutes, 1, 2, 3, 4- and 5-hours post-bolus), repeated-measures ANOVA design with *post-hoc* Bonferroni adjustment, measured differences of SBP, DBP, RMR, cardiac haemodynamic and PWA parameters.

Blood-based biomarkers were analysed using the same model but with a 4 x 3, condition (PLA vs MTCJ vs MTCC vs MTCJ60) by time (1, 3- and 5-hours post-bolus) design on change from pre-bolus values for each condition. A paired-samples *t*-test was used to identify differences between individual data points within conditions and between conditions for the corresponding time point.

Bivariate correlations were performed between SBP and PWA parameters on change from pre-bolus data for each time point measured post-bolus. Coefficients were classified as weak (0.1-0.2), moderate (0.3-0.5), strong (>0.5) (Cohen, 1988).

Statistical analysis was only conducted on data obtained from the same six participants enrolled in Parts A and B. Given the small sample size in this arm, individual physiological responses were compared between interventions in addition to the aforementioned statistical analysis.

5.3. Results

The present study measured variables relevant to MetS, and as the defining aspect is insulin resistance, serum insulin and glucose were regarded as primary endpoints. Other variables focused on the other two main aspects of MetS, cardiovascular dysfunction and lipidaemia. Raw data for all variables from Part A are provided in Appendix 4.

5.3.1. Part A

5.3.1.1. Blood Biomarkers

Analysis of change from pre-bolus responses for serum insulin (Figure 5.3) showed a significant main effect for condition ($F_{(2, 20)}$ = 3.653; P = 0.044, $\eta^2_{partial}$ = 0.27), time ($F_{(1.07, 10.67)}$ = 13.411; P < 0.001, $\eta^2_{partial}$ = 0.57) and interaction ($F_{(4, 40)}$ = 5.837; P = 0.001, $\eta^2_{partial}$ = 0.37). *Post-hoc* comparisons on the interaction indicated PLA (60.84 ± 54.34 pmol.L^{-1}) increased significantly more for the change from pre-bolus to 1-hour post-bolus compared to MTCC (-9.86 ± 22.78 pmol.L^{-1}) (P = 0.016, d = 1.70). A significantly different response was also observed for the change from pre-bolus to 3-hours post-bolus between PLA (-8.99 ± 39.53 pmol.L^{-1}) and MTCJ (-48.80 ± 50.01 pmol.L^{-1}) (P = 0.028, d = 0.88). *Post-hoc* analysis on the main effect for condition showed PLA (12.01 ± 41.60 pmol.L^{-1}) increased significantly more (P = 0.039) than MTCJ (-18.01 ± 44.31 pmol.L^{-1}). The change from 1-hour to 3-hours post-bolus was significant between PLA and MTCC ($t_{(10)}$ = -2.295; P = 0.045, d = 0.85) and between MTCJ and MTCC ($t_{(10)}$ = 3.361; P = 0.007, d = 1.06). The mean post-bolus change was lower in 9/11 participants with MTCC compared to PLA.

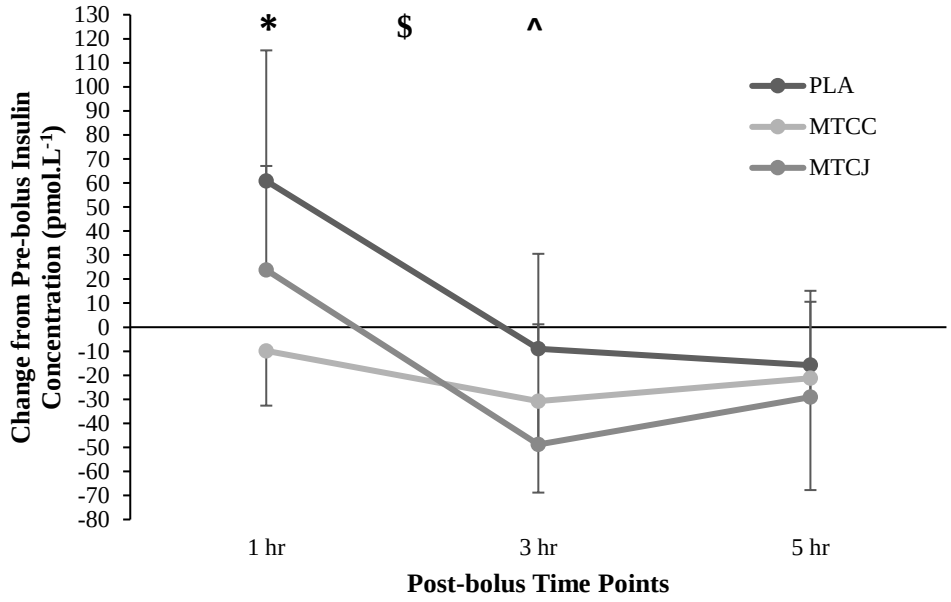


Figure 5.3. Response curves for the mean (±SD) change in insulin concentrations from pre-bolus values to time points post-bolus for each condition. *Denotes significant difference between PLA and MTCC at 1-hour time point. ^Denotes significant difference between PLA and MTCJ at 3-hour time point. $^{\$}$Denotes significant difference for change in insulin from 1-3 hours post-bolus between PLA and MTCJ against MTCC. Statistical analysis performed by a two-way, RM-ANOVA with *post-hoc* Bonferroni adjustment ($P < 0.05$).

A tendency towards significance was observed for the interaction ($F_{(4, 40)} = 2.112$; $P = 0.097$, $\eta^2_{partial} = 0.17$) for LDL (Table 5.7). Statistical analysis for total cholesterol ($F_{(1.159, 11.587)} = 6.470$; $P = 0.023$, $\eta^2_{partial} = 0.39$), indicated a main effect for time only, with *post-hoc* identifying differences between 1-hour ($P = 0.039$, $d = 0.62$) and 3-hours ($P < 0.001$, $d = 0.44$) compared to 5-hours post-bolus (Table 5.7). No significant interaction and main effects for condition or time were detected for glucose, triglycerides, HDL or TC:HDL ($P > 0.05$) (Table 5.7).

Table 5.7. Mean ± SD change from pre-bolus values to post-bolus time points for selected blood-based biomarkers per treatment condition.

		Post-bolus Time Points		
		1 hr	3 hr	5 hr
Glucose ($mmol.L^{-1}$)	PLA	0.35 ± 1.67	-0.58 ± 0.85	-0.63 ± 0.67
	MTCC	-0.11 ± 0.36	-0.22 ± 0.58	-0.28 ± 0.77
	MTCJ	-0.22 ± 0.68	-0.79 ± 0.95	-0.72 ± 1.22
Triglycerides ($mmol.L^{-1}$)	PLA	0.1 ± 0.2	0.1 ± 0.4	0.2 ± 0.4
	MTCC	-0.1 ± 0.2	-0.1 ± 0.4	-0.1 ± 0.3
	MTCJ	-0.1 ± 0.3	0.0 ± 0.2	0.1 ± 0.4
Total Cholesterol*$ ($mmol.L^{-1}$)	PLA	0.04 ± 0.28	0.16 ± 0.47	0.29 ± 0.40
	MTCC	-0.16 ± 0.51	-0.16 ± 0.59	0.15 ± 0.64
	MTCJ	-0.05 ± 0.25	-0.03 ± 0.18	0.13 ± 0.21
HDL ($mmol.L^{-1}$)	PLA	-0.12 ± 0.19	-0.10 ± 0.16	-0.18 ± 0.19
	MTCC	-0.05 ± 0.16	-0.04 ± 0.10	-0.03 ± 0.17
	MTCJ	-0.07 ± 0.12	0.01 ± 0.11	-0.09 ± 0.17
TC:HDL (AU)	PLA	0.27 ± 0.39	0.31 ± 0.54	0.70 ± 0.64
	MTCC	0.30 ± 0.72	0.07± 0.55	0.39 ± 0.64
	MTCJ	0.19 ± 0.43	-0.04 ± 0.40	0.42 ± 0.62
LDL ($mmol.L^{-1}$)	PLA	0.25 ± 0.49	0.36 ± 0.59	0.70 ± 0.56
	MTCC	0.03 ± 0.71	-0.07 ± 0.85	0.06 ± 0.66
	MTCJ	0.08 ± 0.52	0.12 ± 0.33	0.24 ± 0.66

AU (Arbitrary Units); HDL (High-density Lipoprotein); MTCC (Montmorency Tart Cherry Capsules); MTCJ (Montmorency Tart Cherry Juice); PLA (Placebo); TC (Total Cholesterol). *Denotes significant main effect for time with *post-hoc* identifying differences between 1-hour and 5-hours post-bolus. $Denotes significant main effect for time with *post-hoc* identifying differences between 3-hours and 5-hours post-bolus.

5.3.1.2. Cardiac Haemodynamics

No significant main effects for condition, time or the condition by time interaction were detected for SBP, DBP, MAP, SV, CO and TPR ($P > 0.05$) (Table 5.8). However, individual responses for the change from pre-bolus to 2-hours post-bolus showed only 3/11 participants with lower SBP after consuming PLA, while 6/11 participants had lower SBP after ingesting MTCC and 10/11 with MTCJ. The mean change between PLA (3 ± 7 mmHg) and MTCJ (-8 ± 6 mmHg) at 2-hours post-bolus was -11 mmHg (Figure 5.4).

HR responses revealed a significant interaction effect ($F_{(10,\ 100)}$ = 5.301; P < 0.001, $\eta^2_{partial}$ = 0.35). *Post-hoc* analysis displayed a tendency towards significance for HR at 30 minutes post-bolus between PLA (-1 ± 3 beats.min^{-1}) and MTCC (-5 ± 5 beats.min^{-1}) (P = 0.080, d = 0.27), where all 11 participants responded with lower HR at 30 minutes post-bolus compared to pre-bolus with MTCC, whereas only 6 of 11 participants responded the same way with PLA.

Table 5.8. Mean ± SD change from pre-bolus values to post-bolus time points for selected cardiac haemodynamic and PWA parameters per treatment condition.

		Post-bolus Time Points					
		30 mins	1 hr	2 hr	3 hr	4 hr	5 hr
Brachial DBP (mmHg)	PLA	3 ± 4	5 ± 7	5 ± 5	8 ± 8	5 ± 7	4 ± 6
	MTCC	1 ± 9	4 ± 10	2 ± 10	0 ± 6	1 ± 9	3 ± 8
	MTCJ	3 ± 6	6 ± 4	3 ± 4	7 ± 6	6 ± 8	6 ± 5
MAP (mmHg)	PLA	1 ± 5	5 ± 7	5 ± 7	7 ± 9	5 ± 9	5 ± 9
	MTCC	3 ± 11	6 ± 10	2 ± 10	2 ± 6	1 ± 9	5 ± 8
	MTCJ	2 ± 3	2 ± 6	-4 ± 5	4 ± 7	4 ± 8	-2 ± 12
Cardiac Output ($L.min^{-1}$)	PLA	0.11 ± 0.96	-0.02 ± 1.57	-0.31 ± 1.32	-0.58 ± 1.03	-0.53 ± 1.64	-0.35 ± 1.44
	MTCC	-0.10 ± 0.68	-0.24 ± 0.99	-0.13 ± 1.15	0.03 ± 1.06	-0.31 ± 1.48	-0.21 ± 1.37
	MTCJ	-0.33 ± 1.80	-0.46 ± 1.46	-0.04 ± 1.98	-0.34 ± 1.94	-0.62 ± 1.50	-0.61 ± 1.56
Stroke Volume (mL)	PLA	1 ± 15	-1 ± 19	-1 ± 15	-5 ± 19	-5 ± 24	2 ± 24
	MTCC	6 ± 11	-1 ± 17	4 ± 8	8 ± 13	1 ± 18	3 ± 20
	MTCJ	1 ± 27	-4 ± 19	1 ± 27	-4 ± 30	1 ± 24	2 ± 23
TPR ($mmHg \cdot min^{-1} \cdot L$)	PLA	0.00 ± 0.21	0.15 ± 0.37	0.14 ± 0.31	0.26 ± 0.46	0.16 ± 0.27	0.09 ± 0.26
	MTCC	0.01 ± 0.17	0.09 ± 0.28	0.02 ± 0.37	0.08 ± 0.32	0.09 ± 0.39	0.12 ± 0.34
	MTCJ	0.08 ± 0.33	0.07 ± 0.27	-0.03 ± 0.38	0.10 ± 0.34	0.10 ± 0.28	0.11 ± 0.35
HR ($beats.min^{-1}$)	PLA	-1 ± 3	-3 ± 4	-5 ± 7	-5 ± 7	-7 ± 8	-7 ± 7
	MTCC	-5 ± 5	-4 ± 5	-4 ± 7	-6 ± 7	-5 ± 10	-4 ± 10
	MTCJ	0 ± 5	-1 ± 5	-5 ± 6	-7 ± 5	-7 ± 4	-8 ± 6
Aortic SBP (mmHg)	PLA	1 ± 7	3 ± 8	4 ± 9	2 ± 11	4 ± 8	5 ± 5
	MTCC	2 ± 5	4 ± 9	4 ± 14	2 ± 10	2 ± 8	4 ± 6
	MTCJ	-1 ± 8	-1 ± 8	-2 ± 7	1 ± 4	2 ± 7	3 ± 7

Aortic DBP (mmHg)	PLA	0 ± 6	0 ± 7	3 ± 14	3 ± 5	4 ± 7	2 ± 7
	MTCC	5 ± 6	3 ± 5	4 ± 8	0 ± 8	3 ± 7	6 ± 7
	MTCJ	-2 ± 6	-2 ± 6	2 ± 4	1 ± 8	1 ± 7	1 ± 4
Pulse Pressure (mmHg)	PLA	1 ± 8	3 ± 8	1 ± 9	-1 ± 8	0 ± 6	3 ± 5
	MTCC	-3 ± 7	1 ± 9	0 ± 7	2 ± 5	-1 ± 6	-2 ± 7
	MTCJ	1 ± 6	1 ± 5	-4 ± 4	0 ± 8	1 ± 6	2 ± 5
AIx at HR75*^$ (%)	PLA	0 ± 4	-1 ± 3	2 ± 5	2 ± 5	3 ± 5	5 ± 5
	MTCC	0 ± 6	-1 ± 6	-2 ± 6	0 ± 5	2 ± 6	3 ± 7
	MTCJ	0 ± 4	-1 ± 7	-2 ± 7	-2 ± 7	0 ± 8	2 ± 7
SEVR (%)	PLA	-1 ± 33	11 ± 20	15 ± 27	10 ± 21	16 ± 26	3 ± 21
	MTCC	15 ± 23	9 ± 16	17 ± 21	16 ± 22	19 ± 24	9 ± 22
	MTCJ	6 ± 26	8 ± 22	31 ± 32	24 ± 31	21 ± 31	12 ± 20

AIx at HR75 (Augmentation Index at Heart Rate 75 bpm); DBP (Diastolic Blood Pressure); HR (Heart Rate); MAP (Mean Arterial Pressure); mmHg (millimetres of Mercury); mmHg (millimetres of Mercury); MTCC (Montmorency Tart Cherry Capsules); MTCJ (Montmorency Tart Cherry Juice); PLA (Placebo); SEVR (Subendocardial Viability Ratio); TPR (Total Peripheral Resistance) *Denotes significant main effect for time with *post-hoc* identifying differences between 1-hour and 3-hours post-bolus. ^Denotes significant main effect for time with *post-hoc* identifying differences between 1-hour and 5-hours post-bolus. $Denotes significant main effect for time with *post-hoc* identifying differences between 3-hours and 5-hours post-bolus.

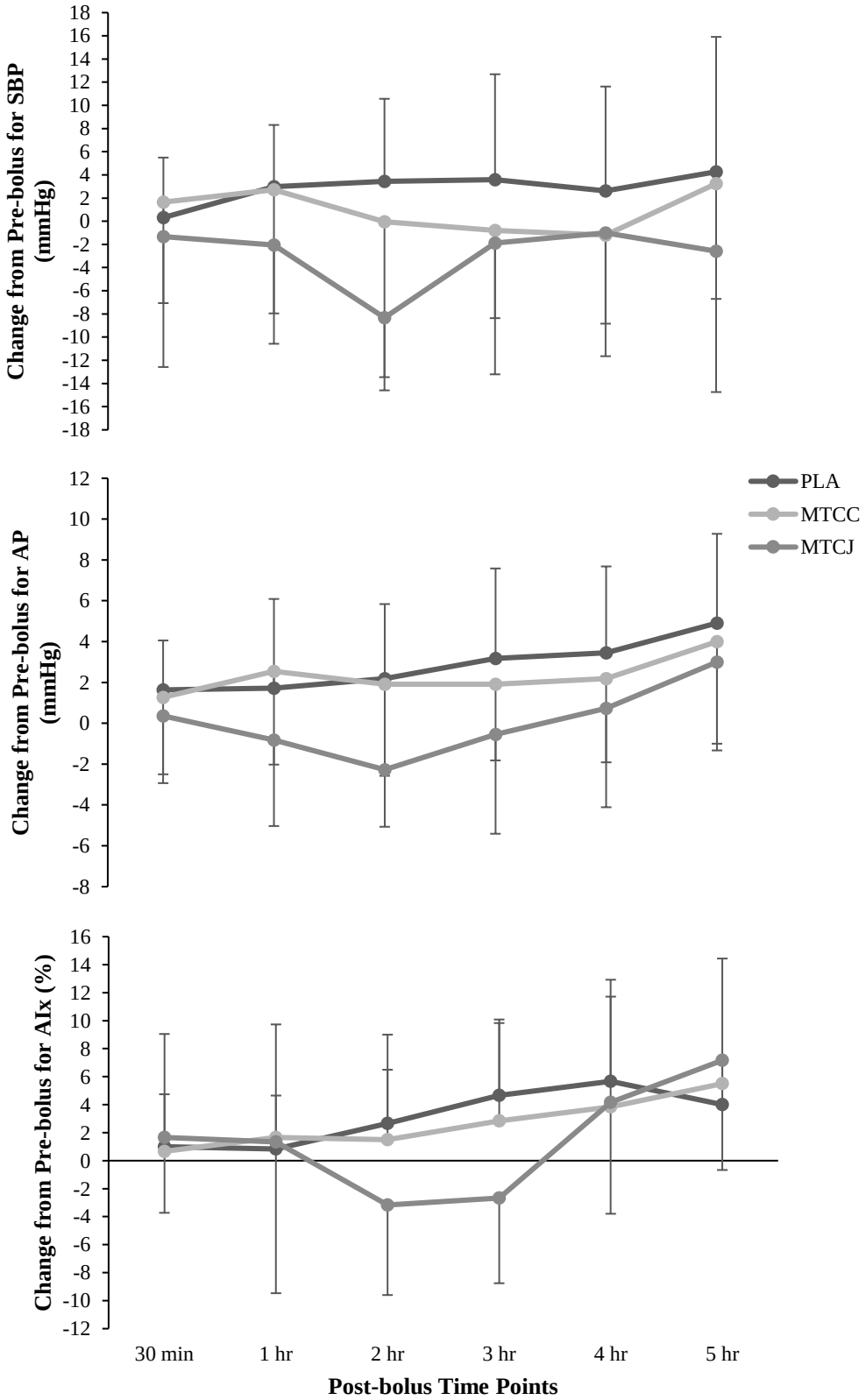


Figure 5.4. Mean (±SD) SBP, augmentation pressure (AP) and augmentation index (AIx) response curves from respective pre-bolus time points for each condition.

5.3.1.3. Pulse Wave Analysis

A main effect for time ($F_{(5, 50)}$ = 7.84; P < 0.001, $\eta^2_{partial}$ = 0.44) only was observed for the change in AP from pre-bolus. *Post-hoc* analysis identified the change in AP was lower at both 1-hour (P = 0.026, d = 0.63) and 2-hours post-bolus (P = 0.041, d = 0.81) against 5-hours post-bolus. However, individual responses showed 7/11 participants responded with lower AP at 2-hours post-bolus with MTCJ, compared to 2/11 participants with PLA (Figure 5.4).

Similarly, only a main effect for time ($F_{(5, 50)}$ = 10.090; P < 0.001, $\eta^2_{partial}$ = 0.50) was observed for the change in AIx from pre-bolus. *Post-hoc* analysis showed AIx to be significantly higher at 5-hours post-bolus than all other post-bolus time points (P < 0.05), except 4-hours post-bolus. Individual responses showed 9/11 participants reduced arterial stiffness at this time point compared to pre-bolus with MTCJ, while only 2/11 responded in this manner with PLA (Figure 5.4). There were no significant correlations between SBP and AIx (ρ = 0.105; P = 0.401) or AP (ρ = 0.145; P = 0.247) at 2-hours post-bolus for MTCJ.

No significant main effects for condition, time or interaction were detected for aortic SBP, aortic DBP, and pulse pressure (P > 0.05). A main effect for time was detected for AIx at HR75 ($F_{(5, 50)}$ = 7.747; P < 0.001, $\eta^2_{partial}$ = 0.44) and SEVR ($F_{(5, 50)}$ = 3.903; P = 0.005, $\eta^2_{partial}$ = 0.28) (Table 5.8). *Post-hoc* analysis identified the change in AP was lower at 30-minutes (P = 0.037, d = 0.64), 1-hour (P = 0.001, d = 0.38) and 2-hours post-bolus (P = 0.001, d = 0.67) compared to 5-hours post-bolus.

5.3.1.4. Resting Metabolic Rate

Resting EE and RER did not show main effects for condition, time or interaction (P > 0.05) (Appendix 4). No main effects for condition or interaction (P > 0.05) were seen, however significant main effects for time were observed for resting fat ($F_{(5, 50)}$ = 18.096; P < 0.001,

$\eta^2_{partial}$ = 0.64) and carbohydrate oxidation ($F_{(5, 50)}$ = 16.750; P < 0.001, $\eta^2_{partial}$ = 0.63). Fat and carbohydrate oxidation were found to be lower at all time points compared to 30-minutes post-bolus (P < 0.05).

5.3.2. Part B

5.3.2.1. Blood Biomarkers

Analysis of change from pre-bolus responses for serum insulin (Figure 5.5) showed a significant main effect for time ($F_{(1.091, 5.454)}$ = 18.99; P = 0.006, $\eta^2_{partial}$ = 0.79) and interaction ($F_{(6, 30)}$ = 4.046; P = 0.004, $\eta^2_{partial}$ = 0.45). The difference between 1-hour to 3-hours post-bolus with change from pre-bolus data was significant between MTCC and MTCJ ($t_{(5)}$ = 3.361; P = 0.020, d = 1.34) and between MTCC and MTCJ60 ($t_{(5)}$ = 3.909; P = 0.011, d = 1.76). Analysis of individual responses for mean post-bolus changes from pre-bolus suggested a greater reduction in insulin concentrations in 4/6 individuals with MTCJ60 compared to MTCJ. Additionally, individual responses showed a greater reduction in insulin concentrations in 4/6 individuals with MTCJ60 compared to PLA.

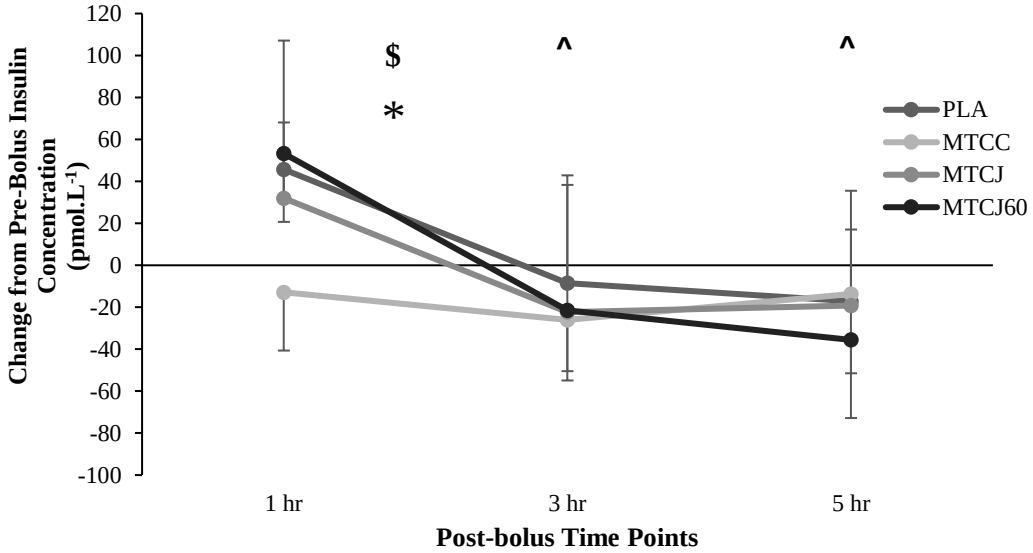


Figure 5.5. Response curves for the mean (±SD) change in insulin concentrations from pre-bolus values to time points post-bolus for each condition. ^Denotes significant difference between 1-hour time point. *Denotes significant difference for change in insulin from 1-3 hours post-bolus between MTCC and MTCJ. $^{\$}$Denotes significant difference for change in insulin from 1-3 hours post-bolus between MTCC and MTCJ60.

Change from pre-bolus responses for serum glucose concentrations did not show any significant main effects for condition, time or interaction ($P > 0.05$). Change from pre-bolus to post-bolus values for selected glycaemic, insulinaemic and lipid variables are presented in Table 5.9.

Table 5.9. Mean ± SD change from pre-bolus values to post-bolus time points for selected blood-based biomarkers per treatment condition.

		Post-bolus Time Points		
		1 hr	3 hr	5 hr
Glucose ($mmol.L^{-1}$)	PLA	0.75 ± 2.23	-0.60 ± 0.87	-0.49 ± 0.56
	MTCC	-0.22 ± 0.37	-0.44 ± 0.56	-0.49 ± 0.41
	MTCJ	0.08 ± 0.49	-0.39 ± 0.62	-0.35 ± 1.10
	MTCJ60	0.24 ± 0.74	-0.50 ± 0.48	-0.43 ± 0.68
Triglycerides ($mmol.L^{-1}$)	PLA	0.1 ± 0.2	0.1 ± 0.3	0.1 ± 0.4
	MTCC	0.1 ± 0.2	0.1 ± 0.2	0.0 ± 0.3
	MTCJ	-0.1 ± 0.2	0.1 ± 0.2	0.2 ± 0.3
	MTCJ60	0.1 ± 0.3	0.3 ± 0.6	0.3 ± 0.6
HDL ($mmol.L^{-1}$)	PLA	-0.16 ± 0.25	-0.16 ± 0.17	-0.24 ± 0.19
	MTCC	-0.05 ± 0.17	-0.03 ± 0.06	0.04 ± 0.17
	MTCJ	-0.04 ± 0.11	0.00 ± 0.14	-0.05 ± 0.17
	MTCJ60	-0.01 ± 0.09	0.04 ± 0.08	0.01 ± 0.17
LDL^ ($mmol.L^{-1}$)	PLA	0.23 ± 0.63	0.41 ± 0.56	0.63 ± 0.44
	MTCC	0.31 ± 0.47	0.34 ± 0.41	0.31 ± 0.34
	MTCJ	-0.11 ± 0.33	0.24 ± 0.30	0.33 ± 0.43
	MTCJ60	-0.12 ± 0.47	-0.01 ± 0.74	0.27 ± 0.99
TC:HDL* (AU)	PLA	0.23 ± 0.50	0.43 ± 0.67	0.85 ± 0.61
	MTCC	0.33 ± 0.82	0.20 ± 0.46	0.38 ± 0.77
	MTCJ	-0.01 ± 0.27	-0.07 ± 0.53	0.30 ± 0.48
	MTCJ60	-0.24 ± 0.26	-0.41 ± 0.44	-0.14 ± 0.52

AU (Arbitrary Units); HDL (High-density Lipoprotein); MTCC (Montmorency Tart Cherry Capsules); MTCJ (Montmorency Tart Cherry Juice – 30 mL dose); MTCJ60 (Montmorency Tart Cherry Juice – 60 mL dose); PLA (Placebo); TC (Total Cholesterol). *Denotes significant main effect for time with *post-hoc* identifying differences between 3-hours and 5-hours post-bolus. ^Denotes significant main effect for time with *post-hoc* identifying differences between 1-hour and 3-hours against 5-hours post-bolus.

No significant interaction or main effects for condition or time were detected for total cholesterol, triglycerides and HDL ($P > 0.05$) (Table 5.9). Individual responses demonstrated a reduction in total cholesterol for all 6 individuals with MTCJ60 compared to MTCJ when analysing mean post-bolus change from pre-bolus data (Figure 5.6). Mean post-bolus change from pre-bolus showed 5/6 individuals responded with higher concentrations of HDL after MTCJ60 consumption compared to PLA. Furthermore, 4/6 individuals responded with higher HDL concentrations after MTCJ60 supplementation compared to MTCJ, while no change was seen in 1 individual.

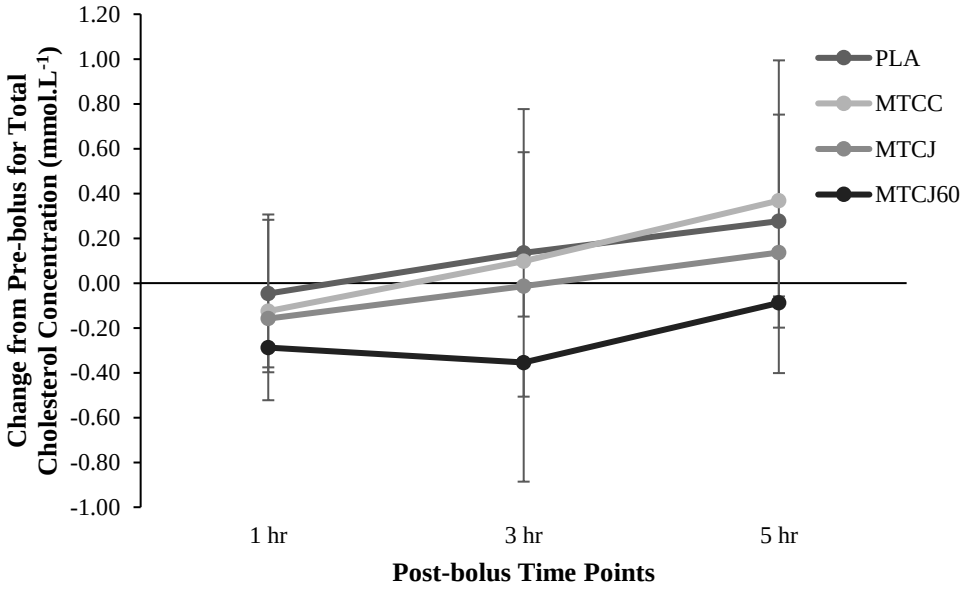


Figure 5.6. Mean (±SD) change in total cholesterol concentrations from pre-bolus for all conditions.

No main effects for condition or interaction were observed ($P > 0.05$) for TC:HDL ratio (Table 5.9), however individual responses showed mean post-bolus change from pre-dose improved in all 6 individuals with MTCJ60 compared to MTCJ, and in 5/6 individuals with MTCJ60 compared to PLA.

No significant main effects for condition or interaction were found for LDL ($P > 0.05$) (Table 5.9).

5.3.2.2. Cardiac Haemodynamics

A significant interaction effect was observed for the change in SBP ($F_{(15, 75)}$ = 1.842; P = 0.044, $\eta^2_{partial}$ = 0.27) with *post-hoc* comparisons identifying a tendency (P = 0.088) towards significance between PLA (4 ± 8 mmHg) and MTCJ (-11 ± 6 mmHg) at 2-hours post-bolus (Figure 5.7). No other main effects for time or condition were detected with SBP (P > 0.05). Examination of individual responses showed 4/6 participants had lower SBP with MTCJ compared to MTCJ60 when averaging change from pre-bolus across all post-bolus time points.

Statistical analysis did not detect any main effects for condition, time or interaction for DBP, MAP, TPR, CO and SV (P > 0.05) (Table 5.10). Heart rate responses only demonstrated a significant main effect for time ($F_{(5, 25)}$ = 13.274; P < 0.001, $\eta^2_{partial}$ = 0.73), with 30-minutes post-bolus decreasing more than 3-hours post-bolus (P = 0.037, d = 0.75). Additionally, heart rate at 1-hour post-bolus reduced significantly more than 2-hours (P = 0.006, d = 0.31), 3-hours (P = 0.022, d = 0.57) and 4-hours post-bolus (P = 0.036, d = 0.57).

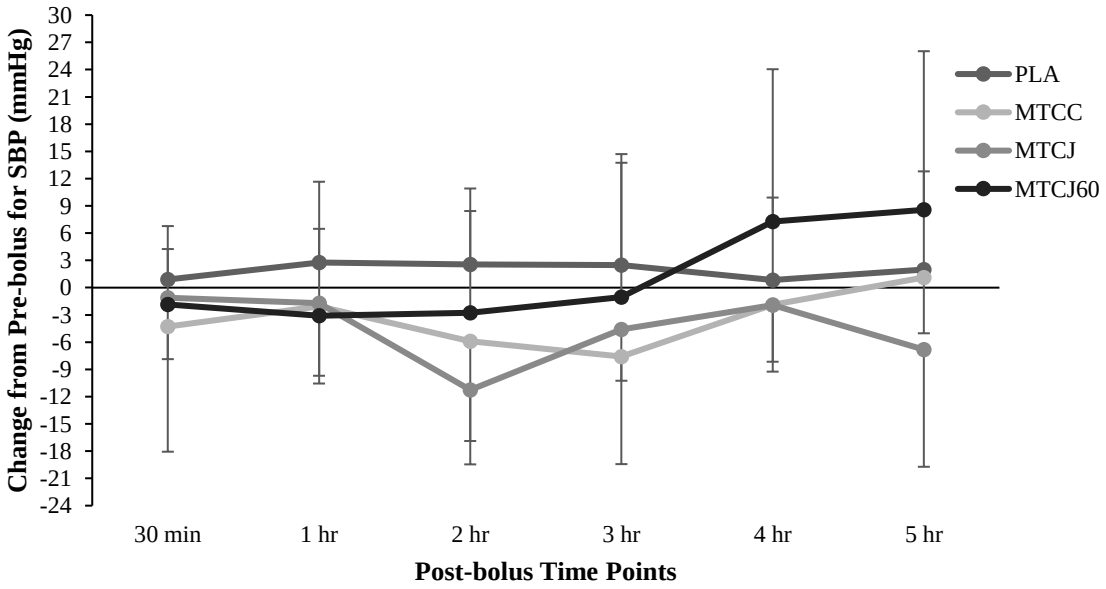


Figure 5.7. Mean (±SD) change in SBP from pre-bolus for all conditions.

Table 5.10. Mean ± SD change from pre-bolus values to post-bolus time points for selected cardiac haemodynamic parameters per treatment condition.

		Post-bolus Time Points					
		30 mins	1 hr	2 hr	3 hr	4 hr	5 hr
Brachial DBP (mmHg)	PLA	4 ± 5	6 ± 8	6 ± 7	8 ± 10	4 ± 8	6 ± 7
	MTCC	-3 ± 9	0 ± 10	-2 ± 6	-2 ± 6	0 ± 10	0 ± 4
	MTCJ	3 ± 5	6 ± 4	3 ± 4	8 ± 6	6 ± 6	3 ± 6
	MTCJ60	0 ± 5	0 ± 7	2 ± 7	1 ± 9	6 ± 6	4 ± 9
MAP (mmHg)	PLA	3 ± 5	9 ± 6	6 ± 7	9 ± 11	6 ± 10	8 ± 10
	MTCC	-2 ± 11	3 ± 9	-3 ± 7	1 ± 7	1 ± 9	3 ± 5
	MTCJ	1 ± 2	2 ± 4	-4 ± 4	5 ± 4	2 ± 8	-4 ± 13
	MTC60	-1 ± 5	-1 ± 8	0 ± 8	1 ± 9	4 ± 10	4 ± 9
Resting HR ($beats.min^{-1}$)	PLA	-2 ± 4	-3 ± 4	-5 ± 4	-7 ± 6	-7 ± 7	-7 ± 6
	MTCC	-6 ± 7	-4 ± 6	-6 ± 8	-8 ± 7	-6 ± 9	-7 ± 7
	MTCJ	-1 ± 5	-3 ± 6	-7 ± 7	-8 ± 6	-8 ± 5	-10 ± 5
	MTCJ60	-1 ± 3	0 ± 5	-4 ± 2	-5 ± 4	-6 ± 7	-4 ± 4
Cardiac Output ($L.min^{-1}$)	PLA	-0.35 ± 0.91	-0.13 ± 2.17	-0.55 ± 1.61	-0.42 ± 0.67	-0.76 ± 0.84	-1.19 ± 0.80
	MTCC	-0.22 ± 0.44	0.12 ± 1.07	0.26 ± 0.48	0.06 ± 0.91	-0.13 ± 1.16	0.06 ± 0.72
	MTCJ	-0.30 ± 1.66	-0.48 ± 1.82	-0.43 ± 1.52	-0.32 ± 1.62	-0.53 ± 1.40	-0.65 ± 1.44
	MTCJ60	0.31 ± 2.47	0.62 ± 3.05	-0.56 ± 2.53	-1.22 ± 2.28	-1.08 ± 2.04	0.01 ± 3.23

Stroke Volume (mL)	PLA	-5 ± 17	-9 ± 22	-6 ± 17	-1 ± 22	-9 ± 18	-11 ± 20
	MTCC	4 ± 7	4 ± 21	9 ± 4	12 ± 15	6 ± 17	13 ± 13
	MTCJ	-3 ± 19	-4 ± 23	2 ± 24	-13 ± 27	4 ± 23	3 ± 25
	MTCJ60	8 ± 28	11 ± 31	-1 ± 28	-6 ± 17	-6 ± 26	12 ± 23
TPR ($mmHg \cdot min^{-1} \cdot L$)	PLA	0.00 ± 0.21	0.15 ± 0.37	0.14 ± 0.31	0.26 ± 0.46	0.16 ± 0.27	0.09 ± 0.26
	MTCC	0.01 ± 0.17	0.09 ± 0.28	0.02 ± 0.37	0.08 ± 0.32	0.09 ± 0.39	0.12 ± 0.34
	MTCJ	0.08 ± 0.33	0.07 ± 0.27	-0.03 ± 0.38	0.10 ± 0.34	0.10 ± 0.28	0.11 ± 0.35
	MTCJ60	-0.19 ± 0.26	-0.13 ± 0.34	0.01 ± 0.26	0.04 ± 0.28	0.11 ± 0.26	-0.06 ± 0.38

DBP (Diastolic Blood Pressure); HR (Heart Rate); MAP (Mean Arterial Pressure); mmHg (millimetres of Mercury); MTCC (Montmorency Tart Cherry Capsules); MTCJ (Montmorency Tart Cherry Juice); MTCJ60 (Montmorency Tart Cherry Juice 60 mL); PLA (Placebo); TPR (Total Peripheral Resistance).

5.3.2.3. Pulse Wave Analysis

No main effects were found for aortic SBP ($P > 0.05$) (Table 5.11). However, 5/6 individuals responded with lower aortic SBP after MTCJ60 consumption compared to PLA when assessing mean post-bolus change from pre-bolus.

Pulse pressure showed a tendency towards significance for the interaction main effect ($F_{(15, 75)} = 1.690$; $P = 0.071$, $\eta^2_{partial} = 0.25$) (Table 5.11). Individual responses highlighting all 6 participants had narrower mean post-bolus pulse pressure with MTCJ60 compared to PLA at 30 minutes post-bolus. Moreover, individual responses showed 4/6 participants with narrower mean post-bolus pulse pressure at 4-hours post-bolus with MTCJ compared to PLA.

Similar to pulse pressure, individual responses for AIx suggested 4/6 participants had more compliant arteries with MTCJ60 compared to PLA when analysing mean post-bolus change from pre-bolus. A significant interaction effect ($F_{(15, 75)} = 1.943$; $P = 0.032$, $\eta^2_{partial} = 0.28$) was observed with AIx (Table 5.11); with differences showing MTCJ (3 ± 11%) to increase more than MTCJ60 (-2 ± 6%) at 30 minutes post-bolus ($P = 0.015$, $d = 0.57$).

A main effect for time was found for AP ($F_{(5, 25)} = 5.994$; $P = 0.001$, $\eta^2_{partial} = 0.55$), although individual responses indicated all 6 participants responded with lower AP with MTCJ compared to PLA at 2-hours post-bolus (Table 5.11). Finally, a main effect of time was observed for SEVR ($F_{(5, 25)} = 3.947$; $P = 0.009$, $\eta^2_{partial} = 0.44$). No other main effects for condition or interaction were found ($P > 0.05$) (Table 5.11). *Post-hoc* analyses were unable to detect differences for the main effect for time with AP and SEVR.

Table 5.11. Mean ± SD change from pre-bolus values to post-bolus time points for selected PWA parameters per condition.

		Post-bolus Time Points					
		30 mins	1 hr	2 hr	3 hr	4 hr	5 hr
Aortic SBP (mmHg)	PLA	6 ± 4	6 ± 9	8 ± 7	9 ± 8	9 ± 5	8 ± 3
	MTCC	1 ± 6	6 ± 11	5 ± 17	1 ± 11	1 ± 9	3 ± 6
	MTCJ	1 ± 9	-1 ± 10	-5 ± 7	1 ± 3	2 ± 9	4 ± 9
	MTCJ60	-1 ± 4	-3 ± 7	1 ± 6	3 ± 7	4 ± 7	2 ± 8
Aortic DBP (mmHg)	PLA	12 ± 13	10 ± 12	14 ± 12	12 ± 16	11 ± 10	11 ± 12
	MTCC	1 ± 3	1 ± 8	-4 ± 8	-1 ± 6	3 ± 8	-3 ± 6
	MTCJ	-3 ± 6	-5 ± 5	1 ± 4	2 ± 9	-2 ± 6	2 ± 5
	MTC60	-1 ± 5	0 ± 8	1 ± 8	1 ± 9	4 ± 10	3 ± 9
Pulse Pressure (AU)	PLA	6 ± 7	-4 ± 8	-6 ± 11	-3 ± 5	-2 ± 5	-3 ± 7
	MTCC	0 ± 7	5 ± 8	1 ± 5	0 ± 3	-2 ± 8	0 ± 9
	MTCJ	-2 ± 7	-6 ± 5	-4 ± 4	-1 ± 6	0 ± 4	2 ± 2
	MTCJ60	-2 ± 6	-3 ± 7	0 ± 7	2 ± 8	0 ± 5	-1 ± 4
AIx at HR75 (%)	PLA	1 ± 4	0 ± 4	5 ± 5	4 ± 5	5 ± 6	6 ± 5
	MTCC	-3 ± 7	-2 ± 5	-2 ± 8	1 ± 6	2 ± 7	3 ± 9
	MTCJ	2 ± 4	1 ± 9	-3 ± 7	-2 ± 8	2 ± 8	5 ± 8
	MTCJ60	-1 ± 5	-1 ± 9	2 ± 9	2 ± 8	3 ± 10	4 ± 11
AIx (%)	PLA	2 ± 4	2 ± 5	6 ± 6	5 ± 7	7 ± 8	6 ± 6
	MTCC	0 ± 8	2 ± 8	1 ± 6	3 ± 7	5 ± 9	6 ± 9
	MTCJ	3 ± 5	3 ± 11	-2 ± 8	-1 ± 7	6 ± 9	7 ± 8
	MTCJ60	-2 ± 6	-2 ± 8	-1 ± 10	3 ± 9	4 ± 10	4 ± 11

Augmentation Pressure (mmHg)	PLA	2 ± 2	2 ± 5	3 ± 5	5 ± 5	5 ± 4	6 ± 4
	MTCC	1 ± 4	3 ± 5	2 ± 4	2 ± 3	3 ± 4	5 ± 6
	MTCJ	2 ± 4	0 ± 5	-2 ± 3	1 ± 6	2 ± 6	5 ± 4
	MTCJ60	-1 ± 3	-1 ± 3	3 ± 3	2 ± 2	3 ± 2	2 ± 4
SEVR (%)	PLA	-6 ± 43	8 ± 25	13 ± 32	10 ± 18	15 ± 28	1 ± 21
	MTCC	13 ± 28	4 ± 20	17 ± 22	15 ± 23	13 ± 23	11 ± 28
	MTCJ	10 ± 32	14 ± 27	41 ± 40	30 ± 41	20 ± 32	21 ± 10
	MTCJ60	-7 ± 9	-2 ± 26	8 ± 17	8 ± 23	-5 ± 31	-5 ± 16

AIx at HR75 (Augmentation Index at Heart Rate 75 bpm); Diastolic Blood Pressure (DBP); mmHg (millimetres of Mercury); MTCC (Montmorency Tart Cherry Capsules); MTCJ (Montmorency Tart Cherry Juice); MTCJ60 (Montmorency Tart Cherry Juice 60 mL); PLA (Placebo); Systolic Blood Pressure (SBP); Subendocardial Viability Ratio (SEVR).

5.4. Discussion

Cardio-metabolic responses to MTC had not previously been examined in humans with MetS, thus providing an aim for this study. Since the inception of the present study, Johnson *et al.* (2017) published an abstract outlining the effects of chronic supplementation of MTCJ on cardiac haemodynamics, arterial stiffness and blood-based cardio-metabolic biomarkers in humans with MetS. However, the present study examined the cardio-metabolic responses of humans with MetS to an acute bolus of MTCC, 30 and 60 mL MTCJ in a randomised, placebo-controlled trial, thus adding further novelty of this research.

The hypotheses were partially accepted as the key findings demonstrated significantly lower post-prandial responses to insulin with MTCJ compared to PLA, and a blunted insulin response with MTCC compared to PLA, 1-hour post-bolus. In accordance with previous research (Keane *et al.,* 2016b), individual responses showed SBP to be lower with MTCJ compared to PLA, 2-hours post-bolus. Individual responses also showed MTCJ60 consumption increased HDL. Finally, responses between MTCC and MTCJ were not statistically different, thus refuting the hypothesis that MTCC may be superior than MTCJ; although physiological differences were apparent at particular time points for certain markers.

5.4.1. Metabolic Responses

Findings from the present research showed the mean post-bolus change for insulin was significantly lower with MTCJ (-18.02 ± 44.31 pmol.L^{-1}) than PLA (12.01 ± 41.60 pmol.L^{-1}), and significantly lower than PLA at 3-hours post-bolus. Also, insulin responses between PLA and MTCC were physiologically different where the mean change from pre-bolus across all post-bolus time points increased with PLA but decreased by 20.61 pmol.L^{-1} with MTCC. However, a caveat of these findings is that they were likely influenced by the high carbohydrate content (99.88% of which sugars) in the placebo, where maltodextrin was added to match the

energy content of the placebo to MTCJ. Consequently, this provoked a contrived response with the placebo on insulin, making comparisons against MTCC and MTCJ difficult. Nevertheless, as this was the first study to assess acute glycaemic and insulinaemic tolerability to MTCC and MTCJ consumption, it is possible to discern novel findings. A blunted insulin response throughout the 5-hour post-bolus period with MTCC was observed, as the 10 capsules ingested contained only 1 gram of carbohydrate. Additionally, in alignment with MTC pharmacokinetics (Keane *et al.*, 2016b), absolute insulin concentrations at 3-hours post-bolus were similar between MTCC (77.42 ± 3.04 pmol.L^{-1}) and MTCJ (76.53 ± 8.95 pmol.L^{-1}). This highlights the role of MTCJ phytochemicals in reducing insulin to a similar concentration as MTCC, despite differences in glycaemic load. Remarkably, data from Part B also showed similar absolute insulin concentrations for MTCC (76.74 ± 41.39 pmol.L^{-1}), MTCJ (70.15 ± 39.28 pmol.L^{-1}) and MTCJ60 (82.97 ± 14.21 pmol.L^{-1}) at 3-hours post-bolus. This indicates the absence of a dose-response effect with MTC phytochemicals but also a boundary level at which insulin concentrations could be reduced to. Interestingly, the similar concentrations suggest no difference between the delivery of MTC phytochemicals in capsule or juice form, indicating no enhancement of phytochemical bioavailability with capsules. However, MTC interventions effectively blunted insulin responses when parent anthocyanin and secondary metabolite plasma concentrations were likely at their greatest.

The high glucose load in the placebo elicited a significantly higher insulin secretion response 1-hour post-bolus compared to MTCC. Additionally, MTC anthocyanins may have inhibited glucose absorption across the apical membrane of enterocytes into systemic circulation via suppression of SGLT-1 (Alzaid, 2013). Furthermore, findings from Part B would support the mechanism that saturation of anthocyanins and secondary metabolites at a threshold (Wallace, Slavin and Frankenfeld, 2016) around 270 mg, limited uptake and subsequently SGLT-1 activity. This likely explains the similar glucose concentrations between MTCJ and MTCJ60,

despite MTCJ60 possessing double the total carbohydrate and sugar content. Likewise, insulinaemic and glycaemic responses to 7 days of New Zealand blackcurrant powder (NZBP) supplementation suggested a 14.3% reduction in fasting insulin compared to control (Willems *et al.*, 2017). Crucially, the authors measured fasting insulin, 1-hour post-consumption of the final bolus, implying an acute effect of NZBP on insulin. Willems *et al.* (2017) ascribed the decline in insulin to improved insulin sensitivity. A similar mechanism may be cited to explain MTCJ and MTCC responses, given baseline HOMA2-IR (2.2 ± 1.4) and HOMA2-%S (60.4 ± 27.3%) values indicated insulin resistance (Queiroz *et al.*, 2009) and low insulin sensitivity, respectively. Lower insulin combined with normal glucose concentrations may suggest MTCJ and MTCC improved insulin sensitivity (Willems *et al.*, 2017). Such as response may subsequently prevent pancreatic β-cell glucotoxicity (Amiot, Riva and Vinet, 2016), thus reducing the burden on β-cells by averting excess insulin secretion (Alvarado *et al.*, 2016). The direction of the insulinaemic response provides evidence that MTCC may be a more tolerable intervention for individuals with dysfunctional glycaemic control and insulin resistance, such as type 2 diabetics. Moreover, it is possible that short-term improvements in insulin control with MTC interventions, may develop into long-term adaptations with consistent use.

5.4.2. Cardiovascular Responses

The most noteworthy cardiovascular response was the reduction in SBP at 2-hours post-bolus with MTCJ compared to PLA, in both Parts A and B. This finding was consistent with previous literature assessing SBP with cherry interventions (Kent *et al.*, 2015a; Keane *et al.*, 2016bc; Chai *et al.*, 2018). Keane *et al.* (2016bc) observed significantly lower SBP with MTCJ compared to PLA for the first 3-hours after ingestion, with peak reductions of 7 mmHg at 2-hours post-bolus (Keane *et al.*, 2016b) and 6 mmHg at 1-hour post-bolus (Keane *et al.*, 2016c). Similarly, the present study observed the greatest reduction in SBP (-8 mmHg) also at 2-hours post-bolus, suggesting MTCJ modulates SBP at this time point as it likely coincides

with parent anthocyanin and metabolite pharmacokinetics (Keane *et al.,* 2016b). Likewise, sweet cherry juice also had its greatest effect on SBP (-5.50 mmHg) 2-hours post-prandially, in old and young adults (Kent *et al.*, 2015b), with the authors attributing the improvement to the parent anthocyanins but not phenolic metabolites, as peak metabolite concentrations did not coincide with the time course of the observed effect. Interestingly, modulation of SBP with MTCC did not occur, despite administration of the same total anthocyanin content. Based on Keane *et al.* (2016b) explaining PCA and VA from MTCJ, likely exerted the dampened SBP response, it may be that these metabolites were not present at physiologically relevant concentrations within systemic circulation with MTCC, potentially due to the variable mechanics of capsule shell disintegration impeding anthocyanin metabolism. Since data from Part B seems not to indicate a dose-response effect for SBP, it is less surprising that the mean reduction in SBP across the 5-hour post-bolus period was greater in 4 of 6 individuals with MTCJ than MTCJ60.

Hypertension and isolated systolic hypertension increase CVD risk (Feresin *et al.*, 2017); however, mean SBP reductions of at least 5-6 mmHg over 5 years has been linked with a lower risk of coronary heart disease and stroke by 20-25% and 35-40%, respectively (Collins *et al.*, 1990). Bundy *et al.* (2017) also reported a reduction in SBP >5 mmHg was clinically relevant and associated with lower CVD/mortality risk. Thus, the magnitude of change with MTCJ indicates clinically relevant reductions in SBP; mean difference between MTCJ and PLA at 2-hours post-bolus of -11 mmHg. Consequently, this emphasises the highly potent hypotensive properties of MTCJ and the effect of a lower MTCJ dosage compared to Keane *et al.* (2016b). This finding is of high importance as approved anti-hypertensive drugs, with associated harmful side effects, lower SBP by a comparable magnitude (Bramlage and Hasford, 2009) as MTCJ, which currently has no known side effects. A limitation of examining acute cardio-metabolic responses is low ecological validity and biological significance of the results

(Rodriguez-Mateos *et al.*, 2013). However, long-term studies supplementing tart (Keane *et al.*, 2016c) and sweet (Kent *et al.*, 2015a) cherries have also been shown to lower SBP, although only in subjects presenting elevated baseline SBP.

The present study demonstrated individual responses for arterial stiffness were improved only at 2-hours post-bolus with MTCJ compared to PLA. However, pulse pressure, AP and TPR as indicators of vascular stiffness were not significantly improved with MTCJ. Furthermore, AIx or AP were not significantly correlated to SBP at 2-hours post-bolus with MTCJ; modulation of arterial stiffness does not explain the reduction in SBP at that time point. These findings align with previous research pertaining to supplementation of MTCJ on arterial stiffness (Lynn *et al.*, 2014; Keane *et al.*, 2016b; Johnson *et al.*, 2017). The rapid effect of AIx and AP improvement with MTCJ60 may be explained by its pharmacokinetics, where Keane *et al.* (2016a) showed significantly greater serum PCA and VA concentrations 1-hour post-bolus of 60 mL MTCJ compared to PLA.

A chemical mechanism involving NO and/or ACE inhibition may explain BP responses. Keane *et al.* (2016b) were unable to demonstrate augmented plasma nitrate/nitrite concentrations after 60 mL MTCJ consumption, rendering NO-mediated vasodilation less likely. Conversely, Kirakosyan *et al.* (2018) demonstrated MTC extract to inhibit ACE, potentially explaining reductions in SBP (MTCJ), aortic SBP and narrower pulse pressure (both with MTCJ60) from the present study; future work should assess ACE inhibition in humans. However, concomitant stimulation of eNOS and ACE inhibition should not be ruled out. Aortic SBP can be used for assessing the effectiveness of anti-hypertensive treatments with respect to cardiovascular risk factors (McEniery *et al.*, 2014), supporting MTCJ60 as a potentially safe, yet effective anti-hypertensive intervention.

MTCC induced vasodilation may have facilitated greater venous return thus explaining HR, SV and CO responses at 30 minutes post-bolus. A similar mechanism was provided by Cook *et al.* (2017a) in response to acute, encapsulated NZBE supplementation. The observed response with MTCC was remarkable, as disintegration of the capsule shell requires 30 minutes (Lown *et al.*, 2017), indicating an immediate response after dissolution. Subsequently, provision of MTC in liquid form (MTCJ) may facilitate faster absorption of phytochemicals (McGhie and Walton, 2007; Bohn, 2014) than MTCC. However, the lack of response with MTCJ would suggest either extensive degradation by salivary amylase (Kamonpatana, 2012) and/or a delayed uptake of secondary metabolites and phase II conjugates (Fernandes *et al.*, 2014). This delayed uptake of metabolites may be the rate-limiting step.

In agreement with responses observed in participants with MetS during the present investigation, Johnson *et al.* (2017) reported 12 weeks MTCJ consumption tended to lower total cholesterol but had no significant effect on other lipids. Despite non-significant statistical differences, individual responses in Part B of the present study showed all 6 participants had lower mean post-bolus total cholesterol concentrations after 60 mL MTCJ consumption compared to 30 mL MTCJ. This suggests total cholesterol may be more sensitive to improvement at higher phytochemical concentrations.

5.4.3. Limitations and Future Work

As with all research, certain limitations are apparent. Firstly, the artificial effect of the placebo on glycaemic and insulinaemic responses influenced the data and nullified comparisons against MTC interventions. This was an oversight on the part of the researcher, despite the initial intention to calorie match the placebo against MTCJ. Subsequent studies should match the placebo for relative percentages of macronutrients to total energy or utilise a water placebo if aiming to evaluate the intervention as a whole, including detrimental effects of juices such as sugars (Peluso and Palmery, 2014).

Secondly, as this was a pilot investigation the sample size was small limiting conclusions. However, this pilot investigation served its purpose in indicating null responses and potential benefits of MTCC, MTCJ and MTCJ60 consumption on cardio-metabolic markers in participants with MetS. This preliminary data serves to inform the design of future clinical trials with larger sample sizes to determine whether there is any clinically relevant benefit of MTC consumption.

Third, the phytochemical pharmacokinetics of MTCC was and remains unknown. Thus, there is a necessity to address this gap to enable better understanding of the data from this study and future work incorporating MTCC. Additionally, any advantageous responses observed with MTC interventions may be due to a residual synergistic effect of habitually consumed polyphenols, as this study aimed to uphold ecological validity by permitting habitual polyphenol intake. The beneficial responses observed with MTCJ and MTCJ60 compared to MTCC and PLA, may be a consequence of other nutrients, such as fibre which was shown to reduce SBP, total cholesterol and post-prandial glucose and insulin (Hodgson, 2004); aligning with findings from the present study. Although much attention has been given to the beneficial effects of anthocyanins and their metabolites, the synergistic influence of other phytonutrients within MTC require consideration.

Finally, acute cardio-metabolic responses were monitored during this study, however to enhance ecological validity and clinical relevance, data from longer-term responses in humans with MetS is required.

5.5. Conclusion

This research is the first to present data that acute administration of MTC interventions in capsule and juice form can modulate certain cardio-metabolic markers in humans with MetS. Further evidence is provided that MTCJ is an effective, low-risk intervention for lowering SBP

in various populations and should be considered for individuals with isolated systolic hypertension. Future work should consider monitoring 24-hour ambulatory BP responses to MTCJ. **Chapter 4** indicated a limited effect of MTCJ in healthy humans, therefore pathological populations should be studied. Despite recruiting humans with MetS in this study, the effects of MTC seemed to only apply to certain variables which were abnormal at baseline. The physiological responses to each MTC intervention differed depending on the cardio-metabolic marker examined, although there were no statistical differences between MTCC and MTCJ. A dose-response effect was not apparent, however individual responses suggested MTCJ60 may reduce atherosclerotic and cardiovascular risk. Consequently, recommendations for which MTC intervention is most suitable should be based on the cardio-metabolic marker that requires most attention, with consideration of the individuals' overall cardio-metabolic health.

Chapter 6

Effects of Short-Term Continuous Montmorency Tart Cherry Juice Supplementation in Participants with Metabolic Syndrome: a pilot study

6.1. Introduction

In keeping with the themes of this thesis, the present chapter examined the effects of 7 days continuous MTCJ supplementation in humans with MetS. **Chapter 5** highlighted for the first time that MTC interventions may exert beneficial effects on certain cardio-metabolic markers in humans with MetS. In acknowledgement of some of the limitations within **chapter 5**, this chapter aimed to assess cardio-metabolic function and potential mechanisms more conclusively in order to further understanding of these responses in this particular population.

The prevention of CVD and T2D would be a major step in retarding the current exponential rise in global prevalence and incidence rates (World Health Organisation, 2017; Cho *et al.*, 2018). MetS augments the incidence of CVD by 2-fold and T2D by 5-fold (Falkner and Cossrow, 2014). Dietary interventions to prevent and mitigate MetS are sought after as they can conveniently be implemented into an individual's lifestyle. Anthocyanins, a sub-class of polyphenols, and their metabolites possess potent anti-oxidative and anti-inflammatory properties and have been shown to improve MetS symptoms (Zhu *et al.*, 2018). Furthermore, *section 2.2.5.2.2.1* highlighted improvements in cardio-metabolic function after consumption of anthocyanin-rich interventions in humans with MetS.

Montmorency tart cherries are rich in phytochemicals including anthocyanins, however their bioefficacy may be attributed to downstream metabolites and synergisms with other nutrients within MTC, which may also induce health benefits (Keane *et al.*, 2016abc; Kirakosyan *et al.*, 2018). Insulin resistance is key to the underlying pathophysiology of MetS and anthocyanin-mediated improvements in insulin and glucose metabolism (Belwal *et al.*, 2017) can ameliorate many of its associated symptoms (Tsuda, 2008). Willems *et al.* (2017) reported 7 days continuous consumption of anthocyanin-rich NZBP likely increased insulin sensitivity in healthy individuals. Furthermore, **chapter 5** suggested the MTC interventions may have elicited improvements in insulin sensitivity and thus pancreatic β-cell function based on

glycaemic and insulinaemic responses observed amongst participants with MetS. Thus, to test this hypothesis the present study assessed surrogate markers of insulin sensitivity, insulin resistance and pancreatic β-cell function after short-term, continuous MTCJ supplementation.

Chapters 4 and **5** discussed provision of small anti-oxidant dosages, based on the concept of hormesis, may be more effective at inducing cellular, molecular and thus functional changes to improve health; as supported by Wallace (2011) and Wallace, Slavin and Frankenfeld (2016). Greater improvements of functional and blood-based cardio-metabolic biomarkers previously measured in **chapter 5** may be induced after multiple days' supplementation of a low anti-oxidant dosage. Thus, a 30 mL dose of MTC concentrate (270 mg anthocyanins) was provided for 7 days, as **chapter 5** did not indicate any further improvements in cardio-metabolic function with 60 mL MTC concentrate or MTCC. Additionally, the lower dosage is thought to retain more anthocyanins and secondary metabolites in systemic circulation for longer; maximising their bioactive properties (Seymour *et al.*, 2014). Lastly, the lower dosage is more economically viable for consumers, making this research more ecologically valid.

Centred around **chapter 5**, cardio-metabolic responses were also observed after an acute bolus of MTCJ, on the 7th day of supplementation, when bioavailability of MTC phytochemicals are thought to be greatest. Subsequently, this may explain the clinically-relevant reduction in SBP at 2-hours post-consumption of tart (Keane *et al.*, 2016b) and sweet (Kent *et al.*, 2015b) cherry juice, due to the pharmacokinetic profile of their anthocyanins and secondary metabolites. A caveat of recommending MTCJ as an intervention for managing hypertension is that greater clinical evidence is required which can be applied to normal daily-living conditions. This can be achieved through 24-hour ambulatory blood pressure monitoring (ABPM), instead of the lab-based BP measurements obtained by previous studies involving cherry interventions (Ataie-Jafari *et al.*, 2008; Kelley *et al.*, 2013; Lynn *et al.*, 2014; Kent *et al.*, 2015ab; Keane *et al.*, 2016bc; Johnson *et al.*, 2017; Chai *et al.*, 2018), which can be affected by phenomena such

as white coat syndrome (Hodgkinson *et al.*, 2011). Moreover, 24-hour ABPM is deemed to be the reference standard for accurate assessment of cardiovascular risk in adults (Hodgkinson *et al.*, 2011; Hermida *et al.*, 2015). Hence, this study measured 24-hour ABPM before and after 7 days of MTCJ supplementation, as Stote *et al.* (2017) demonstrated a trend towards reduced SBP after 7 days blueberry juice supplementation. Furthermore, **chapter 5** corroborated findings from previous literature of sweet (Kent *et al.*, 2015ab) and tart (Keane *et al.*, 2016a; Keane *et al.*, 2016b; Chai *et al.*, 2018) cherries' anti-hypertensive properties, specifically in participants with MetS; however, the mechanism of action has yet to be elucidated. Keane *et al.* (2016b) were unable to demonstrate increased NO bioavailability, whilst Lynn *et al.* (2014), Keane *et al.* (2016b) and data from **chapter 5** did not indicate significant modulation of arterial stiffness with MTCJ. Therefore, an alternative mechanism involving ACE inhibition was hypothesised in the present study, since Kirakosyan *et al.* (2018) observed 88.7% ACE inhibition *in vitro* with MTC extract.

The present study aimed to expand upon Johnson's *et al.* (2017) study by examining cardio-metabolic responses after short-term (7 days), continuous MTCJ supplementation and acute responses to a single-bolus, in humans with MetS. The study also aimed to provide data using clinically-relevant methodologies and explain mechanisms of action for observed responses with MTCJ. It was hypothesised that MTCJ would improve glycaemic and insulinaemic function through increasing insulin sensitivity. Moreover, MTCJ would maintain reductions in SBP 2-hours post-consumption and lower 24-hour SBP after 7 days supplementation, through ACE inhibition. Lastly, 6 days prior loading of MTCJ would improve acute cardio-metabolic responses more than a single, acute bolus.

6.2. Methods

6.2.1. Participants

Twelve (6 males and 6 females) participants with Metabolic Syndrome (Tables 6.1 and 6.2) volunteered for this pilot study. All participants were screened for MetS prior to formal inclusion onto the study according to the harmonised criteria outlined by Alberti *et al.* (2009), where 3 of the 5 qualifying criteria [Waist Circumference: ethnicity and sex specific criteria; Fasting Triglycerides: ≥1.69 mmol.L^{-1}; Fasting High-Density Lipoprotein: <1.03 mmol.L^{-1} (men), <1.29 mmol.L^{-1} (women); Blood Pressure: ≥130 mmHg SBP or ≥85 mmHg DBP; Fasting Glucose: ≥5.6 mmol.L^{-1}] had to be met. Ethical approval was obtained from the University of Hertfordshire Health, Science, Engineering and Technology Ethics Committee and informed consent was provided by all participants prior to enrolment. This study was registered as a clinical trial on clinicaltrials.gov (NCT03619941).

Refer to **chapter 5**, *section 5.2.1.1*, for further details regarding recruitment (Figure 6.1) and exclusion criteria.

Table 6.1. Selected baseline characteristics obtained during screening (n = 12).

Characteristics	Mean ± SD
Age (years)	50 ± 10
Stature (m)	1.73 ± 0.12
Body Mass (kg)	94.1 ± 23.1
BMI ($kg.m^{-2}$)	31 ± 7
Whole-body Fat (%)	39 ± 11
Trunk Fat (%)	32 ± 10
Fat Mass (kg)	41 ± 17
Fat Free Mass (kg)	55 ± 17
Fasting Total Cholesterol ($mmol.L^{-1}$)	4.17 ± 1.21
24-hour SBP (mmHg)	128 ± 10
24-hour DBP (mmHg)	77 ± 8
Fasting Insulin ($pmol.L^{-1}$)	118.99 ± 68.14
HOMA2-IR (AU)	2.2 ± 1.4
HOMA2-β (%)	137.9 ± 49.5
HOMA2-%S (%)	63.2 ± 40.5
ACE ($pg.mL^{-1}$)	8627 ± 8702

AU (Arbitrary Units); BMI (Body Mass Index); HOMA2-IR (Homeostatic Model Assessment of Insulin Resistance); HOMA2-β (Homeostatic Model Assessment of pancreatic β-cell function); HOMA2-%S (Homeostatic Model Assessment of Insulin Sensitivity).

Table 6.2. Individual baseline characteristics of MetS criteria obtained during screening (n = 12).

Characteristics	Mean ± SD	Participant											
		1	2	3	4	5	6	7	8	9	10	11	12
Waist Circumference (cm)	101.0 ± 19.3	102*	125*	80*	85	125.4*	119*	88*	82*	74.5	100*	104*	127*
Fasting Glucose ($mmol.L^{-1}$)	5.32 ± 1.04	6.15*	4.89	5.42	4.20	6.90*	6.38*	3.94	4.90	3.95	5.04	5.26	6.08*
Fasting Triglycerides ($mmol.L^{-1}$)	1.6 ± 0.3	1.7*	2.0*	1.2	1.8*	2.2*	1.3	1.7*	1.9*	1.8*	1.7*	1.1	1.3
Fasting HDL ($mmol.L^{-1}$)	1.41 ± 0.32	1.95	1.32	1.38	1.69	1.09	1.31	1.19*	1.58	1.88	1.52	1.01*	1.01*
SBP (mmHg)	135 ± 16	122	131*	131*	158*	149*	161*	104	131*	136*	135*	131*	125
DBP (mmHg)	77 ± 8	66	75	88*	91*	85*	70	75	70	85*	79	69	76

DBP (Diastolic Blood Pressure); HDL (High-density Lipoprotein); SBP (Systolic Blood Pressure).

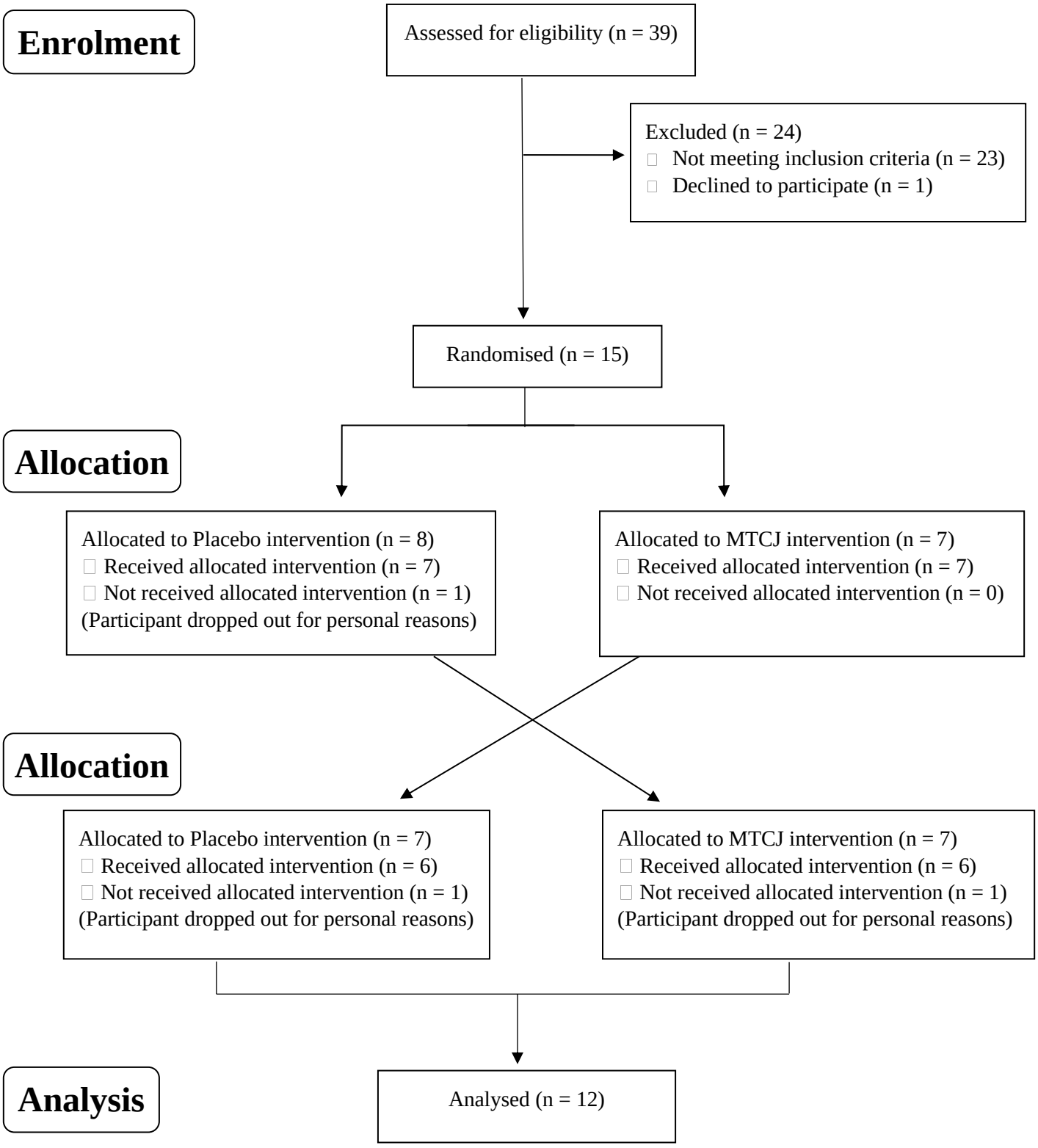


Figure 6.1. CONSORT flow diagram of the participants recruited, screened, tested, analysed and excluded during the course of the study.

6.2.2. Procedures

6.2.2.1. Research Design

A single-blind (blinded to participant), placebo-controlled, randomised, crossover design was utilised; each participant acted as their own control. During the 6-week study duration, participants completed both testing sessions during which placebo (PLA) or Montmorency Tart Cherry Juice (MTCJ) supplements were consumed for a continuous period of 7 days. As in **chapters 4** and **5**, a 14-day washout period (Howatson *et al.*, 2012; Cook *et al.*, 2015; Keane *et al.*, 2016b) was incorporated prior to crossover to the opposing condition.

Participants attended 4 testing sessions in total, in the laboratory. Schematics of the overall study design and specific procedures during a testing session are depicted in Figure 6.2. The first testing session lasted 1 hour; baseline anthropometric (stature, body mass, waist circumference, percentage body fat), functional (PWA, cardiac haemodynamics, RMR) and fasting blood-based biomarkers were measured. Participants were then provided with supplements to consume over the next 6 days at home, with the final dose on the 7th day being consumed in the laboratory during testing session two. Testing session two lasted 6 hours; anthropometric, functional and fasting blood-based biomarkers were measured prior to consumption of the supplement (6 days post-supplementation), after which functional measurements were recorded at 30 minutes, 1, 2, 3, 4- and 5-hours post-bolus. Blood sampling was performed at pre-bolus, 1, 3- and 5-hours post-bolus only. Testing session three was the same as session one, however participants were provided with the opposing supplement to which they consumed between sessions one and two. Lastly, session four was identical to session two.

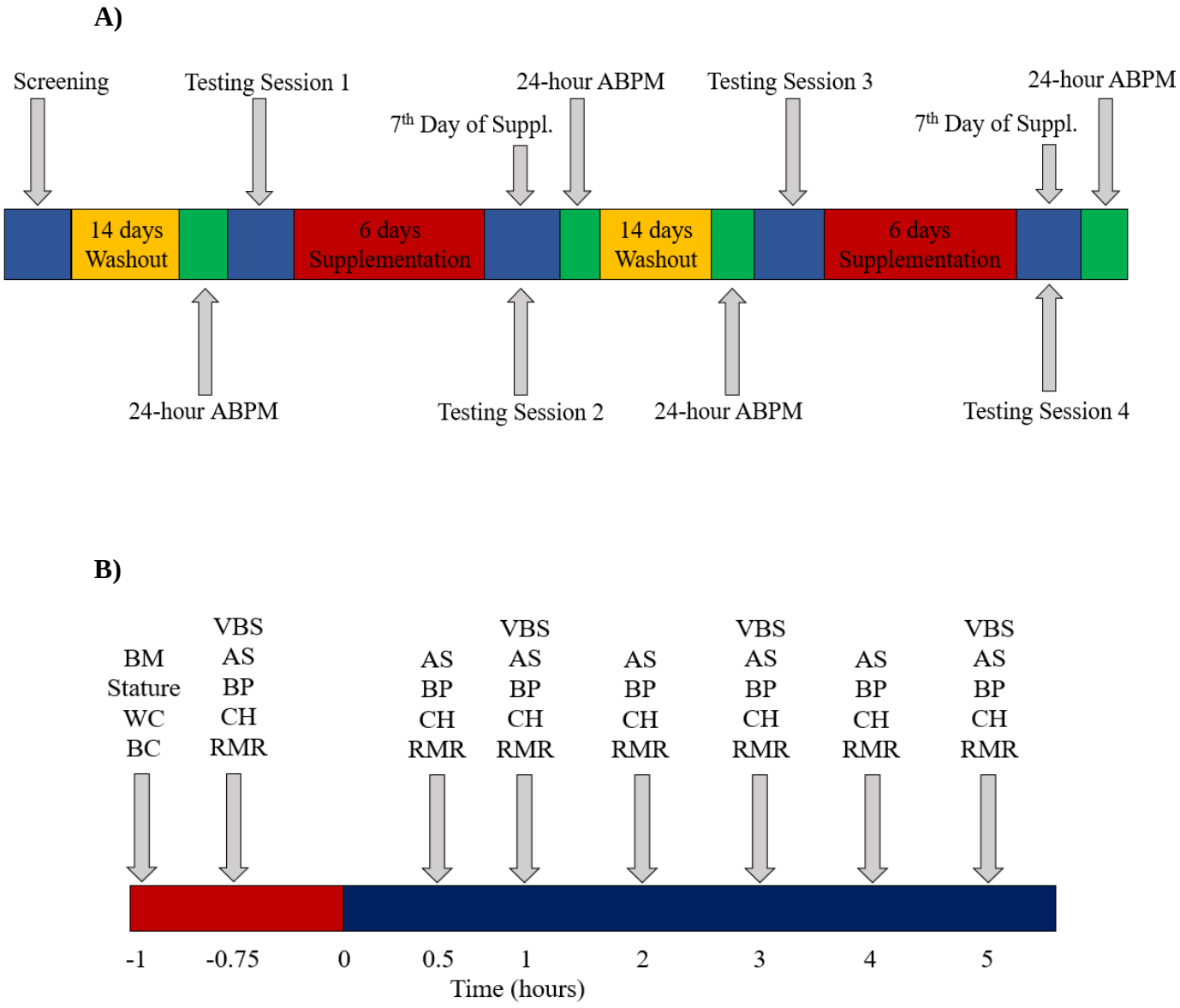


Figure 6.2. (A) Schematic representation of the overall study design. (B) Schematic representation of the specific procedures during each testing session. 'Suppl.' denotes supplementation.

6.2.2.2. Dietary Guidelines

Refer to general methodology *section 3.2* for further details on dietary guidelines employed in this study.

All participants complied with dietary guidelines upon analysis for percentage contributions of macronutrients to total energy intake [(protein 14 ± 22%), (CHO 48 ± 40%), (fat 38 ± 41%)], total polyphenols (62 ± 70 mg) and anthocyanins (20 ± 17 mg).

6.2.2.3. Supplementation

This study acutely administered two different supplements including a placebo which acted as the control condition and one experimental condition, MTCJ. MTCJ consisted of 30 mL Montmorency tart cherry concentrate (Cherry Active, Active Edge Ltd, Hanworth, UK) and 100 mL water. It was identified that MTC concentrate contained glucose (55.73% of total sugars) and fructose (44.27% of total sugars). The placebo was composed of 30 mL commercially available fruit-flavoured cordial (Cherries and Berries, Morrisons, Bradford, UK) mixed with 100 mL water. The placebo drink was matched against MTCJ for percentage contribution of carbohydrate to total energy and contribution of sugars to total carbohydrate, energy content, taste and visual appearance by adding dextrose (My Protein Ltd, Northwich, UK), fructose (Fruit Sugar, Morrisons, Bradford, UK), cornflour (Morrisons, Bradford, UK), citric acid (100% Pure Citric Acid, VB and Sons, UK), red and black food colouring (Morrisons, Bradford, UK). Nutritional analysis of both beverages is detailed in Table 6.3. Anonymity of the supplementation was ensured by blinding the participants to the source of anthocyanins. This was achieved by explaining that an ‘anthocyanin-rich supplement’ would be provided rather than disclosing Montmorency tart cherries as the specific source. Only 2/12 participants correctly distinguished the supplements provided (intervention or placebo) and none identified the intervention as ‘cherry’ or ‘containing cherries (either sweet or tart)’. Refer to *section 3.3* for more information on supplementation.

Table 6.3. Nutritional information of each supplement provided.

	Montmorency tart cherry concentrate (per 30 mL)	Placebo (per 130 mL)
Energy (kcal)	102	102
Carbohydrate (g)	24.5	25
of which sugars (g)	17.9	18
Glucose (g)	9.98	10.03
Fructose (g)	7.92	7.97
Protein (g)	1.1	0.5
Fat (g)	0	0
of which saturates (g)	0	0
Fibre (g)	2.6	Trace
Total Anthocyanins (mg)	270	0

6.2.2.4. Measures and Equipment

6.2.2.4.1. Anthropometrics

Refer to general methodology *section 3.5.1* for details on procedures of measuring stature, body mass and waist circumference.

Refer to general methodology *section 3.5.2* for details on procedures of measuring segmental body composition.

6.2.2.4.2. Cardiac Haemodynamics

Refer to general methodology *section 3.5.3.1* for details on procedures for obtaining cardiac haemodynamic measurements.

6.2.2.4.3. Blood Pressure

Refer to general methodology *section 3.5.3.2* for details on procedures for obtaining blood pressure measurements.

6.2.2.4.4. 24-hour Ambulatory Blood Pressure

In addition to clinic blood pressure measurements in the laboratory using an automated sphygmomanometer, 24-hour ambulatory blood pressure monitoring (ABPM) (Meditech ABPM-04, Meditech, Hungary) was also conducted through an oscillometric method. Barna, Keszei and Dunai (1998), have clinically validated the use of ABPM-04 monitors against British Hypertension Society guidelines. Moreover, ABPM-04 monitors have been used in interventional studies in participants with MetS (Johnston *et al.*, 2010). ABPM provides a more accurate representation of blood pressure as measurements are obtained under normal daily-living conditions, negating white coat syndrome; and mean pressures are taken from multiple readings over a 24-hour period, thus ABPM has become the reference standard for measuring BP non-invasively and diagnosing hypertension (Hodgkinson *et al.*, 2011).

Participants underwent a familiarisation period of wearing the ABPM device prior to data collection. A day before and after the 7 day supplementation period, participants were fitted with a 24-hour ABPM on the non-dominant arm (Drawz, Abdalla and Rahman, 2012). The ABPM was programmed (CardioVisions Software 1.15.2, Meditech, Hungary) to take readings every 30 minutes during the day (07:00-22:00) and every hour during the night (22:00-07:00) (Gupta *et al.*, 2008; Stull *et al.*, 2015). Participants were encouraged to follow their normal daily living pattern but maintain similar activities during each day of 24-hour ABPM measurement, and were advised to keep still and relax their arm whenever the monitor recorded measurements (Igwe *et al.*, 2017). A minimum of 14 day-time readings and 7 night-time readings were considered for a valid 24-hour ABPM (O'Brien *et al.*, 2003). Dietary consumption during 24-hour ABPM measurements coincided with the enforced 3-day dietary guidelines, thus participants were instructed to keep the same dietary intake on all 4 days of measurement.

The following data was obtained from the ABPM monitor: mean 24-hour, day-time and night-time SBP, DBP, MAP and PP (Fagard *et al.*, 2008). The difference between mean day and night SBP, DBP, MAP and PP was also calculated.

6.2.2.4.5. Pulse Wave Analysis

Refer to general methodology *section 3.5.3.3* for details on procedures for obtaining PWA measurements.

6.2.2.4.6. Resting Metabolic Rate

Refer to general methodology *section 3.5.4* for details on procedures for obtaining RMR measurements.

In addition to values for RER and EE ($kcal.day^{-1}$), resting fat and carbohydrate oxidations rates were also calculated at all time points.

6.2.2.5. Blood Sampling and Analysis

6.2.2.5.1. Blood Sampling

Refer to general methodology *section 3.6.1* for details on general blood sampling procedures.

Refer to **chapter 5**, *section 5.2.1.2.5.1* for specific details regarding blood sampling within this study.

6.2.2.5.2. Glucose

Refer to general methodology *section 3.6.2* for details on performing glucose analysis.

Overall intra-individual and inter-individual CV were 2.13% and 10.05%, respectively.

6.2.2.5.3. Insulin and Insulin Resistance and Sensitivity Indexes

Refer to general methodology *section 3.6.8* for details on performing insulin analysis.

Inter- and intra-plate CV were 6.1% and 5.5%, respectively.

Refer to general methodology *section 3.6.8.1* for details on insulin resistance and sensitivity analysis.

6.2.2.5.4. Triglycerides

Refer to general methodology *section 3.6.4* for details on performing triglyceride analysis. Overall intra-individual and inter-individual CV were 2.94% and 5.45%, respectively.

6.2.2.5.5. Total Cholesterol

Refer to general methodology *section 3.6.5* for details on performing total cholesterol analysis. Overall intra-individual and inter-individual CV were 4.45% and 5.63%, respectively.

6.2.2.5.6. HDL

Refer to general methodology *section 3.6.6* for details on performing HDL analysis. Overall intra-individual and inter-individual CV were 4.22% and 5.04%, respectively.

6.2.2.5.7. Total Cholesterol:HDL Ratio

Refer to general methodology *section 3.6.7* for details on performing TC:HDL analysis.

6.2.2.5.8. LDL

LDL was determined indirectly using the Friedewald formula (Friedewald, Levy and Fredrickson, 1972).

6.2.2.5.9. Angiotensin-I-Converting Enzyme Assay

Human ACE (CD 143) (peptidyl-dipeptidase A, EC 3.4.15.1) protein concentrations were measured according to manufacturer's guidelines, from serum samples in duplicates using a 96-well ELISA [Human ACE ELISA (CD 143) ab119577, Abcam, Cambridge, UK]; based on the 'sandwich immunoassay' principle.

An ACE specific mouse monoclonal antibody was pre-coated onto 96-well plates. A 7-point standard curve (Figure 6.3) was generated after serial dilution of lyophilised recombinant human ACE standard (50000 $pg.mL^{-1}$). Following serial dilution, 100 µL of standards (50000,

25000, 12500, 6250, 3125, 1562.5, 781.25 pg.mL^{-1} and blank control containing no protein) and serum samples were then added to wells and incubated for 90 minutes at 37ºC (Combi-H12 Hybridisation Incubator, FINEPCR, South Korea). A biotinylated detection polyclonal antibody from goat, specific for ACE was then added (100 µL) to each well and incubated for 60 minutes at 37ºC, followed by washing with phosphate buffered saline buffer (0.01 M, pH 7.4). Secondary enzyme-linked antibody, Avidin-Biotin-Peroxidase Complex (100 µL), was added to each well and incubated for 30 minutes at 37ºC, unbound conjugates were then washed away with phosphate buffered saline buffer. The colour developing agent, TMB, was then added (90 µL) to visualise the HRP enzymatic reaction and the plate incubated for 20 minutes at 37ºC in the dark. Catalysis of TMB by HRP produced a blue colour product. After incubation, acidic stop solution (100 µL) was added to each well, producing an immediate colour change from blue to yellow. The plate was then immediately read on a plate reader (Multiskan™ FC Microplate Photometer, Thermo Scientific, USA) at 450 nm to obtain absorbances of each well. ACE concentrations (pg.mL^{-1}) were then calculated using the standard curve according to Beer-Lambert's law. The intensity of yellow colouration was directly proportional to the amount of human ACE protein in the sample.

A linear standard curve was generated (Figure 6.3) using standard curve fitting software (SigmaPlot, Systat Software Inc, San Jose, USA). The kit inter-assay using a quality control and intra-assay precision, range and sensitivity were 2.1% (quality control), 7.5%, 780-50000 pg.mL^{-1} and <5 pg.mL^{-1}, respectively. Inter- and intra-plate precision were 12.3% and 9.2%, respectively. A common sample measured on each plate was used for determining inter-plate CV.

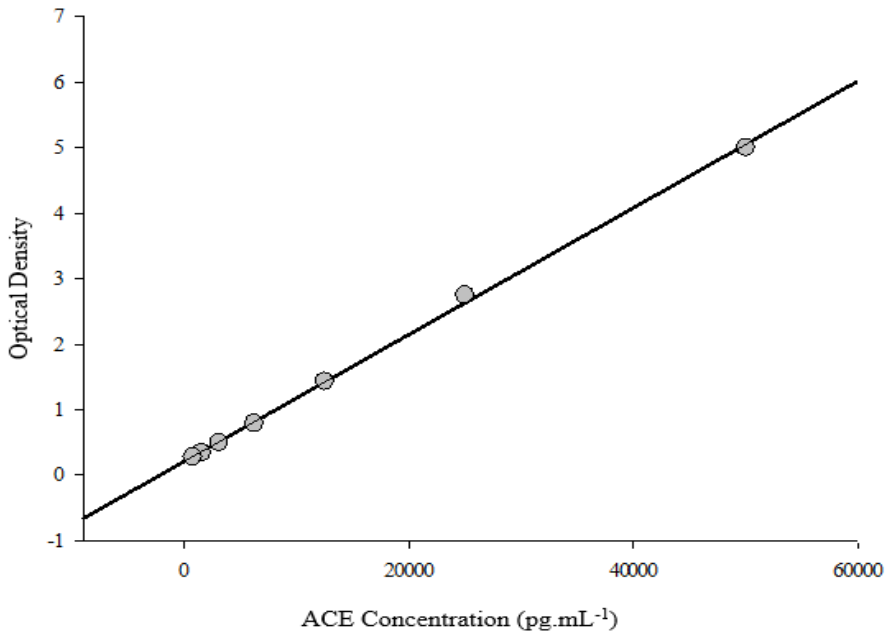


Figure 6.3. Representative seven-point standard curve used to determine ACE concentrations from serum samples.

6.2.3. Data Analysis

Statistical analysis was performed using SPSS v22.0 (IBM, Chicago, USA) where data are reported as mean ± standard deviation (±SD). Data normality was checked using a Shapiro-Wilk test. Greenhouse-Geisser correction was applied upon violation of Mauchly's test of sphericity for ANOVAs ($P < 0.05$). Statistical significance was set at $P < 0.05$. Based on data from **chapter 5** for the interaction effect between condition (PLA and MTCJ) and time for serum insulin, *a priori* power ($\alpha = 0.05$; $1\text{-}\beta = 0.8$) analysis indicated a sample size of 8 would be sufficient to detect a significant difference pre-post 6 days supplementation for serum insulin. Therefore, a minimum of 12 participants were recruited assuming a 20% dropout rate (Nogueira *et al.*, 2012).

A within-group two-way, 2 x 2, condition (PLA vs MTCJ) x time (Pre-Load and Post-Load), repeated-measures ANOVA design with *post-hoc* Bonferroni adjustment, measured differences for all blood-based biomarkers, cardiac haemodynamic, PWA, RMR and 24-hour BP parameters.

To account for day-to-day physiological variances at pre-bolus between conditions for each variable, data was analysed as change from pre-bolus for each time point measured post-bolus during testing sessions 2 and 4. This enabled a fair assessment of the post-bolus responses to each condition, from pre-bolus across all variables. The pre-bolus time point was not included as a covariate, as one-way ANOVA analysis indicated no significant differences ($P > 0.05$) between conditions for all variables at the pre-bolus time point, hence two-way repeated-measures ANOVA was performed. A within-group two-way, 2 x 6, condition (PLA vs MTCJ) x time (30 minutes, 1, 2, 3, 4- and 5-hours post-bolus), repeated-measures ANOVA design with *post-hoc* Bonferroni adjustment, measured differences of cardiac haemodynamic, PWA and RMR parameters on change from pre-bolus values. Blood-based biomarkers were analysed using the same model but with a 2 x 3, condition (PLA vs MTCJ) by time (1, 3- and 5-hours post-bolus) design on change from pre-bolus values for each condition.

Comparisons between acute responses observed after MTCJ consumption in **chapters 5** and **6** were conducted to determine any differential effects after 6 days of prior loading **(chapter 6)** compared to a single, acute bolus **(chapter 5)**. A 2 x 3, supplementation strategy (acute v 6 days loading) by time (1, 3- and 5-hours post-bolus) mixed-model ANOVA was performed on change from pre-bolus data for all blood-based variables except ACE. The same model was used for all cardiac haemodynamic, PWA and RMR parameters but with a 2 x 6, supplementation strategy (acute v 6 days loading) by time (30 minutes, 1, 2, 3, 4- and 5-hours post-bolus) design.

Partial Eta-Squared ($\eta^2_{partial}$) was used to report effect sizes for ANOVA where effects were classified as small (0.01-0.08), moderate (0.09-0.25) and large (>0.25) (Cohen, 1998). Cohen's *d* effect size was used for paired-samples *t*-test and *post-hoc* interaction comparisons where

effects were classified as no effect (0-0.1), small (0.2-0.4), moderate (0.5-0.7) and high (≥0.8) (Cohen, 1998).

Bivariate correlations were performed to check for relationships between ACE and all cardiac haemodynamic, PWA and 24-hour BP parameters on change from pre- to post-supplementation data for PLA and MTCJ. Bivariate correlations were also performed between the aforementioned variables on change from pre-bolus data for each post-bolus time point. Coefficients were classified as weak (0.1-0.2), moderate (0.3-0.5), strong (>0.5) (Cohen, 1988).

6.3. Results

Based on results from **chapter 5**, serum insulin was regarded as the primary endpoint for the present study. HOMA2-IR, acute SBP and 24-hour SBP were regarded as secondary variables. Tertiary endpoints focused on other aspects of MetS including hyperlipidaemia, hyperglycaemia and cardiovascular dysfunction.

Post-hoc power analysis indicated sufficient power (1-β = 0.99; α = 0.05; n = 12) to detect a significant main effect for the interaction between condition and time (pre-post 6 days supplementation) for insulin, HOMA2-IR and 24-hour SBP. Acute SBP also demonstrated sufficient power (1-β = 0.86; α = 0.05; n = 12) to detect a significant interaction effect.

6.3.1. Blood Biomarkers

6.3.1.1. Glucose

Responses to glucose pre and post 6 days supplementation (Figure 6.4) demonstrated a significant interaction effect ($F_{(1, 11)}$ = 5.534; P = 0.038, $\eta^2_{partial}$ = 0.34) and main effect for time ($F_{(1, 11)}$ = 8.077; P = 0.016, $\eta^2_{partial}$ = 0.42) only. *Post-hoc* indicated a significant difference between PLA and MTCJ at pre-supplementation time point (P = 0.023, d = 2.85). Fasting glucose was significantly ($t_{(11)}$ = 3.506; P = 0.005, d = 0.56) lower 6 days after supplementation

(5.40 ± 0.95 mmol.L^{-1}) compared to pre-supplementation (5.90 ± 0.86 mmol.L^{-1}) with MTCJ. Individual responses showed 10/12 individuals with lower fasting glucose after 6 days supplementation of MTCJ compared to PLA.

Mixed-model ANOVA demonstrated a tendency for the main effect of supplementation strategy ($F_{(1, 21)} = 4.289$; $P = 0.051$, $\eta^2_{partial} = 0.17$), where a single acute bolus induced a larger reduction in glucose from pre-bolus compared to 6 days prior loading. Individual responses showed 8/11 participants with lower glucose concentrations for the mean post-bolus change from baseline after a single bolus of MTCJ. In comparison, 4/12 participants responded in this manner after 6 days consumption of MTCJ. Moreover, at 1-hour post-bolus glucose concentrations after 6 days MTCJ consumption increased in 11/12 participants, compared to 4/11 participants after acute MTCJ supplementation (Figure 6.5).

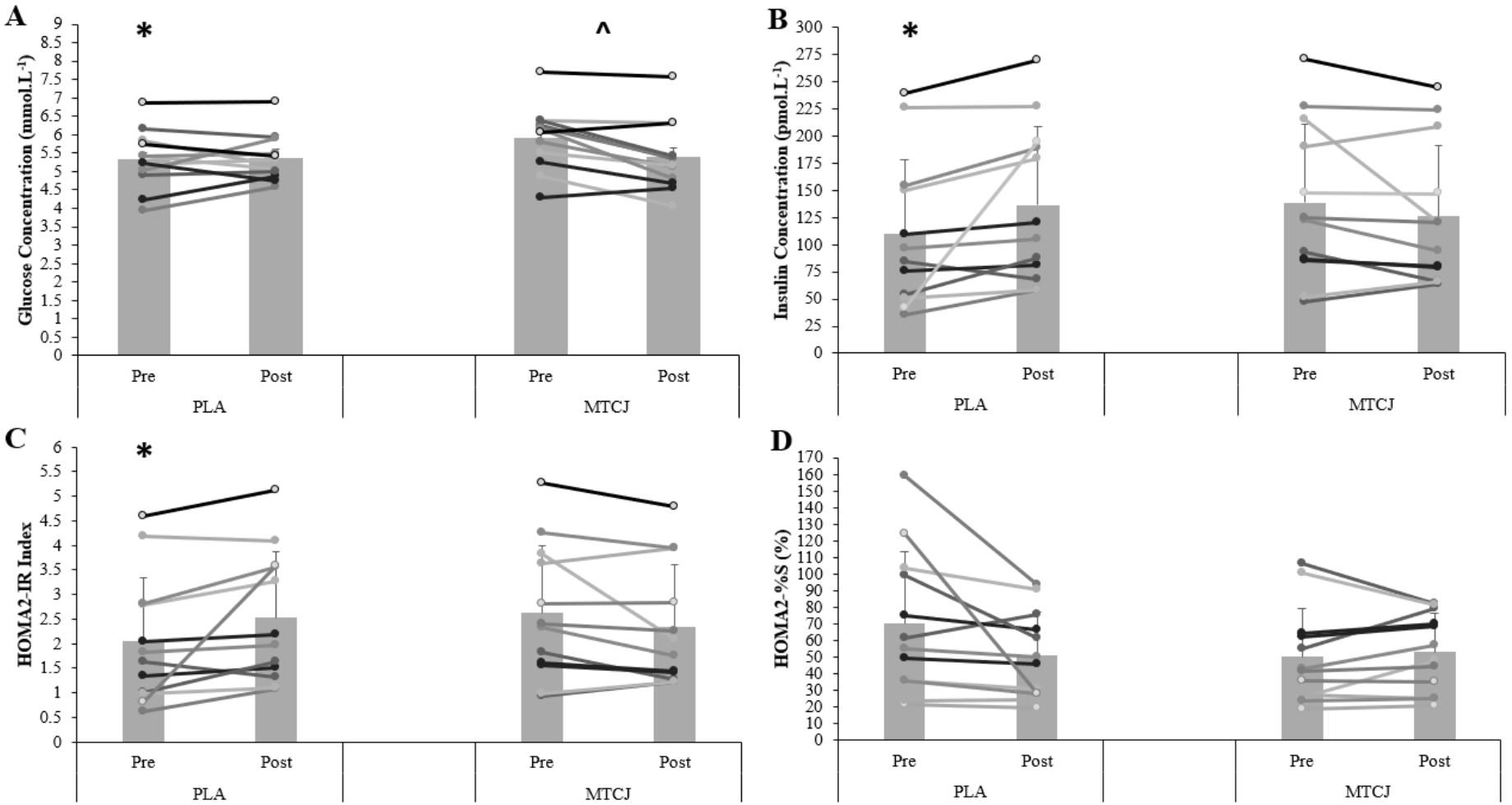


Figure 6.4. (A) Glucose, (B) Insulin, (C) HOMA2-IR and (D) HOMA2-%S responses before and after supplementation of PLA and MTCJ. Bar graphs depict mean (±SD) group values for each condition, pre and post 6 days supplementation. Lines depict individual responses for all 12 participants. *Denotes significant difference between conditions at respective time point. ^Denotes significant difference between pre- and post-supplementation time points for MTCJ. Statistical analysis performed by a two-way, RM-ANOVA with *post-hoc* Bonferroni adjustment ($P < 0.05$).

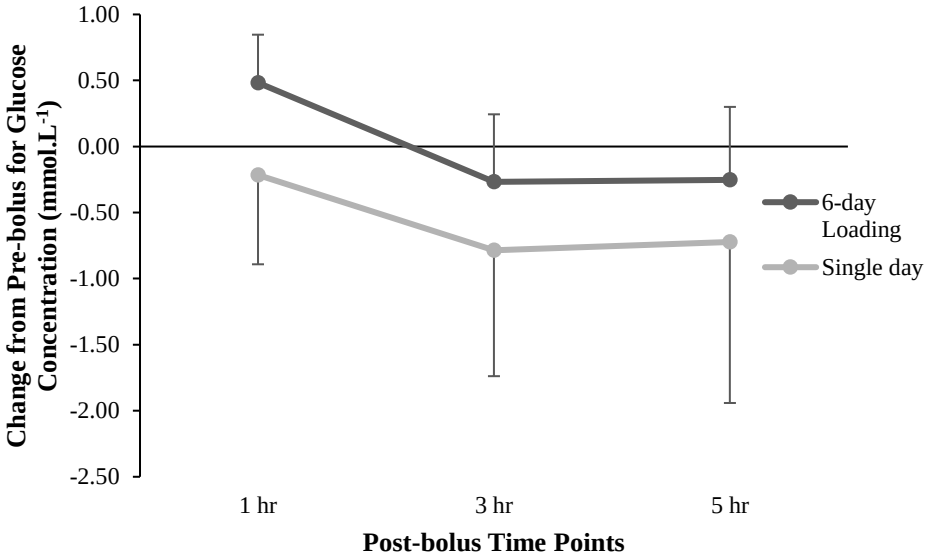


Figure 6.5. Mean (±SD) change from pre-bolus responses for glucose after MTCJ supplementation with 6 days prior loading compared to a single, acute bolus.

6.3.1.2. Insulin

Insulin responses to 6 days supplementation (Figure 6.4) only showed a significant interaction effect ($F_{(1, 11)} = 7.293$; $P = 0.021$, $\eta^2_{partial} = 0.40$). Pairwise comparisons indicated a significant difference between PLA and MTCJ at pre-supplementation time point ($P = 0.049$, $d = 0.41$). However, physiological responses showed after 6 days the mean change pre- to post-supplementation was 27.05 ± 42.42 pmol.L^{-1} with PLA and -12.42 ± 30.50 pmol.L^{-1} with MTCJ. Moreover, individual responses showed 10/12 individuals with lower fasting insulin after 6 days supplementation of MTCJ compared to PLA, indicating a tendency for lower fasting insulin after MTCJ consumption compared to PLA.

Similar to glucose, serum insulin only demonstrated a main effect of time ($F_{(2, 22)} = 16.828$; $P < 0.001$, $\eta^2_{partial} = 0.61$) on change from pre-bolus data (Table 6.4). *Post-hoc* showed insulin to be significantly higher at 1-hour post-bolus compared to both 3-hours ($P = 0.005$, $d = 1.27$) and 5-hours post-bolus ($P = 0.004$, $d = 1.34$). Insulin responses were not significantly different between MTCJ supplementation strategies or the strategy by time interaction (mixed-model

ANOVA) ($P > 0.05$), however there was a main effect for time ($F_{(2, 21)} = 26.151$; $P < 0.001$, $\eta^2_{partial} = 0.56$) (Appendix 8). *Post-hoc* showed insulin to be significantly higher at 1-hour post-bolus compared to both 3-hours ($P < 0.001$, $d = 1.56$) and 5-hours post-bolus ($P < 0.001$, $d = 1.42$).

Table 6.4. Mean ± SD change from pre-bolus values to post-bolus time points for selected blood-based biomarkers per treatment condition.

		Post-bolus Time Points		
		1 hr	3 hr	5 hr
Glucose* (mmol.L^{-1})	PLA	0.47 ± 0.96	-0.09 ± 0.60	0.12 ± 0.66
	MTCJ	0.48 ± 0.36	-0.27 ± 0.51	-0.25 ± 0.55
Insulin* (pmol.L^{-1})	PLA	9.70 ± 44.24	-32.34 ± 37.38	-38.82 ± 41.38
	MTCJ	27.26 ± 42.20	-28.53 ± 29.30	-32.20 ± 32.60
Triglycerides* (mmol.L^{-1})	PLA	0.2 ± 0.2	0.3 ± 0.3	0.4 ± 0.4
	MTCJ	0.0 ± 0.2	0.2 ± 0.1	0.3 ± 0.2
HDL (mmol.L^{-1})	PLA	0.00 ± 0.05	-0.02 ± 0.08	-0.03 ± 0.08
	MTCJ	-0.01 ± 0.05	0.00 ± 0.04	-0.04 ± 0.07
LDL (mmol.L^{-1})	PLA	0.34 ± 0.41	0.50 ± 0.53	0.65 ± 0.53
	MTCJ	0.06 ± 0.49	0.20 ± 0.29	0.12 ± 0.39
TC:HDL (AU)	PLA	-0.01 ± 0.10	0.13 ± 0.35	0.17 ± 0.44
	MTCJ	0.08 ± 0.40	-0.06 ± 0.28	-0.07 ± 0.44
ACE (pg.mL^{-1})	PLA	447 ± 4048	581 ± 1801	376 ± 1776
	MTCJ	420 ± 1929	332 ± 2325	22 ± 3250

ACE (Angiotensin-I-converting Enzyme); AU (Arbitrary Units); HDL (High-density Lipoprotein); HOMA2-IR (Homeostatic Model Assessment of Insulin Resistance); HOMA2-β (Homeostatic Model Assessment of pancreatic β-cell function); HOMA2-%S (Homeostatic Model Assessment of Insulin Sensitivity); LDL (Low-density Lipoprotein); MTCJ (Montmorency Tart Cherry Juice); PLA (Placebo); TC (Total Cholesterol). *Denotes significant main effect for time with *post-hoc* identifying differences between 1-hour and both 3-hours and 5-hours post-bolus.

6.3.1.3. HOMA2

As with insulin, HOMA2-IR ($F_{(1, 11)} = 8.115$; $P = 0.016$, $\eta^2_{partial} = 0.43$) and HOMA2-%S ($F_{(1, 11)} = 6.332$; $P = 0.029$, $\eta^2_{partial} = 0.37$) demonstrated a significant interaction after 6 days supplementation (Figure 6.4), with pairwise comparisons showing a significant difference

between PLA and MTCJ at pre-supplementation time point for HOMA2-IR (P = 0.039, d = 0.43). Individual responses for HOMA2-IR showed insulin resistance increased in 10/12 participants with PLA, whereas insulin resistance was reduced in 8/12 participants after 6 days MTCJ consumption. Moreover, HOMA2-%S showed 9/12 participants increased insulin sensitivity with 6 days continuous MTCJ consumption; 2/12 increased with PLA.

A main effect for time ($F_{(1, 11)}$ = 7.720; P = 0.018, $\eta^2_{partial}$ = 0.41) only was observed for HOMA2-β with pre-post 6 days supplementation data (Table 6.5). *Post-hoc* analysis highlighted greater pancreatic β-cell function post-supplementation compared to pre-supplementation (P = 0.018, d = 0.37). No significant main effects were found on change from pre-bolus data for HOMA2-β (P > 0.05).

Table 6.5. Mean ± SD responses for selected blood-based biomarkers before and after 6 days supplementation of PLA and MTCJ.

		Pre-Supplementation	Post-Supplementation
HOMA2-β* (%)	PLA	131 ± 43	152 ± 49
	MTCJ	132 ± 53	148 ± 56
Triglycerides ($mmol.L^{-1}$)	PLA	1.2 ± 0.1	1.3 ± 0.1
	MTCJ	1.4 ± 0.1	1.3 ± 0.1
ACE ($pg.mL^{-1}$)	PLA	8706 ± 8748	9334 ± 10363
	MTCJ	10161 ± 11474	9127 ± 10915

ACE (Angiotensin-converting Enzyme); HOMA2-β (Homeostatic Model Assessment of pancreatic β-cell function); MTCJ (Montmorency Tart Cherry Juice); PLA (Placebo). *Denotes significant main effect for time.

6.3.1.4. Lipids

Reponses to total cholesterol after 6 days supplementation showed significant main effects for time ($F_{(1, 11)}$ = 5.097; P = 0.045, $\eta^2_{partial}$ = 0.32) and interaction ($F_{(1, 11)}$ = 5.700; P = 0.036, $\eta^2_{partial}$ = 0.34) (Figure 6.6). *Post-hoc* identified a significant difference between PLA and MTCJ at pre-supplementation (P = 0.030, d = 2.14). Total cholesterol was significantly

($t_{(11)}$ = 3.724; P = 0.003, d = 0.39) lower 6 days after supplementation (4.1 ± 1.0 mmol.L^{-1}) compared to pre-supplementation (4.5 ± 1.0 mmol.L^{-1}) with MTCJ.

A significant interaction effect was observed for HDL ($F_{(1, 11)}$ = 5.212; P = 0.043, $\eta^2_{partial}$ = 0.32) and LDL ($F_{(1, 11)}$ = 7.004; P = 0.023, $\eta^2_{partial}$ = 0.39) after 6 days supplementation (Figure 6.6). A significant difference between PLA and MTCJ was found at post-supplementation time point for HDL (P = 0.049, d = 0.42), while pre-supplementation was significantly different for LDL (P = 0.031, d = 0.42). LDL was significantly ($t_{(11)}$ = 3.681; P = 0.004, d = 0.21) lower 6 days after supplementation (2.71 ± 1.62 mmol.L^{-1}) compared to pre-supplementation (3.07 ± 1.69 mmol.L^{-1}) with MTCJ.

Individual responses showed 6/12 participants increased HDL concentrations after 6 days MTCJ supplementation, whereas 2/12 increased with PLA. Moreover, 10/12 participants reduced LDL concentrations after MTCJ supplementation, compared to 5/12 with PLA.

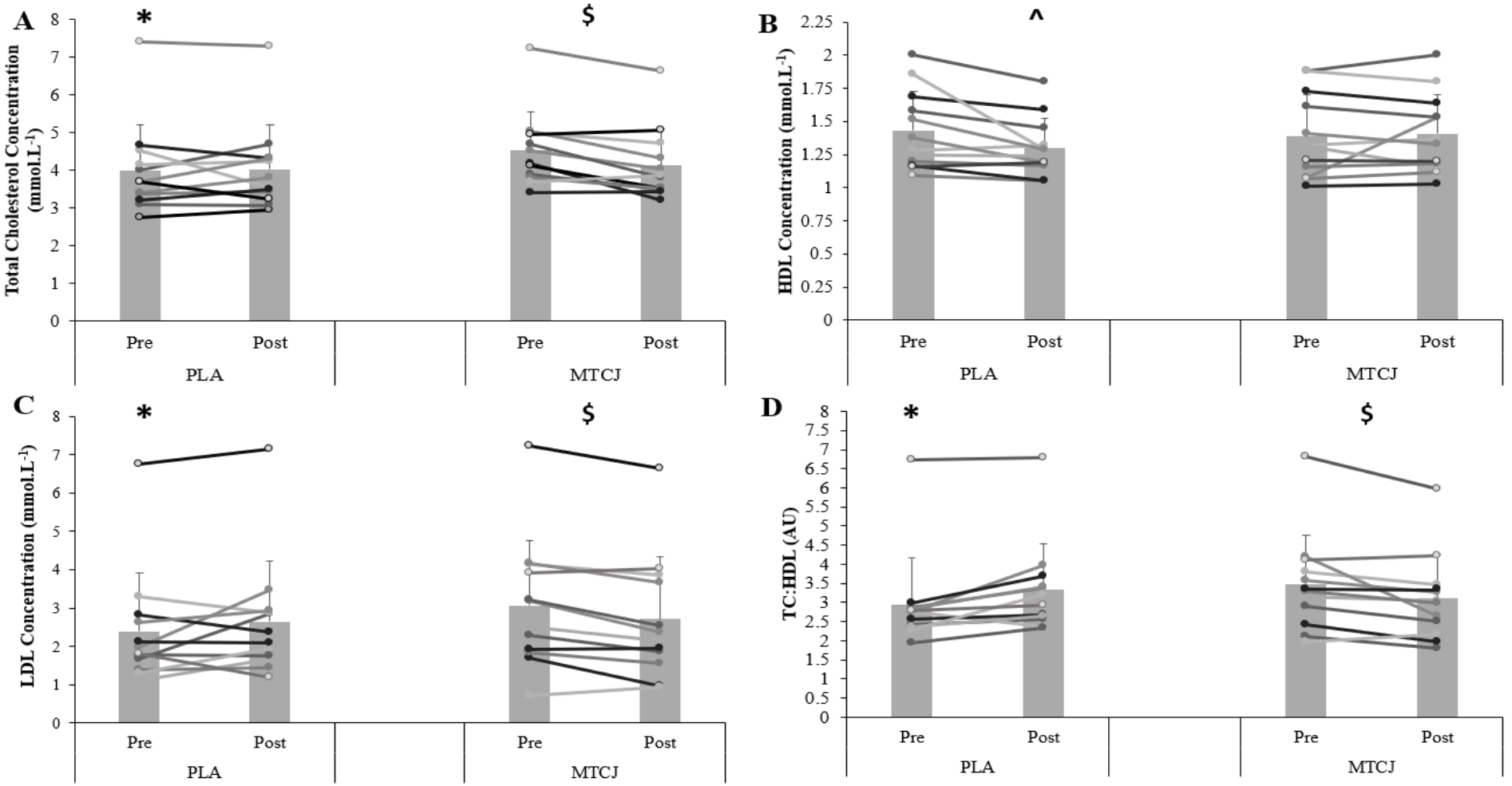


Figure 6.6. (A) Total cholesterol, (B) HDL, (C) LDL and (D) TC:HDL responses before and after supplementation of PLA and MTCJ. Bar graphs depict mean (±SD) group values for each condition, pre and post 6 days supplementation. Lines depict individual responses for all 12 participants. *Denotes significant difference between conditions at pre-supplementation time point. ^Denotes significant difference between conditions at post-supplementation time point. $^{\$}$Denotes significant difference between pre- and post-supplementation time points for MTCJ. Statistical analysis performed by a two-way, RM-ANOVA with *post-hoc* Bonferroni adjustment ($P < 0.05$).

A main effect for condition ($F_{(1, 11)} = 5.328$; $P = 0.041$, $\eta^2_{partial} = 0.33$) showed total cholesterol concentrations increased significantly more with PLA than MTCJ for the mean change from pre-bolus (Figure 6.7). Individual responses for the change from pre-bolus showed 10/12 participants with lower total cholesterol concentrations at 3-hours post-bolus after MTCJ consumption compared to PLA. Similarly, 7/12 participants responded with lower total cholesterol concentrations 5-hours post-bolus with MTCJ compared to PLA.

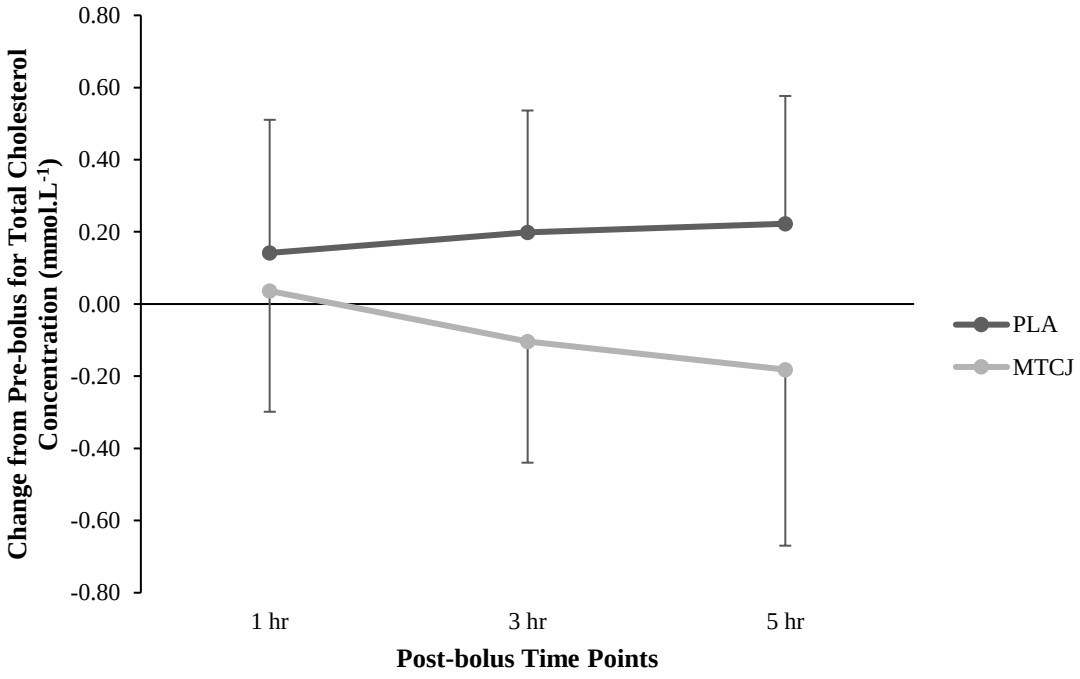


Figure 6.7. Mean (±SD) change in total cholesterol from pre-bolus for PLA and MTCJ.

Change from pre-bolus data indicated a significant main effect of condition ($F_{(1, 11)} = 5.874$; $P = 0.034$, $\eta^2_{partial} = 0.35$) and time ($F_{(2, 22)} = 4.110$; $P = 0.030$, $\eta^2_{partial} = 0.27$) for LDL, with higher LDL concentrations at pre-supplementation compared to post-supplementation ($P = 0.034$, $d = 0.08$) (Table 6.4). No main effects for condition, time or interaction were found for HDL or TC:HDL ratio with change from pre-bolus data ($P > 0.05$) (Table 6.4).

Mixed-model analysis showed the change from pre-bolus at 5-hours post-bolus tended to be significantly different (main interaction effect: $F_{(2, 42)} = 2.592$; $P = 0.087$, $\eta^2_{partial} = 0.11$), with

total cholesterol concentrations increasing after a single acute MTCJ bolus, whereas a decrease was observed after 6 days prior loading (Appendix 8). Individual responses showed 9/11 participants to have higher total cholesterol concentrations than baseline at 5-hours post-bolus after consumption of a single, acute bolus of MTCJ, compared to 5/11 participants after 6 days prior loading.

A main effect of time only was found for HDL with mixed-model analysis between acute and 6 days prior loading strategies ($F_{(2, 21)}$ = 4.117; P = 0.023, $\eta^2_{partial}$ = 0.16) (Appendix 8). *Post-hoc* comparisons showed HDL concentrations were significantly lower at 5-hours post-bolus compared to 3-hours post-bolus (P = 0.037, d = 0.70). No main effects for mixed-model analysis were found with LDL (P > 0.05) (Appendix 8).

After 6 days supplementation, an interaction effect was observed for TC:HDL ($F_{(1, 11)}$ = 13.681; P = 0.004, $\eta^2_{partial}$ = 0.55) (Figure 6.6). *Post-hoc* comparisons showed PLA to be significantly higher than MTCJ at pre-supplementation time point (P = 0.011, d = 2.67). TC:HDL ratio was significantly ($t_{(11)}$ = 2.690; P = 0.021, d = 0.30) lower 6 days after supplementation (3.47 ± 1.28 mmol.L^{-1}) compared to pre-supplementation (3.11 ± 1.13 mmol.L^{-1}) with MTCJ. Mixed-model analysis did not indicate any main effects for supplementation strategy, time or the interaction for TC:HDL (P > 0.05) (Appendix 8).

No significant interaction or main effects for condition and time were observed for triglycerides with pre-post 6 days supplementation data (P > 0.05) (Table 6.5). However, change from pre-bolus data showed a main effect of time ($F_{(2, 22)}$ = 11.649; P < 0.001, $\eta^2_{partial}$ = 0.51) and a tendency for a significant interaction ($F_{(2, 22)}$ = 3.148; P = 0.063, $\eta^2_{partial}$ = 0.22) (Table 6.4). *Post-hoc* comparisons for the main effect of time showed triglyceride concentrations increased

more at 3-hours (P = 0.020, d = 1.50) and 5-hours post-bolus (P = 0.010, d = 2.50) compared to 1-hour post-bolus.

A main effect for time only was found with mixed-model analysis for triglycerides ($F_{(2,\ 21)}$ = 5.901; P = 0.006, $\eta^2_{partial}$ = 0.22) (Appendix 8). *Post-hoc* analysis showed triglyceride concentrations increased more at 3-hours post-bolus compared to 1-hour post-bolus (P = 0.012, d = 0.91). Individual responses showed all 12 participants to have higher triglyceride concentrations than baseline at 3-hours post-bolus after 6 days prior loading of MTCJ, whereas only 7/11 participants responded in this manner after a single, acute bolus.

6.3.1.5. ACE

ACE did not show any main effects for condition, time or interaction with pre-post 6 days supplementation data (Table 6.5) or change from pre-bolus data (Table 6.4) (P > 0.05).

6.3.2. Cardiac Haemodynamics

No main effects for condition, time or interaction were detected for SBP, DBP, MAP, HR, SV, CO and TPR (P > 0.05) for pre-post 6 days supplementation data (Appendix 6). Despite there being no main effects for condition or time, a tendency towards significance was detected for the interaction ($F_{(5,\ 55)}$ = 2.128; P = 0.076, $\eta^2_{partial}$ = 0.16) with SBP. Individual responses for the change from pre-bolus to 2-hours post-bolus showed 4/12 participants decreased SBP with PLA (4 ± 13 mmHg), while 10/12 participants reduced with MTCJ (-7 ± 10 mmHg) (Figure 6.8).

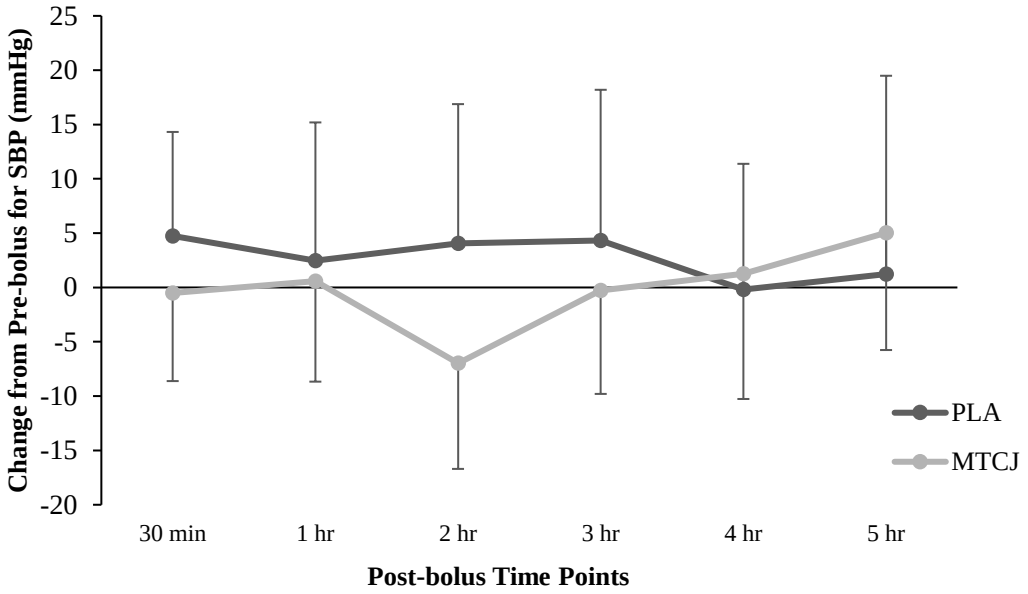


Figure 6.8. Mean (±SD) change in SBP from pre-bolus for PLA and MTCJ.

Mixed-model analysis indicated only a main effect for time with SBP ($F_{(5,\ 21)}$ = 5.469; P < 0.001, $\eta^2_{partial}$ = 0.21), DBP ($F_{(5,\ 21)}$ = 3.026; P = 0.014, $\eta^2_{partial}$ = 0.13), MAP ($F_{(5,\ 21)}$ = 3.802; P = 0.003, $\eta^2_{partial}$ = 0.15) and HR ($F_{(5,\ 21)}$ = 27.038; P < 0.001, $\eta^2_{partial}$ = 0.56) (Appendix 9).

There were no main effects for condition, time or interaction on change from pre-bolus data with DBP, MAP, CO and TPR (P > 0.05) (Appendix 6). Responses to CO, SV and TPR did not indicate significant differences for the main effect of supplementation strategy, time or interaction with mixed-model analysis (P < 0.05) (Appendix 9).

6.3.3. Pulse Wave Analysis

No significant main effects for condition, time or interaction were detected for aortic SBP, aortic DBP, AP, pulse pressure, AIx, AIx at HR75 and SEVR (P > 0.05) for pre-post 6 days supplementation data (Appendix 6).

No main effects for condition, time or interaction (P > 0.05) were found for aortic SBP. However, individual responses for the change from pre-bolus to 2-hours post-bolus showed 4/12 participants decreased aortic SBP with PLA (3 ± 9 mmHg), while 9/12 participants responded with lower aortic SBP after consuming MTCJ (-4 ± 8 mmHg) (Figure 6.9).

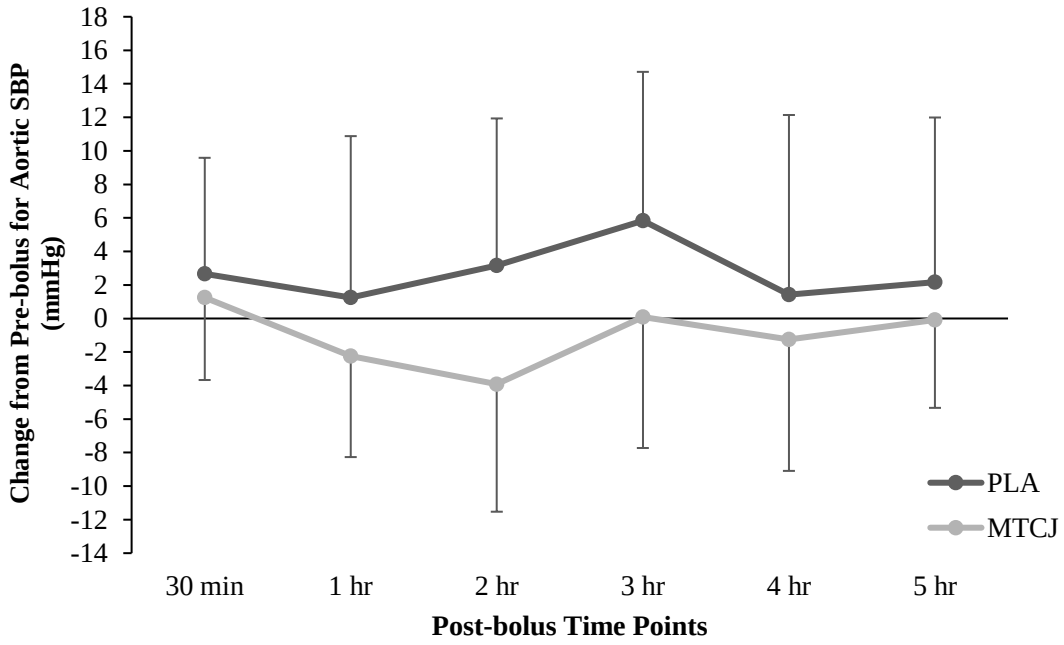


Figure 6.9. Mean (±SD) change in aortic SBP from pre-bolus for PLA and MTCJ.

Main effects for time with mixed-model analyses were found for AIx ($F_{(5, 21)}$ = 5.885; P < 0.001, $\eta^2_{partial}$ = 0.22), AP ($F_{(5, 21)}$ = 8.238; P < 0.001, $\eta^2_{partial}$ = 0.28) and SEVR ($F_{(5, 21)}$ = 6.363; P = 0.001, $\eta^2_{partial}$ = 0.23) (Appendix 9).

There were no main effects for condition, time or interaction on change from pre-bolus data with aortic DBP and pulse pressure (P > 0.05) (Appendix 6). No main effects of supplementation strategy, time or interaction with mixed-model analysis were found for aortic DBP, pulse pressure or AIx at HR75 (P > 0.05) (Appendix 9).

6.3.4. Resting Metabolic Rate

No significant main effects for condition, time or the condition by time interaction were detected for resting energy expenditure after 6 days MTCJ consumption ($P > 0.05$).

Significant interaction effects were found for resting RER ($F_{(1, 11)} = 10.045$; $P = 0.009$, $\eta^2_{partial} = 0.48$), fat oxidation rate ($F_{(1, 11)} = 9.394$; $P = 0.011$, $\eta^2_{partial} = 0.46$) and carbohydrate oxidation rate ($F_{(1, 11)} = 5.644$; $P = 0.037$, $\eta^2_{partial} = 0.34$) between PLA and MTCJ after 6 days supplementation (Figure 6.10). *Post-hoc* identified a significant difference between conditions at pre-supplementation time point for fat ($P = 0.024$, $d = 0.68$) and carbohydrate ($P = 0.027$, $d = 0.81$) oxidation rates. RER was significantly ($t_{(11)} = 2.823$; $P = 0.017$, $d = 0.70$) lower 6 days after supplementation (0.83 ± 0.04) compared to pre-supplementation (0.86 ± 0.04) with MTCJ.

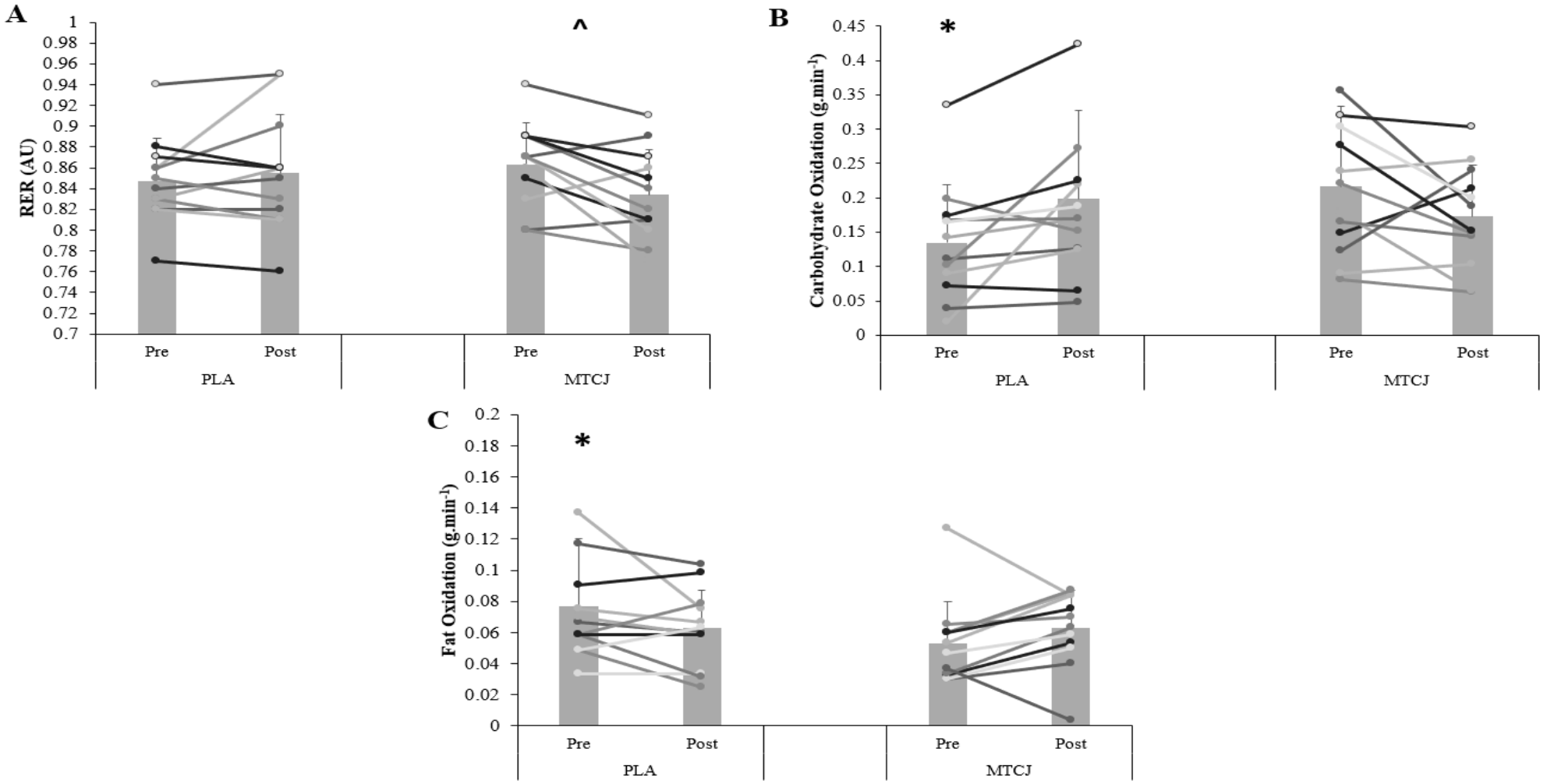


Figure 6.10. (A) Resting RER, (B) Carbohydrate Oxidation and (C) Fat Oxidation responses before and after supplementation of PLA and MTCJ. Bar graphs depict mean (±SD) group values for each condition, pre and post 6 days supplementation. Lines depict individual responses for all 12 participants. *Denotes significant difference between conditions at pre-supplementation time point. ^Denotes significant difference between pre- and post-supplementation time points for MTCJ. Statistical analysis performed by a two-way, RM-ANOVA with *post-hoc* Bonferroni adjustment ($P < 0.05$).

A significant main effect for time only was observed for resting energy expenditure ($F_{(5, 55)}$ = 2.788; P = 0.026, $\eta^2_{partial}$ = 0.20), RER ($F_{(5, 55)}$ = 43.536; P < 0.001, $\eta^2_{partial}$ = 0.80), fat ($F_{(5, 55)}$ = 14.183; P < 0.001, $\eta^2_{partial}$ = 0.56) and carbohydrate ($F_{(5, 55)}$ = 15.936; P < 0.001, $\eta^2_{partial}$ = 0.59) oxidation on change from pre-bolus data (Appendix 7).

Mixed-model analyses for resting RER ($F_{(5, 21)}$ = 37.794; P < 0.001, $\eta^2_{partial}$ = 0.64), fat ($F_{(5, 21)}$ = 17.798; P < 0.001, $\eta^2_{partial}$ = 0.46) and carbohydrate ($F_{(5, 21)}$ = 20.075; P < 0.001, $\eta^2_{partial}$ = 0.49) oxidation indicated significant main effects for time and supplementation strategy only (Appendix 9). No significant main effects for supplementation strategy, time or interaction were found for resting energy expenditure (P > 0.05) (Appendix 9).

6.3.5. 24-hour ABPM

A significant interaction ($F_{(1, 11)}$ = 9.941; P = 0.016, $\eta^2_{partial}$ = 0.59) and main effect for condition ($F_{(1, 11)}$ = 7.916; P = 0.026, $\eta^2_{partial}$ = 0.53) was observed for mean 24-hour SBP after 7 days of supplementation (Figure 6.11). A significant difference between PLA and MTCJ was identified with *post-hoc* analysis at the post-supplementation time point (P = 0.024, d = 0.44). Individual responses showed 11/12 participants reduced 24-hour SBP after 7 days MTCJ supplementation, compared to 2/12 with PLA.

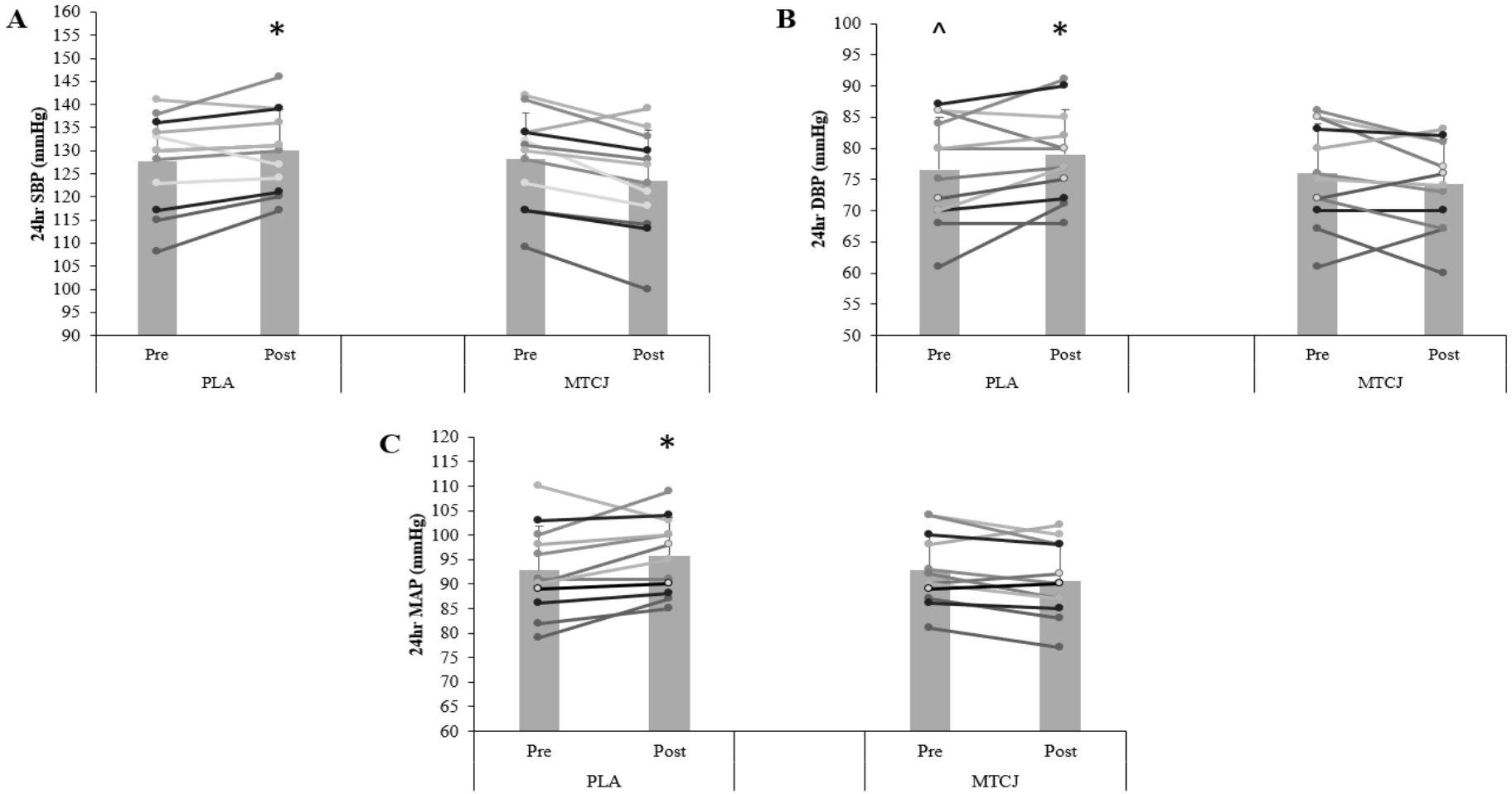


Figure 6.11. (A) Mean 24-hour SBP, (B) Mean 24-hour DBP and (C) Mean 24-hour MAP responses before and after supplementation of PLA and MTCJ. Bar graphs depict mean (±SD) group values for each condition, pre and post 7 days supplementation. Lines depict individual responses for all 12 participants. ^Denotes significant difference between conditions at pre-supplementation time point. *Denotes significant difference between conditions at post-supplementation time point. Statistical analysis performed by a two-way, RM-ANOVA with *post-hoc* Bonferroni adjustment ($P < 0.05$).

Likewise, mean 24-hour DBP only showed significant main effects for condition ($F_{(1, 11)}$ = 12.321; P = 0.010, $\eta^2_{partial}$ = 0.64) and interaction ($F_{(1, 11)}$ = 12.789; P = 0.009, $\eta^2_{partial}$ = 0.65) (Figure 6.11). At pre-supplementation (P = 0.049, d = 0.14) and post-supplementation (P = 0.008, d = 0.66) time points, *post-hoc* comparisons demonstrated a significant difference between PLA and MTCJ. Individual responses showed 10/12 participants reduced mean 24-hour DBP after 7 days MTCJ supplementation, compared to 2/12 with PLA.

A significant interaction ($F_{(1, 11)}$ = 6.236; P = 0.041, $\eta^2_{partial}$ = 0.47) and tendency towards significance for the main effect of condition ($F_{(1, 11)}$ = 5.122; P = 0.058, $\eta^2_{partial}$ = 0.42) was detected for mean 24-hour MAP (Figure 6.11). *Post-hoc* analysis indicated PLA was significantly higher than MTCJ at the post-supplementation time point (P = 0.010, d = 0.58). Individual responses showed 9/12 participants reduced mean 24-hour MAP after 7 days MTCJ supplementation, compared to 1/12 with PLA.

A tendency towards a significant interaction effect ($F_{(1, 11)}$ = 3.995; P = 0.086, $\eta^2_{partial}$ = 0.36) was found for mean 24-hour pulse pressure. No main effects for time or condition were detected (P > 0.05).

Mean day-time SBP demonstrated a tendency towards significance for the interaction ($F_{(1, 11)}$ = 4.499; P = 0.072, $\eta^2_{partial}$ = 0.39) and a significant main effect for condition only ($F_{(1, 11)}$ = 6.507; P = 0.038, $\eta^2_{partial}$ = 0.48) (Table 6.6). Three of 12 participants were found to have lower mean day-time SBP with PLA after 7 days supplementation, whilst 11/12 participants were found to have lower mean day-time SBP with MTCJ.

Significant main interaction ($F_{(1, 11)}$ = 5.725; P = 0.048, $\eta^2_{partial}$ = 0.45) and condition ($F_{(1, 11)}$ = 5.876; P = 0.046, $\eta^2_{partial}$ = 0.46) effects were observed for mean day-time DBP (Table 6.6). Pairwise comparisons for the interaction effect showed significantly higher mean day-time DBP with PLA compared to MTCJ at post-supplementation time point (P = 0.020, d = 0.67). Individual responses showed 8/12 participants had lower mean day-time DBP after 7 days MTCJ supplementation, compared to 2/12 with PLA.

There were no significant main effects for condition, time or interaction with mean day-time MAP (P > 0.05) (Table 6.6). A main effect for time was observed for mean day-time pulse pressure ($F_{(1, 11)}$ = 13.661; P = 0.008, $\eta^2_{partial}$ = 0.66), with *post-hoc* showing pre-supplementation to be higher than post-supplementation (P = 0.008, d = 0.42) (Table 6.6). Analysis on night-time SBP, DBP, MAP and pulse pressure demonstrated no significant main effects for condition, time or interaction (P > 0.05) (Table 6.6).

The day-night difference for SBP ($F_{(1, 11)}$ = 7.355; P = 0.030, $\eta^2_{partial}$ = 0.51) and pulse pressure ($F_{(1, 11)}$ = 7.199; P = 0.031, $\eta^2_{partial}$ = 0.51) only showed a significant main effect for condition, where PLA was larger than MTCJ (Table 6.6). Day-night differences for DBP and MAP indicated no significant main effects for condition, time or interaction (P > 0.05) (Table 6.6).

Table 6.6. Mean ± SD day-time, night-time and day-night differences from 24-hour ABPM responses before and after 7 days supplementation of PLA and MTCJ.

		Pre-Supplementation	Post-Supplementation
Day SBP* (mmHg)	PLA	132 ± 8	133 ± 8
	MTCJ	132 ± 9	127 ± 11
Day DBP$ (mmHg)	PLA	79 ± 7	81 ± 7^
	MTCJ	79 ± 6	76 ± 6
Day MAP (mmHg)	PLA	92 ± 8	97 ± 7
	MTCJ	92 ± 10	94 ± 7
Day PP§ (mmHg)	PLA	53 ± 6	51 ± 5
	MTCJ	53 ± 5	50 ± 8
Night SBP (mmHg)	PLA	113 ± 13	117 ± 7
	MTCJ	117 ± 12	117 ± 13
Night DBP (mmHg)	PLA	68 ± 9	69 ± 8
	MTCJ	69 ± 10	68 ± 8
Night MAP (mmHg)	PLA	82 ± 11	85 ± 7
	MTCJ	81 ± 14	84 ± 11
Night PP (mmHg)	PLA	46 ± 8	48 ± 5
	MTCJ	48 ± 7	49 ± 7
D/N SBP* (mmHg)	PLA	19 ± 12	16 ± 6
	MTCJ	15 ± 10	10 ± 8
D/N DBP (mmHg)	PLA	11 ± 8	12 ± 8
	MTCJ	10 ± 9	9 ± 7
D/N MAP (mmHg)	PLA	10 ± 8	13 ± 7
	MTCJ	11 ± 11	10 ± 9
D/N PP* (mmHg)	PLA	7 ± 7	4 ± 4
	MTCJ	5 ± 5	1 ± 3

DBP (Diastolic Blood Pressure); D/N (Day/Night Difference); MAP (Mean Arterial Pressure); mmHg (millimetres of Mercury); MTCJ (Montmorency Tart Cherry Juice); PLA (Placebo); PP (Pulse Pressure); SBP (Systolic Blood Pressure). *Denotes significant main effect for condition. §Denotes significant main effect for time. $Denotes significant main effect for interaction. ^Denotes significant difference between conditions at corresponding time point.

6.3.6. ACE Correlations

Bivariate correlations did not reveal any significant relationships between ACE and cardiac haemodynamic, PWA or 24-hour BP parameters with change from pre- to post-supplementation data ($P > 0.05$) or change from pre-bolus data for either PLA or MTCJ ($P > 0.05$).

6.4. Discussion

In succession to the research carried out in **chapter 5**, this study aimed to examine blood-based and functional cardio-metabolic responses to an acute bolus of MTCJ but also to short-term continuous (7 days) MTCJ supplementation in a human, MetS population. The present study was the first to assess 24-hour ABPM responses and measure ACE concentrations *ex vivo*, to explain cardiovascular responses to cherry (sweet or tart) consumption, in a human trial. The hypotheses of the study were partially accepted as individual responses suggested a tendency for potential improvements in the underlying pathophysiology of MetS, insulin resistance and sensitivity, after 6 days MTCJ consumption compared to PLA. Findings also indicated a significant reduction in glucose, total cholesterol and LDL concentrations with concomitant lower resting RER values after 6 days MTCJ consumption compared to PLA. Of great clinical relevance, MTCJ significantly improved 24-hour BP after 7 days consumption compared to PLA. However, the present study was unable to confirm the hypothesis that ACE inhibition was responsible for the anti-hypertensive effect of MTCJ. Lastly, individual responses indicated 6 days prior loading of MTCJ to improve total cholesterol concentrations, compared to a single, acute bolus; although RER and glucose individual responses were improved with a single, acute bolus, suggesting the hypothesis was partially accepted.

6.4.1. Metabolic Responses

Reductions in glucose concentrations after 6 days of MTCJ supplementation were observed suggesting improved glycaemic function. As there was a tendency for lower fasting insulin concentrations with concomitant normal fasting glucose concentrations, this may be suggestive of improved insulin sensitivity as mentioned by Willems *et al.* (2017), after 6 days supplementation of MTCJ compared to PLA. However, HOMA2-%S was not found to be higher after 6 days MTCJ intake, although 9/12 participants did report a physiological increase in HOMA2-%S. Hence, with a larger sample size these findings may corroborate the physiological theory postulated in **chapter 5**, that MTCJ may confer improvements in insulin sensitivity. Willems *et al.* (2017) demonstrated a reduction in fasting insulin by 14.3% after 7 days consumption of NZBP and attributed this to heightened insulin sensitivity. Similarly, fasting insulin was reduced by 9.3% after 6 days consumption of MTCJ and reduced by 14% when comparing against placebo. Individual responses suggested insulin resistance tended to be improved after 6 days intake of MTCJ (HOMA2-IR change: -0.27 ± 0.56) compared to PLA (HOMA2-IR change: 0.48 ± 0.78), likewise Willems *et al.* (2017) indicated 7 days anthocyanin-rich NZBP consumption tended to improve insulin resistance. Together, these findings highlight short-term continuous supplementation of cyanidin- (MTC) and delphinidin-rich (NZBP) interventions may have the capacity to improve insulin sensitivity/resistance in healthy (Willems *et al.*, 2017) and MetS populations. However, larger datasets are required to justify these initial observations. Moreover, it remains to be seen how long any beneficial effects last, and whether intermittent supplementation for 6-7 days over a longer duration may be a more physiologically effective, ecologically valid and economically viable supplementation strategy.

Stote *et al.* (2017) were unable to show significant reductions in glucose, insulin and insulin sensitivity after 7 days blueberry juice consumption due to normal baseline values. It was noted

that glucose, insulin and HOMA2-IR were significantly higher with MTCJ than PLA at pre-supplementation in the present study due to the high day-to-day inter-individual variance of individuals with MetS. Thus, higher baseline values may have facilitated improvements in these markers based on individual responses compared to Stote *et al.* (2017) over 7 days. Notably, analysis indicated no supplementation order effect for these markers.

Given that tart cherry concentrate is naturally high in sugar, accumulation of glucose and fructose likely contributed to raised glucose concentrations at 1-hour post-bolus, by 0.7 $mmol.L^{-1}$, with 6 days prior loading of MTCJ compared to a single, acute bolus. Chai *et al.* (2018) also showed a similar glucose response after 12 weeks MTCJ supplementation. Yet, Ataie-Jafari *et al.* (2008) reported reduced HbA_{1c} after 6 weeks tart cherry concentrate consumption, and an 8% reduction in fasting glucose. Similarly, the present study showed MTCJ significantly reduced fasting glucose by 9% after 6 days consumption, highlighting the potential efficacy of tart cherry lowering hyperglycaemia in insulin resistant subjects. However, a caveat of such a finding is that chronic consumption of dietary sources rich in glucose and fructose facilitates extensive apical GLUT-2 recruitment (Fernandes *et al.*, 2015); enhancing post-prandial glycaemic stress (Alzaid *et al.*, 2013). The accumulation of fructose and subsequent alterations in gut bacteria (Payne, Chassard and Lacroix, 2012) may also explain the individual response data where triglyceride concentrations (Schaefer, Gleason and Dansinger, 2009) were greater at 3-hours post-bolus after 6 days prior loading of MTCJ, compared to the single bolus. Likewise, Chai *et al.* (2018) reported significantly higher triglyceride concentrations with prolonged MTCJ consumption. Mechanistically, the probable improvements in insulin sensitivity (HOMA2-%S) with MTCJ may have been through receptor-interactions, specifically increased insulin-receptor substrate 1 phosphorylation prompting glucose uptake through increased GLUT-4 translocation (Belwal *et al.*, 2017).

The significant reduction in RER after 6 days MTCJ supplementation, may be explained by the tendency for heightened insulin sensitivity; also suggested by Solverson *et al.* (2018) after 7 days blackberry feeding. The interaction between insulin sensitivity and fat oxidation may be mediated by anthocyanin-induced AMPK activation (Solverson *et al.*, 2018). MTC has been shown to activate PPAR transcription factors, downstream of AMPK, in rodent (Seymour *et al.*, 2008, 2009) and nematode **(Chapter 7)** models. Therefore, activation of PPARα and PGC-1α may account for preferential oxidation of fat as a substrate, while PPARγ agonism may explain the tendencies for insulin sensitising effects of MTCJ in the present study. This finding supports the hypothesis first proposed in **chapter 4** that MTC may act as a CRM.

As fasting glucose and insulin responses were complementary of each other, the change after 6 days indicates MTCJ may normalise glucoregulatory control (Belwal *et al.*, 2017), in a population with early insulin resistance (baseline HOMA2-IR: 2.2 ± 1.4). The significance of which may be highlighted when considering insulin resistance is the underlying cause of impaired cardio-metabolic function in humans with MetS.

6.4.2. Lipid Responses

This study suggests MTCJ may improve aspects of the lipid profile in individuals with MetS. The significant reduction in total cholesterol found in the present study agrees with findings from Ataie-Jafari *et al.* (2008), but similar responses were not observed in other studies providing tart cherries (Martin *et al.*, 2010; Chai *et al.*, 2018), although a trend for lower concentrations was observed in MetS adults (Johnson *et al.*, 2017). The reduction in total cholesterol with MTCJ may be explained by lower LDL fractions after 6 days supplementation. Similarly, reductions in LDL were reported by Ataie-Jafari *et al.* (2008) and Chai *et al.* (2018) in subjects with elevated baseline LDL after tart cherry juice consumption; aligning with findings from other human trials supplementing anthocyanin-rich interventions that hyperlipidaemia is a prerequisite to observe improvements (Wallace, Slavin and Frankenfeld,

2016). Clinically, LDL responses after 6 days MTCJ consumption may correspond to an 8% relative risk reduction of major vascular events (Silverman *et al.*, 2016).

Chapter 5 postulated a prolonged dampening effect due to accumulation, and likely greater retention, of MTC phytochemicals in tissues and systemic circulation. This may explain the individual responses where total cholesterol concentrations were lower at 5-hours post-bolus, after 6 days prior loading. This suggests total cholesterol was amenable to change in the presence of greater tart cherry anthocyanin and secondary metabolite concentrations, as supported by findings from Ataie-Jafari *et al.* (2008) (720 $mg.day^{-1}$ anthocyanins for 6 weeks), individual responses with 60 mL MTCJ consumption in **chapter 5** and 12 weeks MTCJ consumption in humans with MetS (Johnson *et al.*, 2017).

The present study is the first to report significantly greater HDL concentrations after cherry consumption compared to a control condition, however this result was more an effect of reductions in HDL with placebo. Moreover, the change after 6 days MTCJ consumption was not significant as baseline HDL was normal, agreeing with previous studies supplementing tart cherries (Ataie-Jafari *et al.*, 2008; Martin *et al.*, 2010; Lynn *et al.*, 2014; Johnson *et al.*, 2017; Chai *et al.*, 2018) and **chapters 4** and **5**.

Bing sweet cherry consumption had no effect on TC:HDL ratio in healthy adults (Kelley *et al.*, 2006). In the present study, TC:HDL ratio was found to be lower after 6 days consumption of MTCJ and individual responses showed 10/12 participants with lower TC:HDL ratios after MTCJ supplementation compared to 1/12 participants with placebo. Furthermore, TC:HDL ratio was shown to be a good predictor of cardiovascular risk reduction when assessing interventions (Millán *et al.*, 2009), thus improvements in TC:HDL after 6 days MTCJ consumption highlight its clinical efficacy against cardiovascular events.

Lastly, changes in the lipid profile induced by MTCJ after 6 days indicate a state of lower atherogenic and cardiovascular risk, aligning with findings in patients with MetS after MTCJ consumption (Johnson *et al.*, 2017), and potentially explaining the improved 24-hour ABPM responses in the current study.

6.4.3. Cardiovascular Responses

The efficacy of sweet and tart cherry interventions on improving blood pressure, particularly SBP, had been demonstrated numerous times in various populations (Ataie-Jafari *et al.*, 2008; Kent *et al.*, 2015ab; Keane *et al.*, 2016bc), including MetS as shown in **chapter 5**. However, these studies used lab-based measurements which is clinically inferior to 24-hour ABPM (Hermida *et al.*, 2011; Hodgkinson *et al.*, 2011). Therefore, the present study was the first to demonstrate reductions in mean 24-hour SBP, DBP and MAP after cherry supplementation in any population. Cocoa flavanols were also found to reduce day-time DBP, mean 24-hour SBP and DBP in participants with features of MetS (Grassi *et al.*, 2008).

A clinically significant reduction in mean 24-hour SBP was observed after 7 days MTCJ consumption (-5 mmHg); which would be associated with prevention of all-cause and cardiovascular mortality by 20% (Banegas *et al.*, 2018). This finding adds greater clinical and biological relevance to the individual responses reported for acute SBP reductions in the present study, **chapter 5** and by Keane *et al.* (2016b) after an acute, single-bolus of MTCJ. In this study, a reduction of 11 mmHg was observed after 6 days MTCJ consumption compared to PLA for acute SBP at 2-hours post-bolus. Comparative reductions of 8 mmHg were seen with 7 days blueberry juice consumption compared to PLA in individuals with pre- and stage 1 hypertension (Stote *et al.*, 2017), highlighting the efficacy of 6-7 days consumption of anthocyanin-rich juices (~270-314 $mg.day^{-1}$ total anthocyanins). As in **chapter 5**, the magnitude of acute SBP reduction with MTCJ was comparable to approved anti-hypertensive drugs associated with harmful side effects (Bramlage and Hasford, 2009). Moreover, the

2 mmHg reduction in mean 24-hour DBP after 7 days MTCJ consumption would be associated with a risk reduction of CHD and stroke by 6 and 15%, respectively (Cook *et al.*, 1995). This response was facilitated by significant day-time DBP reductions with MTCJ, which has been shown to be a significant predictor of CVD, CHD and stroke (Fagard *et al.*, 2008).

Despite supplementing a similar daily anthocyanin dosage as the present study (270 $mg.day^{-1}$), Stull *et al.* (2015) reported no effect on 24-hour BP after consuming anthocyanin-rich (290.3 $mg.day^{-1}$ anthocyanins) blueberry smoothie for 6 weeks compared to PLA, in individuals with MetS. This finding supports the short-term, low-dose anti-oxidant hypothesis. The pharmacokinetics of PCA and VA may explain the acute lab-based SBP reductions with MTCJ compared to PLA, 2-hours post-bolus, based on individual responses. The hypotensive effects of VA may also explain the 24-hour ABPM responses to MTCJ consumption as VA is structurally similar to apocynin, a vasodilatory drug (Rodriguez-Mateos *et al.*, 2013; Igwe *et al.*, 2017). Lab-based DBP responses were similar to observations reported by other MTCJ supplementation studies (Lynn *et al.*, 2014; Johnson *et al.*, 2017; Chai *et al.*, 2018), including **chapter 5**. Differences between lab-based and 24-hour ABPM may be due to the provision of an additional bolus of MTCJ prior to ABPM and/or greater variability in measurement of lab-based BP monitoring compared to 24-hour ABPM, due to phenomena such as white coat syndrome (Drawz, Abdalla and Rahman, 2012; O'Brien *et al.*, 2013).

In comparison to Keane *et al.* (2016b) (-7 mmHg) and **chapter 5** (-11 mmHg), the magnitude of acute, lab-based SBP reduction in the present study (-11 mmHg) was similar after 2-hours compared to placebo. Thus, indicating no additional benefit of 6 days prior loading of MTCJ in individuals with MetS exhibiting early-hypertension at baseline. This suggests an elevated baseline SBP and the attainment of a physiologically relevant threshold of phytochemicals are required to elicit a reduction in SBP; beyond which further reductions are not observed. Individual responses indicated lower aortic SBP 2-hours post-bolus of MTCJ compared to

PLA. A similar mechanism may be cited as the brachial SBP response at 2-hours post-bolus, through PCA and VA mediated endothelium-dependent vasodilation. Additionally, the brachial and aortic SBP responses concur with a cross-sectional study highlighting significantly lower brachial and aortic SBP with higher habitual anthocyanin intakes (Jennings *et al.*, 2012).

Aviram and Dornfeld (2001) demonstrated reductions in SBP were correlated with significantly lower ACE activity after 2 weeks pomegranate juice consumption. Furthermore, Kirakosyan *et al.* (2018) demonstrated 88.7% ACE inhibition *in vitro*, with MTC extract applied in physiologically relevant concentrations. However, in the present study, as no correlations were observed between serum ACE concentrations and changes in cardiovascular parameters, ACE inhibition may not explain the hypotensive effects of MTCJ; potentially because PCA and VA are weak ACE inhibitors (Hidalgo *et al.*, 2012). Moreover, the present study would have benefitted from assessment of ACE activity rather than ACE protein concentrations, to explain the hypotensive mechanism of MTCJ. Yet, individual responses showed 8/12 participants had lower ACE concentrations 6 days after MTCJ consumption compared to 4/12 with PLA. Overall, ACE inhibition may still explain the hypotensive effects of MTCJ, however future research should incorporate more rigorous methodologies to elucidate mechanisms.

6.4.4. Strengths, Limitations and Future Work

Despite the prospective nature of the study, a limitation would be the small sample size on which conclusions are based, therefore larger clinical trials are required assessing individuals with MetS, but also other clinical populations. Due to financial constraints surrogate indices of pancreatic β-cell function, insulin resistance and sensitivity were used. Future work should consider using tolerance tests or hyperinsulinaemic-euglycaemic clamps (Stull, 2016) to assess the efficacy of MTCJ on post-prandial responses, which provides more ecologically valid

conclusions. However, the present study addressed the artificial placebo effect encountered in **chapter 5** by using an energy and carbohydrate matched placebo to assess the impact of the phytochemicals in MTCJ.

A key strength of the study was the use of 24-hour ABPM as a clinically relevant measure of assessing the effect of MTCJ on blood pressure and subsequently cardiovascular risk. Another strength was the use of a dietary supplement made of a whole food; upholding ecological validity due to the simplicity of incorporating such an intervention into habitual diets. Further understanding of the efficacy of purified anthocyanins alone compared to a whole dietary supplement on cardio-metabolic markers is required, to inform dietary and clinical practice. Lastly, although not objectively measured, participants did not report any adverse effects from MTCJ consumption indicating a degree of tolerance; a major benefit over pharmacological interventions.

6.5. Conclusion

The present study has provided novel findings and revealed for the first time the ability of an MTC intervention (specifically juice) to significantly improve 24-hour BP, fasting glucose, total cholesterol and TC:HDL ratio, and also lower resting RER compared to a control in any human population. LDL concentrations were also found to be lower in individuals with MetS for the first time, after 6 days consumption of MTCJ compared to PLA. Moreover, individual responses also indicated tendencies for improvements in fasting insulin, insulin resistance and insulin sensitivity after consuming MTC, for the first time in any human population. Together, these responses demonstrated clinically relevant improvements on aspects of cardio-metabolic function, emphasising the potential efficacy of MTCJ in preventing further cardio-metabolic dysregulation in an ‘at risk’ population. An overarching theme from the comparison between

supplementation strategies with MTCJ on acute cardio-metabolic responses, demonstrated 6 days prior loading had a limited beneficial impact compared to a single, acute bolus.

This research was unable to confirm ACE inhibition as a mechanism of the hypotensive properties of MTCJ, thus further work is required to elucidate mechanisms for BP responses, but also other cardio-metabolic improvements shown here. It remains to be seen how long the reductions in 24-hour BP last with MTCJ, however with further research MTCJ could perhaps replace or be used as an adjuvant to anti-hypertensive drugs in the future. Nevertheless, the evidence presented is promising for individuals with elevated cardiovascular risk particularly, pre-hypertension.

Chapter 7

Elucidating Mechanisms of Action of Montmorency Tart Cherry Juice Using the Model Organism *Caenorhabditis elegans*

The study presented within this chapter was conducted in collaboration with Dr. Samantha Hughes and colleagues at the HAN BioCentre, HAN University of Applied Sciences, Nijmegen, The Netherlands. Contributions towards this study are highlighted on page *xi*.

7.1. Abstract

Montmorency Tart Cherries, MTC, (*Prunus cerasus* L.) possess a high anthocyanin content as well as one of the highest oxygen radical absorbance capacities of fruits at common habitual portion sizes. MTC have been shown to contribute to reducing plasma lipids, plasma glucose and fat mass in rats and strikingly, similar effects are observed in humans. However, there is a paucity of research examining the molecular mechanisms by which such MTC effects are induced. Here, we show that when exposed to MTC, *Caenorhabditis elegans* display a significant extension to lifespan, and these worms are healthier. Using RNA interference, it is possible to silence genes involved in fat metabolism and provide insight into the molecular pathways through which MTC acts. We have identified that MTC functions via the PPAR signalling pathway, specifically *nhr-49* and *daf-22*. Our data provides encouraging evidence that MTC may be operating as a calorie restriction mimetic via metabolic pathways.

7.2. Introduction

Globally, approximately 1.75 billion people have MetS, a cluster of cardio-metabolic criteria including obesity, hyperglycaemia, dyslipidaemia and elevated blood pressure (Holubková *et al.*, 2012). MetS is often a precursor to type 2 diabetes and cardiovascular disease, which together place a significant burden on health services and are the leading causes of reduced lifespan and increased morbidity worldwide (O'Neill *et al.*, 2016). Given the social challenges faced by the prevalence of MetS, obesity, cardiovascular disease and diabetes, non-pharmacological interventions are desperately needed to safely prevent and mitigate the development of these diseases.

Recently, there has been renewed interest into diets supplemented with "functional foods" particularly rich in polyphenols for health and exercise benefits, namely beetroot juice (Ferreira and Behnke, 2010), purple sweet potatoes (Liu *et al.*, 2010), blueberries (Wilson *et al.*, 2006; McAnulty *et al.*, 2011), pomegranate juice (Trombold *et al.*, 2011), green tea (Jówko *et al.*, 2011) and cherries (Traustadottir *et al.*, 2009; Bell *et al.*, 2014b). Ensuring a diet rich in such foods results in significant health benefits to humans, specifically related to their anti-oxidative, anti-inflammatory, anti-obesity and anti-cancer properties (Ghosh, 2005; Wu *et al.*, 2006; Seymour *et al.*, 2009). Montmorency Tart Cherries (*Prunus cerasus* L.), MTC, possess a high anthocyanin content and has one of the highest oxygen radical absorbance capacities of fruits consumed at common habitual portion sizes (Ou *et al.*, 2012). The health benefit of MTC is likely due to the presence of polyphenols, mainly anthocyanins, which are commonly found in the skin of the fruit and are responsible for its dark red pigmentation (Khoo *et al.*, 2017).

Human studies have established that MTC has anti-inflammatory (Bell *et al.*, 2014b), anti-oxidative (Bell *et al.*, 2014b), anti-hypertensive (Keane *et al.*, 2016b) and anti-hyperuricaemic (Bell *et al.*, 2014b) properties. Correspondingly, rats fed MTC displayed significantly improved lipid profiles and reduced fat mass, hyperinsulinaemia and hyperglycaemia compared to control animals (Seymour *et al.*, 2008, 2009). Additionally, tart cherries have been shown to have an anti-diabetic effect in diabetic rats, via the reduction of plasma glucose (Tahsini and Heydari, 2012). Together, although this is promising evidence that MTC can thwart MetS development, there is a paucity of research examining mechanisms of action by which these effects are induced. Previous studies investigating the mechanisms underpinning MTC used animal models and showed that MTC induced gene expression of PPARα/γ and downregulated both IL-6 and TNF-α (Seymour *et al.*, 2008, 2009), all major pathways involved in fat metabolism and insulin signalling. It is likely that the phytochemicals in MTC may alter the transcription of other genes involved in the response to oxidative stress (Kirakosyan *et al.*, 2018), but these have yet to be

fully elucidated. It is therefore obvious that a detailed understanding of the effect of MTC on these, and other, pathways that lead to positive health effects is lacking.

Caenorhabditis elegans is a powerful model organism to study the molecular pathways that underpin human disease (Markaki and Tavernarakis, 2010; Shaye and Greenwald, 2011). *C. elegans* is a small, transparent nematode worm with a short life span of just 4 weeks, a large brood size of 250 genetically identical offspring and simple anatomy. Strikingly, the nematode has many well-studied molecular networks and tissue systems that are also found in vertebrates, including intestine, skin (cuticle) and a nervous (autonomic and somatic) system. The worm has been fully sequenced and mapped (Hillier *et al.*, 2005), with 80% of human genes possessing homologues in *C. elegans* (Kaletta and Hengartner, 2006). In addition, feeding behaviour, nutritional uptake and fat metabolism are conserved between *C. elegans* and humans (Hashmi *et al.*, 2013). Lastly, *C. elegans* is extremely powerful in terms of technical methodology, such as RNA interference (RNAi) (Ashrafi *et al.*, 2003; Kamath and Ahringer, 2003; Rual *et al.*, 2004), and together with easy access to a vast number of genetic mutants, facilitates molecular pathway dissemination. Taken together, these factors emphasise the translational relevance of this model species and its potential to predict effects in higher animals and inform clinical nutrition practice in humans.

This study examined the response of *C. elegans* to various dilutions of MTC concentrate that relate to the recommended dilution for human consumption. Due to the significant genetic homology between nematodes and humans, it is possible to silence genes involved in fat metabolism in nematodes and observe the effect in the presence and absence of MTC. Such single gene knockdown provides insight into the molecular pathways by which MTC is able to exert its health promoting effects.

7.3. Materials and Methods

7.3.1. Strains and Maintenance of Worms

Strains were derived from the wild type *N2* Bristol strain and maintained at 20°C, as described previously (Brenner, 1974). *C. elegans* were maintained on Nematode Growth Media (NGM) agar prepared according to standard protocols (Brenner, 1974) and plates seeded with *OP50 E. coli* as a bacterial food source.

To synchronise worm populations, gravid worms were washed from plates with M9 buffer (Brenner, 1974) and dissolved using alkaline hypochlorite solution (4 mL 5% sodium hypochlorite, 1 mL 4M sodium hydroxide, 5 mL dH_2O) to obtain the eggs. Hypochlorite solution was removed by washing the eggs three times with M9 buffer. Eggs were left to hatch overnight at 15°C in M9 buffer in the absence of a food source, giving rise to a population of synchronised L1 larvae which could then be placed directly onto NGM to develop to L4 stage at a similar rate.

7.3.2. MTC concentrate seeded NGM

Montmorency tart cherry concentrate (Cherry Active, Active Edge Ltd, Hanwell, UK) has a recommended human consumption of 30 mL MTC concentrate mixed with 240 mL water, daily. This is equivalent to a daily dose of 125 $\mu L.mL^{-1}$.

MTC was added directly to the cooled molten NGM prior to pouring plates. The final concentrations of MTCJ in the NGM plates were 16.7 $\mu L.mL^{-1}$, 66.7 $\mu L.mL^{-1}$, 83.3 $\mu L.mL^{-1}$, 116.77 $\mu L.mL^{-1}$ and 150 $\mu L.mL^{-1}$ in NGM. The pH of all preparations was checked, substituting water for NGM. For each MTC spiked water sample, there was no change in pH compared to the control.

7.3.3. RNA interference

RNA interference experiments were performed using standard protocols (Ashrafi *et al.*, 2003; Kamath and Ahringer, 2003). NGM for RNAi was supplemented with 116.7 $\mu L.mL^{-1}$ of MTCJ in NGM, similar to the recommended daily human dose, or 116.7 $\mu L.mL^{-1}$ distilled water as the control.

The RNAi clones were chosen based on their homology to human genes involved in different aspects of fat metabolism (Ashrafi *et al.*, 2003; Shaye and Greenwald, 2011). Clones were obtained from the Vidal ORFeome-based RNAi library (Rual *et al.*, 2004) and those used in this research are listed in Table 7.1. All nematode experiments were conducted using the *N2* strain. dsRNA was delivered by feeding to age-synchronised L1 stage animals, which were incubated at 20°C and phenotypes were observed 48 hours later, when control animals had reached the L4 stage. Control RNAi was performed using *HT115* bacteria transformed with an empty *L4440* vector.

Table 7.1. List of worm genes, and their human homologues, used for RNAi analysis

Human Gene / description	Worm gene name
Isoform 1 of Hepatocyte nuclear factor 4-gamma	*nhr-49*
gastric triacylglycerol lipase isoform 1	*lipl-1*
fatty acid desaturase 1	*fat-4*
Isoform 1 of Tubby protein homologue	*tub-1*
fatty acid desaturase 1	*fat-3*
Isoform SCPx of Non-specific lipid-transfer protein	*daf-22*
Elongation of very long chain fatty acids protein 3	*elo-4*
One of the nematode cytochrome P450s	*cyp-13A11*
4-trimethylaminobutyraldehyde dehydrogenase	*alh-11*
Isoform HNF4-Alpha-2 of Hepatocyte nuclear factor 4-alph	*nhr-80*
highly similar to Krueppel-like factor 5	*klf-1*
SREBP	*sbp-1*
Lipase member M precursor	*lipl-4*
Glutaminyl-peptide cyclotransferase-like protein	*H27A22.1*
Acyl-coenzyme A thioesterase 8	*C37H5.13*
Fatty acyl-CoA reductase 1	*fard-1*
Isoform 5 of Phospholipase B1, membrane-associated	*F09C8.1*
Acyl-coenzyme A thioesterase 8	*F25E2.3*
Acyl-CoA:lysophosphatidylglycerol acyltransferase 1	*acl-12*
Isoform 5 of Phospholipase B1, membrane-associated	*T19D7.7*
O-linked N-acetylglucosamine (O-GlcNAc)-selective N-acetyl-beta-D-glucosaminidase (O-GlcNAcase)	*oga-1*
Acyl-coenzyme A thioesterase 8	*C17C3.3*
Glycerol-3-phosphate acyltransferase 3	*M79.2*
Gene involved in fat metabolism	*daf-7*
Neuronal acetylcholine receptor subunit alpha-7	*eat-2*

7.3.4. Lifespan Assay

To assess lifespan, wild type *N2* worms were used. Animals were reared on *OP50* seeded NGM at 19°C following standard protocols. When gravid, animals were bleached and the resulting age synchronised L1 animals were placed onto seeded NGM. When animals were L4, this was counted as Day 0 of the survival assay. During the reproductive period, the worms were transferred to a new plate and observed every day for death. Animals were scored as dead if they failed to respond to a touch by a platinum wire. Survival curves were generated and data analysed using the OASIS software (Yang *et al.*, 2011). For multiple analysis, $P < 0.0055$ was considered as significant (Bonferroni corrections).

7.3.5. Microscopy

To take images, worms were mounted onto 2% agarose pads in 0.1% sodium azide. Fluorescent imaging was carried out using a Zeiss Imager.M2 microscope and photomicrographs were taken using a x40 objective (Zeiss) and Zeiss Zen 2012 Blue Software. All images of animals were compiled using Adobe Photoshop 7.0 and backgrounds merged.

7.3.6. Obesity Assay

The obesity assay was adapted from Mak *et al.* (2006) and Ashrafi *et al.* (2003). A stock solution of Nile Red (Roth) was prepared at 0.5 $mg.mL^{-1}$ in acetone and diluted in 1xPBS to 1 $\mu g.mL^{-1}$, both solutions were stored in the dark at 4°C. Nile Red was added to molten NGM (1 mL of working solution in 50 mL NGM) and when solid the plates were seeded with *E. coli OP50*. Age synchronised L1 worms were added to the plates and allowed to develop to L4, at which point the worms were sacrificed for microscopy.

Images were taken using the same gain and exposure settings of animals on the same focal plane (around the grinder in the second pharyngeal bulb). Using ImageJ software, the fluorescence in a 20x20 pixel box placed at the grinder was calculated, to give a value

corresponding to level of staining of the Nile Red vital dye. The arbitrary values for "red" are equivalent to the level of Nile Red staining, which are normalised to the control worms and plotted.

7.4. Results and Discussion

7.4.1. Lifespan is increased following exposure to MTC

Studies have shown that downregulation of the evolutionarily conserved insulin/IGF-1 signalling pathway causes a metabolic shift away from glucose metabolism to lipid oxidation and is associated with longevity (van Heemst, 2010). Here, MTC was tested to see if it was able to extend lifespan, indicative of a positive effect on the insulin signalling pathway. Wild type animals that were not exposed to MTC lived for a maximum of 17 days post L4, while animals exposed to MTC displayed a significant increase in lifespan (Figure 7.1, Table 7.2). This extension to lifespan was most significant at lower MTC dilutions, 16.7 $\mu L.mL^{-1}$ of MTC, the longest-lived animal was 23 days old and at 150 $\mu L.mL^{-1}$, the maximum lifespan was 20 days (Figure 7.1, Table 7.2). Interestingly, the days at which 50% of the population died was only extended for the worms exposed to 16.7 $\mu L.mL^{-1}$ MTC. In addition, lifespan of animals at 66.7 and 33.3 $\mu L.mL^{-1}$ of MTC showed a similar lifespan to those of 16.7 $\mu L.mL^{-1}$ MTC and lifespan of worms exposed to 66.7 and 83.3 $\mu L.mL^{-1}$ of MTC were comparable (data not shown).

Together, data from this study shows there is an extension to lifespan following exposure to MTC, with lower dilutions more beneficial to the worms than elevated dilutions. It is possible that a hormetic effect of MTC is occurring, where the benefit provided by anti-oxidants in MTC, is to scavenge free radicals more effectively, while at higher concentrations the pro-oxidant nature of MTC phytochemicals reverses some health-promoting effects (Blando, Gerardi and Nicoletti, 2004; Konczak and Zhang, 2004).

Such a lifespan extension as observed here is similar to that of blueberry polyphenols, which were able to increase lifespan and delay ageing in *C. elegans* (Wilson *et al.*, 2006). This may be related to the fact that despite tart cherries containing sugar, which would be expected to reduce lifespan, the fruit is able to reduce glycaemic stress by modulating the insulin signalling pathway (Seymour *et al.*, 2009). Such a reduction in glycaemic stress may be mediated by the presence of MTC anthocyanins, phenolic acids and/or their secondary metabolites (Kirakosyan *et al.*, 2009; Scazzocchio *et al.*, 2011), which may reduce the concentration of ligands available to activate the insulin signalling cascade.

Table 7.2. Statistical analysis of lifespan. Raw lifespan data was analysed using the OASIS software (Yang *et al.*, 2011). The control animals (no MTC exposure), lived for a maximum of 17 days, which was extended in all MTC exposed worms. Of the animals exposed to 16.7 $\mu L.mL^{-1}$ MTC, 50% mortality was extended from 10 to 13 days, and was the only dilution of MTC to show this. For each condition, over 100 animals were used, bar 150 $\mu L.mL^{-1}$ where 80 animals were used. For lifespan plots, see Figure 7.1.

MTC Concentration	Age in days at:		Bonferroni *p* value
	50% mortality	100% mortality	
0 $\mu L.mL^{-1}$	10	17	-
16.7 $\mu L.mL^{-1}$	13	23	0.0001
66.7 $\mu L.mL^{-1}$	9	20	0.2358
150 $\mu L.mL^{-1}$	8	20	0.0001

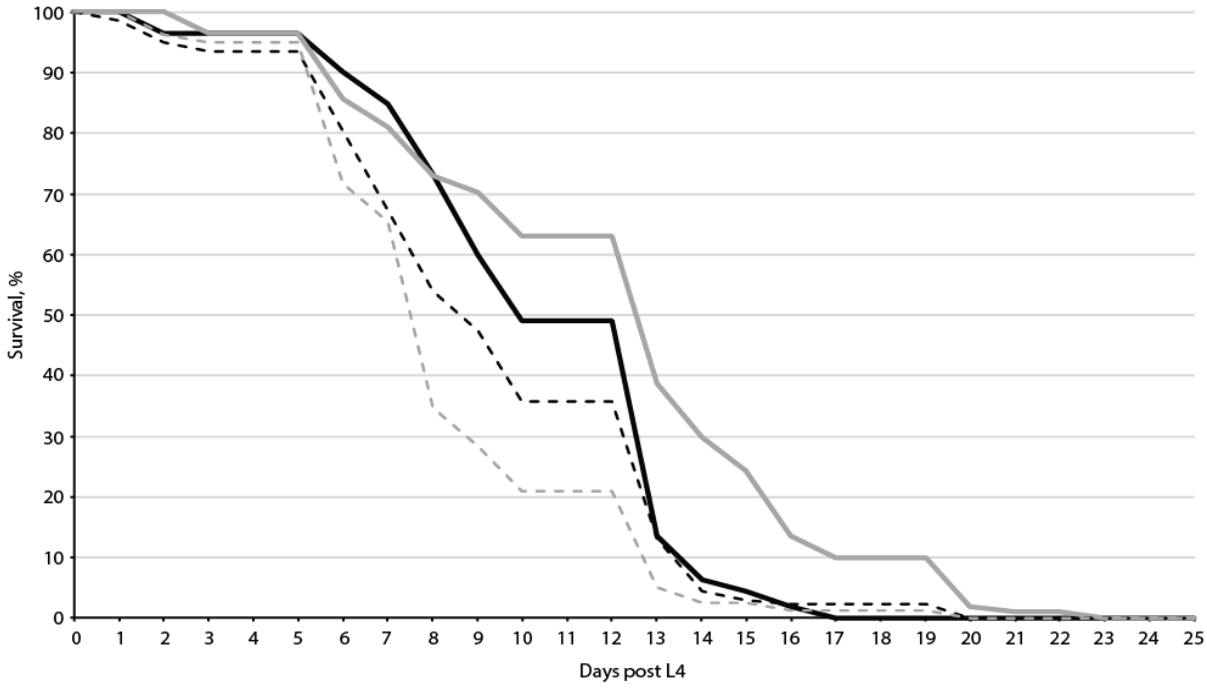


Figure 7.1. Lifespan of worms was extended following exposure to MTC. Wild type worms lived for a maximum of 17 days post L4 (solid black lines). Animals exposed to 16.7 µL.mL^{-1} displayed an extension to lifespan throughout (solid grey lines). In contrast, animals exposed to 66.7 µL.mL^{-1} (dashed black line) and 150 µL.mL^{-1} (dashed grey line) showed a faster death rate compared to the control animals but did have a slight extension to maximum lifespan. For each condition, over 100 animals were used, bar 150 µL.mL^{-1} where 80 animals were used. For statistical analysis, see Table 7.2.

7.4.2. Exposure to High MTC Dilutions Reduces Lipid Staining

Fat metabolism and lifespan are intertwined and likely to be directly coupled (Hansen, Flatt and Aguilaniu, 2013), therefore the fat content of MTC exposed animals using the vital dye, Nile Red was measured (Ashrafi *et al.*, 2003; Mak *et al.*, 2006). Strikingly, animals exposed to the lower doses of MTC (16.7 µL.mL^{-1} and 66.7 µL.mL^{-1}) displayed a significantly increased level of Nile Red staining compared to controls (Figures 7.2 and 7.3). In contrast, elevated levels of MTC significantly reduced Nile Red staining (83.3 µL.mL^{-1} and 116.7 µL.mL^{-1}). It is of interest to note that worms exposed to the 116.7 µL.mL^{-1} dilution of MTC had a developmental delay of 24 hours compared to all other dilutions (data not shown).

Long-lived *daf-2* mutant *C. elegans* display an increase in fat accumulation compared to wild type animals (O'Rourke *et al.*, 2009), suggesting that this is the cause of the increased lifespan observed with low dilutions of MTC. Although MTC has an elevated carbohydrate content, this would prompt the activation of transcription factors, such as SKN-1, that promote the expression of anti-oxidant or detoxification enzymes (Pang *et al.*, 2014), thus allowing animals to promote longevity, as shown in blueberries (Wilson *et al.*, 2006) (refer to *section 7.3.1*).

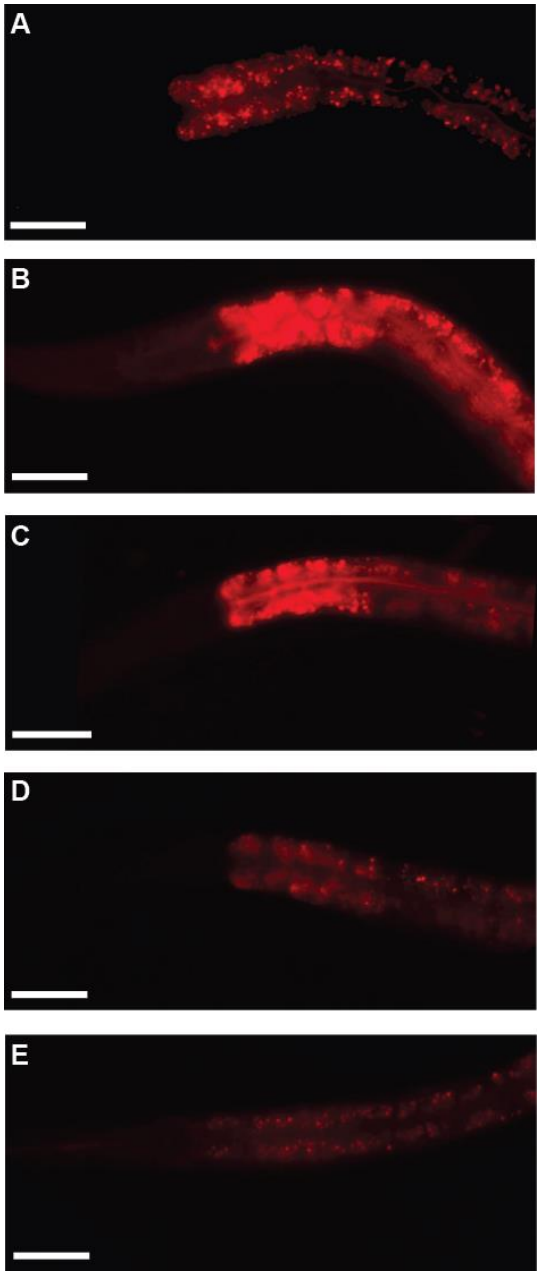


Figure 7.2. Representative images of worms exposed to MTC. In all cases, animals were grown from L1 to L4 on NGM supplemented with increasing dilutions of MTC and the vital dye Nile Red. At L4 stage, worms were mounted for microscopy and imaged using identical settings. Images were taken at the anterior of the animal, with the focal plane always on the valve of the grinder. (A) Control animals, (B) 16.7 µL.mL^{-1} MTC, (C) 66.7 µL.mL^{-1} MTC, (D) 83.3 µL.mL^{-1} MTC and (E) 116.7 µL.mL^{-1} MTC. Anterior is to the left, ventral to the bottom. Scale bar is 500 µm.

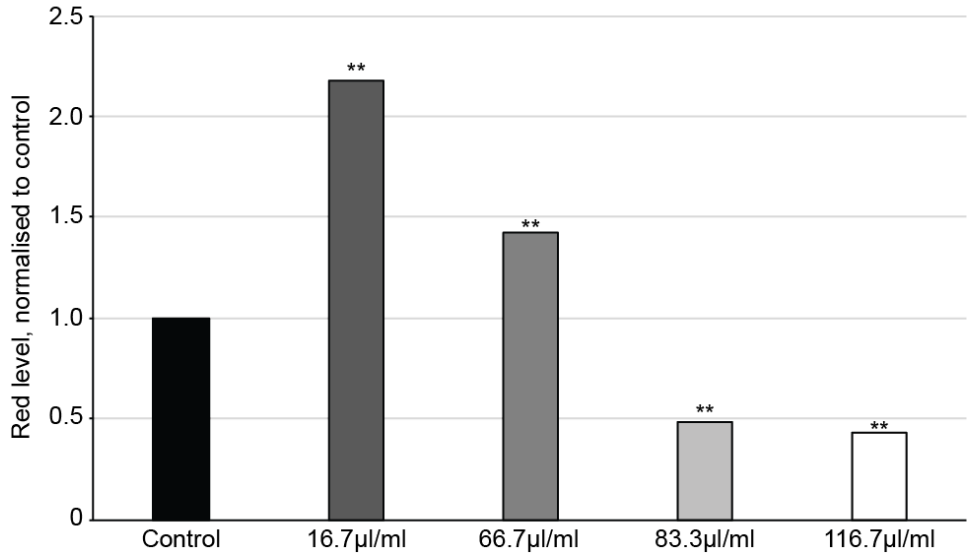


Figure 7.3. Graph to show the relative fat staining in worms exposed to MTC. Images of worms exposed to MTC were analysed using ImageJ and the values of Nile Red staining are normalised to the control, and this value plotted. Control animals (black bars) show some Nile Red staining of the upper intestine. There is a significant increase in fat staining in the animals exposed to 16.7 µL.mL^{-1} and 66.7 µL.mL^{-1} MTC (dark grey bars) while those worms exposed to 83.3 µL.mL^{-1} and 116.7 µL.mL^{-1} MTC (light grey and white bars) have a significant reduction in fat staining. In all cases ** is $P < 0.01$ and $n = 26$.

7.4.3. MTC Acts Through the PPAR Signalling Pathway

Unravelling the pathways through which MTC acts, will shed light on the mechanisms by which MTC provide their many health benefits. As there is significant homology between the mechanisms of fat storage and regulation in *C. elegans* and mammals (Ashrafi, 2007), it is possible to elucidate which pathways are involved with MTC. A number of candidate genes were chosen based on their homology to key human fat metabolism processes.

During the lifespan experiments, it was noticed that there was a small developmental delay in the time it took animals exposed to elevated MTC dilutions to reach L4 stage compared to controls. The majority of the genes tested showed a similar effect to the control RNAi, *L4440*, in the presence of MTC, in that all animals reached the L4 stage at approximately the same time. However, 7 genes (*tub-1, daf-22, elo-4, sbp-1, lipl-4, H27A22.1* and *eat-2*) that were silenced in the presence of MTC showed a similar stage of development as the control in the absence of

MTC i.e. a reversal of the delay (Figure 7.4A). Strikingly, RNAi of *nhr-49*, *lipl-1*, *fat-3*, *klf-1*, *F09C8.1*, *C17C3.3* and *daf-7* caused an enhancement of the developmental delay caused by MTC alone (Figure 7.4B).

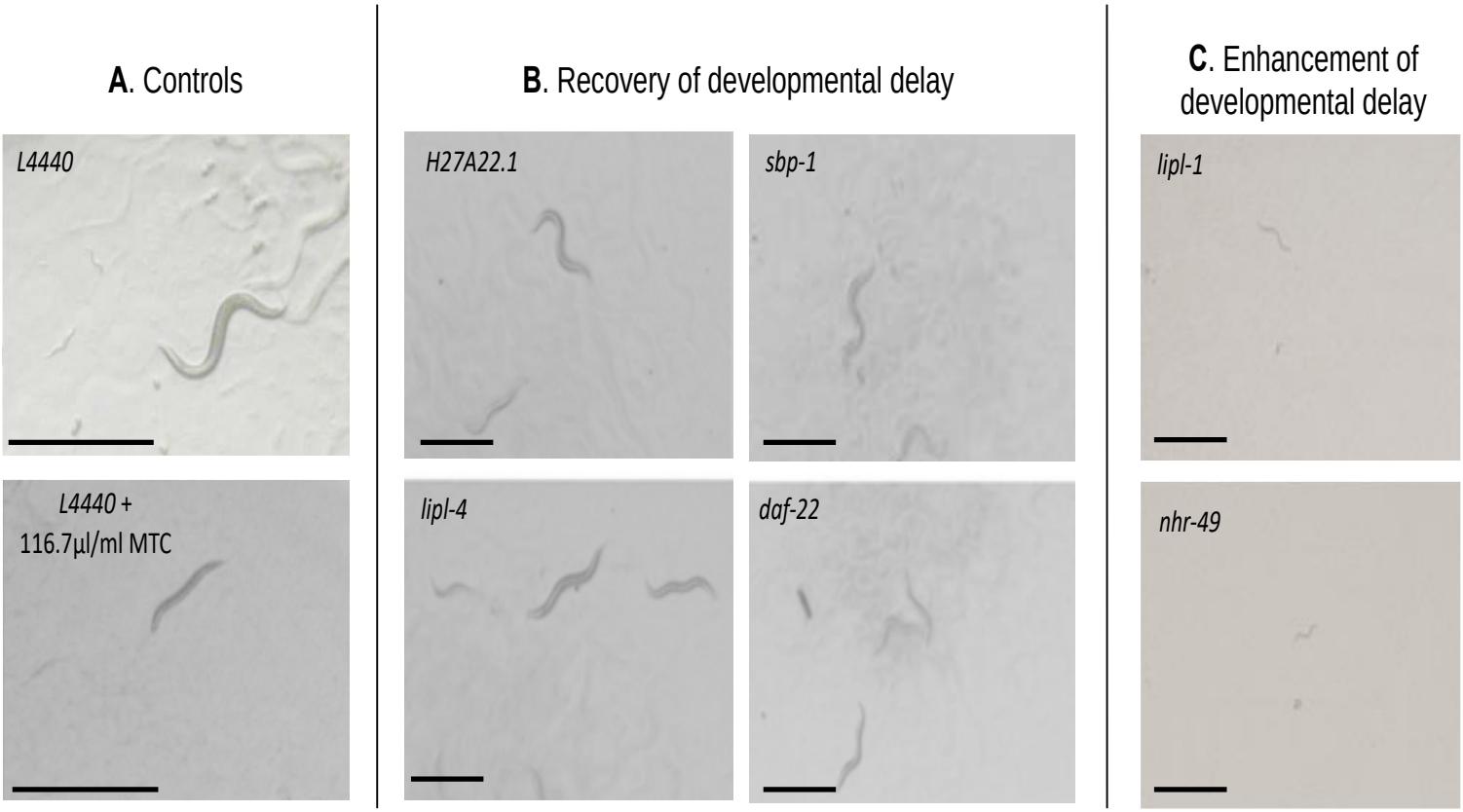


Figure 7.4. RNAi knockdown affects development of animals exposed to MTC. Wild type animals were placed onto RNAi NGM seeded with, or without, 116.7 µL.mL^{-1} MTC, and after 48 hours at 20°C the developmental progress of the animals was compared. (A) Control animals that are not exposed to MTC and are fed control RNAi (empty vector *L4440*) reach the L4 stage (top panel). In contrast MTC exposed worms lag 1 day behind the non-exposed animals (bottom panel). (B) Representative images of worms, which when genes of interest were silenced in the presence of MTC showed a recovery of the developmental delay. Shown are *H27A22.1*, *sbp-1*, *lipl-4* and *daf-22*. (C) Representative images of animals where RNAi silencing of genes enhanced the developmental delay. Two genes are shown, *lipl-1* and *nhr-49*.

It was found that the RNAi knockdown of *daf-22,* the nematode homologue of human SCP2, in the presence of MTC reversed the developmental delay that is caused by exposure to MTC. In contrast, in the presence of MTC RNAi knockdown of *nhr-49,* the HNF4G/PPARα homologue, further enhanced the developmental delay compared to the control. Such an effect is intriguing, as both *daf-22* and *nhr-49* function in the PPAR signalling pathway (Burgering *et al.*, 2004; van Gilst *et al.*, 2005), which are central regulators of fat metabolism [reviewed in (Nunn, Bell and Barter, 2007)]. Strikingly, cyanidin-3-O-β-glucoside and its metabolite protocatechuic acid, also found in MTC, have been shown to activate PPARs thus providing an insulin-sensitising effect (Scazzocchio *et al.*, 2011). Indeed, here we confirm that MTC influences the PPAR pathway (Seymour *et al.*, 2008) and thus is likely to operate as a calorie restriction mimetic (Corton *et al.*, 2004).

It is striking that when *sbp-1* (nematode homologue of SREBP-1c, Sterol regulatory element binding protein) is silenced in the presence of elevated MTC dilutions there is a recovery of the developmental delay. In contrast, there is an enhancement of the MTC induced developmental delay when *lipl-1* (a lysosomal lipase) is knocked down. Both *sbp-1* and *lipl-1* have recently been shown to be regulated by the MAX-3 transcription factor, to modulate lipid metabolism in nematodes (Moreno-Arriola *et al.*, 2017). It is likely, that these genes form part of a complex network modulating the response to carbohydrates (including glucose) and fat, as suggested previously.

However, ultimately, regulation of longevity and lipid metabolism is complex, involving multiple genes and signalling pathways and how longevity and fat accumulation are uncoupled from diet remains elusive.

7.5. Conclusion

To date, there is promising evidence that MTC can contribute to preventing MetS development, although there is a lack of research examining the mechanism of action by which such effects are induced. The data from this study provides encouraging evidence that MTC may be operating as a CRM via metabolic pathways and it is shown here that the nematode *C. elegans* is a useful model for dissecting pathways that correlate to humans. Together, this data highlights the use of *C. elegans* to dissect the molecular pathways through which MTC act and will allow for further detailed pathway dissemination. Due to the large number of genetic mutants available, it is possible to replicate studies in nematodes that have defects in the insulin signalling pathway to further understand how MTC might act to alleviate MetS symptoms. Indeed, such data can then be used to design and perform more targeted longitudinal clinical trials in humans with MetS. In addition, it is possible that genes identified in *C. elegans* as having a role in obesity or diabetes are likely to be implicated in human disease as well or, at the very least, provide candidates for anti-obesity and anti-diabetic drugs.

Chapter 8

General Discussion

8.1. Thesis Overview

Current and projected, global, prevalence and incidence rates of cardio-metabolic diseases are alarming. Hence, there is an urgent need to identify safe interventions to prevent the accentuation of this major problem. This thesis constituted a series of studies examining the efficacy of safe lifestyle interventions, including dietary supplementation of Montmorency tart cherry juice and capsules with and without FATMAX exercise, on cardio-metabolic function in healthy humans and those with MetS. The emphasis of the thesis was around preventing and reducing cardio-metabolic dysfunction using these interventions in populations susceptible to greater risk of developing cardio-metabolic diseases. Lastly, limited research had identified mechanisms for the purported benefits of MTC, therefore various *C. elegans* models were used to identify mechanistic pathways through which MTC may act.

8.1.1. Key Findings

This thesis tested the hypotheses outlined in *section 2.3.2*. This thesis identified numerous novel findings and corroborated previously reported results through the four experimental studies conducted, including:

H_1: Montmorency tart cherry supplementation will significantly improve resting cardio-metabolic biomarkers compared to the placebo-controlled condition, in healthy and MetS human populations.

i. MTC supplementation did not significantly improve cardio-metabolic markers compared to the placebo-controlled condition, in healthy humans.

ii. MTC supplementation significantly improved certain cardio-metabolic markers compared to the placebo-controlled condition, in humans with MetS.

H_2: Montmorency tart cherry supplementation with FATMAX exercise will improve cardio-metabolic biomarkers compared to the placebo-controlled condition, in a healthy human cohort **(Chapter 4)**.

iii. Combination of 20 days MTCJ supplementation and FATMAX exercise did not augment fat oxidation at rest or during exercise, nor did it improve cardio-metabolic biomarkers in healthy humans.

    a. Continued supplementation of MTCJ beyond 10 days did not induce a concomitant elevation in total anti-oxidant status, rather levels returned to baseline. Hence, short-term anti-oxidant supplementation from MTCJ seemed to be more effective at improving cardio-metabolic function and suggests MTCJ may be a hormetin.

H_3: A single, acute bolus of Montmorency tart cherry juice and capsules will improve acute cardio-metabolic biomarkers compared to the placebo-controlled condition, in humans with MetS **(Chapter 5)**.

iv. A single, acute bolus of MTCJ and MTCC acutely improved certain cardio-metabolic biomarkers in humans with MetS.

    a. MTCJ induced a clinically relevant reduction in SBP, 2-hours after consumption in individuals with MetS. The magnitude of change was comparable to pharmacological anti-hypertensive drugs.

    b. MTCC and MTCJ reduced insulin concentrations, 1-hour and 3-hours after consumption, respectively. A health benefit of MTCC consumption was shown for the first time in any human population. Data also indicated a potential improvement in insulin sensitivity mediated by MTCC and MTCJ.

c. Acute cardio-metabolic responses were not statistically different between acute MTCJ and MTCC supplementation in individuals with MetS. Physiological responses indicated MTCC reduced insulin more than MTCJ, 1-hour post-bolus. Although, MTCJ reduced SBP more than MTCC at 2-hours post-bolus.

d. Dose-response effect was not apparent with MTCJ. However, individual responses suggested HDL, TC:HDL ratio and arterial stiffness were improved after consumption of 60 mL MTC concentrate compared to 30 mL MTC concentrate in humans with MetS.

H_4: Short-term, continuous supplementation of Montmorency tart cherry juice in individuals with MetS will improve cardio-metabolic biomarkers compared to the placebo-controlled condition **(Chapter 6)**.

v. Short-term, continuous supplementation of MTCJ improved certain cardio-metabolic biomarkers in individuals with MetS.

a. Revealed for the first time an MTC intervention improved fasting glucose, total cholesterol, TC:HDL ratio and lowered resting RER in any human population, after 6 days of MTCJ supplementation compared to a control group. 24-hour BP was improved after 7 days of MTCJ supplementation.

b. Also, for the first time in individuals with MetS, a reduction in LDL concentrations was observed after consuming an MTC intervention compared to a control group.

c. Individual responses indicated physiological tendencies for improved fasting insulin, insulin sensitivity and insulin resistance after 6 days of MTCJ supplementation compared to the placebo group, in humans with underlying insulin resistance (MetS).

d. Acute SBP following 7 days of continuous MTCJ supplementation was reduced 2-hours post-bolus by a clinically relevant magnitude, corroborating results from **chapter 5**, Keane *et al.* (2016bc) and Kent *et al.* (2015). Moreover, individual responses showed a physiological reduction in aortic SBP 2-hours post-bolus, aligning with MTC anthocyanins and secondary metabolites pharmacokinetics.

e. However, 6 days prior loading of MTCJ had no further beneficial effect compared to a single, acute bolus on acute cardio-metabolic responses.

f. Unable to confirm ACE inhibition as a mechanism for hypotensive properties of MTCJ, therefore further work is required.

H_5: Montmorency tart cherry acts as a calorie restriction mimetic and exerts its beneficial cardio-metabolic responses through evolutionary conserved mechanisms related to calorie restriction, in *C. elegans* **(Chapter 7)**.

vi. Exposure to various dilutions of MTC concentrate resulted in a significant extension to lifespan in *C. elegans*, and these worms were healthier.

a. Elucidation of the molecular mechanisms of action suggested MTC operates through the PPAR signalling pathway, corroborating findings from Seymour *et al.* (2008, 2009).

b. This study provided encouraging evidence that MTC may be functioning as a calorie restriction mimetic via metabolic pathways.

8.1.2. Thesis Development

The opening study **(Chapter 4)** in this thesis aimed to examine the effect of combining FATMAX exercise with MTCJ supplementation on fat oxidation rate, body composition and cardio-metabolic biomarkers in healthy human participants. Rationale for this study was based on

Venables *et al.* (2008) and Cook *et al.* (2015; 2017b) demonstrating greater fat oxidation rates with green tea polyphenols and anthocyanin-rich blackcurrants, respectively.

Chapter 5 addressed some of the issues encountered in **chapter 4** and acted upon the null results obtained in the first experimental study. Specifically, recruitment of participants with MetS possessing some cardio-metabolic dysfunction in **chapter 5**, rather than healthy participants, as in **chapter 4**; as anthocyanin-rich dietary interventions have been shown to improve symptoms associated with MetS in this population (Basu and Lyons, 2012; Amiot, Riva and Vinet, 2016; Vendrame *et al.*, 2016). Secondly, acute administration of MTCJ and MTCC to coincide with MTC phytochemical pharmacokinetics, as previously conducted by Keane *et al.* (2016b). MTCC was provided to determine whether cardio-metabolic function could be improved due to its lower carbohydrate content and potential to augment phytochemical bioavailability, compared to MTCJ. Lastly, to establish whether a dose-response effect on cardio-metabolic markers was apparent with acute MTCJ supplementation.

Chapter 5 highlighted acute improvements in cardio-metabolic function after acute supplementation of MTC interventions, but no dose-response effect. Consequently, **chapter 6** supplemented 30 mL MTC concentrate for 7 days, due to superior practicality, feasibility and economic viability compared to MTCC and 60 mL MTC concentrate. Based on findings from **chapter 4**, Solverson *et al.* (2018) and Willems *et al.* (2017), it was reasoned short-term, low-dose, exogenous anti-oxidant supplementation, would improve cardio-metabolic function. To provide greater clinical relevance, **chapter 6** assessed responses after 7 days continuous, MTCJ supplementation. Thus, **chapter 6** also enabled ascertainment of whether 6 days prior loading of MTCJ enhances acute cardio-metabolic function compared to a single, acute bolus. Additionally, **chapter 6** attempted to elucidate *ex vivo*, a mechanism of action for the hypotensive properties of MTCJ observed in **chapter 5** and by Keane *et al.* (2016bc), through ACE inhibition; based on data reported by Kirakosyan *et al.* (2018).

The last experimental chapter **(Chapter 7)**, undertaken in collaboration with the HAN University of Applied Sciences, Nijmegen, aimed to delineate the mechanistic pathways through which MTC interventions may exert their cardio-metabolic effects. A model organism, *Caenorhabditis elegans*, was used to observe developmental responses after RNAi of target genes of interest associated with lipid and glucose metabolism. Then, in the presence and absence of various dilutions of MTC concentrate, responses to lifespan and fat content were observed and mechanisms subsequently discussed compared to development read-outs after RNAi. The lifespan assay showed worms exposed to lower dilutions of MTC concentrate lived significantly longer than those exposed to higher MTC dilutions and non-exposed worms. Paradoxically, the obesity assay indicated greater lipid accumulation in the longest-lived worms exposed to lower dilutions, compared to controls. Equally, worms exposed to higher MTC dilutions presented significantly less lipid accumulation. In rodents, mild calorie restriction was shown to increase fat mass (Li *et al.*, 2010). A plausible explanation may be that due to lower nutrient availability, worms were more efficient in extracting nutrients at lower MTC dilutions. Hence metabolism of carbohydrates from MTC may have facilitated lipid accretion; and activated transcription factors such as SKN-1, that promote anti-oxidant enzyme expression (GPx and glutathione-S-transferase) and subsequently longevity (Sykiotis *et al.*, 2011). Moreover, improved extraction and thus metabolism of MTC phytochemicals may have mediated lifespan extension. Overall, these findings highlighted MTC may be operating as a calorie restriction mimetic via the PPAR pathway, as previously shown in rodent models (Seymour *et al.*, 2008, 2009).

8.2. Common Concepts Throughout Thesis

Common emerging themes arising from this series of studies support the observations made in response to MTC consumption. These concepts include the anti-oxidant hypothesis based on the action of MTC as a hormetin; the influence of baseline values on responses to MTC; the high carbohydrate content of MTCJ and the supplementation strategy of MTCJ.

8.2.1. Anti-oxidant Theorem

A consistent finding from this series of studies has shown low-dose, short-term, anti-oxidant supplementation to exert beneficial effects on cardio-metabolic health. This was demonstrated in **chapter 4** where fat oxidation responses corresponded to total anti-oxidant status which was elevated during the first 10 days of supplementation but returned to baseline between days 10-20. **Chapter 6** showed provision of anti-oxidants for a short time (~7 days) was effective at improving aspects of cardio-metabolic function. Lastly, **chapter 7** postulated a hormetic effect of MTCJ, as lower concentrations extended lifespan compared to higher concentrations which hastened death rate; potentially through increased toxicity associated with high anti-oxidant concentrations. Lifespan extension with low anti-oxidant concentrations support the CRM actions of MTC, and other dietary hormetins (Calabrese *et al.*, 2010). It is postulated that excessive exogenous anti-oxidant supplementation, inhibits endogenous anti-oxidant production to maintain a homeostatic balance (Poljsak, Šuput and Milisav, 2013). However, provision of low-dose exogenous anti-oxidant concentrations heightens the total anti-oxidant balance but not as to induce toxic effects. Therefore, enhancing the defence mechanism to stressors and mediating improved health (Martucci *et al.*, 2017), with MTC. In summation, this series of studies has put further scrutiny on the efficacy of costly, prolonged continuous supplementation trials; as low-dose, short-term, anti-oxidant supplementation was shown to be effective.

8.2.2. Carbohydrate Content of MTC

This thesis and Chai *et al.* (2018) identified MTC concentrate to possess a high carbohydrate content, potentially contributing to greater glycaemic, insulinaemic and lipaemic stress. It was identified total carbohydrate accounted for 96% of total energy, of which 70% were sugars, in MTC concentrate. Glucose and fructose contributed 55.73% and 44.27% of total sugars, respectively. Relative to the same anthocyanin content in 30 mL MTC concentrate, MTCC provided approximately 8 and 18 times less total carbohydrate and sugars, respectively. Hence,

Chapter 5 used MTCC to assess acute cardio-metabolic responses to acute supplementation of an MTC intervention with a low carbohydrate load.

Accumulation of fructose with prolonged consumption of MTCJ may have negated its benefits by exacerbating some of the deleterious effects of fructose such as hyperlipidaemia, hyperuricaemia and low-grade inflammation (Karim, Adams and Lalor, 2012). Hence, explaining the tendency for improved LDL with MTCC compared to MTCJ in **chapter 5**, and greater glucose and triglyceride concentrations after continuous MTCJ supplementation compared to a single acute bolus in **chapter 6**, as also reported by Chai *et al.* (2018). **Chapters 4** and **6** also showed heightened carbohydrate oxidation, signifying MTCJ induced greater glycolytic flux after continuous MTCJ supplementation.

Despite MTC concentrate possessing a high carbohydrate content, the anthocyanins, phenolic acids and/or their secondary metabolites may have modulated insulin signalling pathways, through reducing the concentration of ligands available to activate the insulin signalling cascade; as discussed in **chapter 7** with *C. elegans*. Reduced ligand availability may have been mediated through anthocyanins inhibiting glucose absorption into systemic circulation via suppression of SGLT-1 (Alzaid *et al.*, 2013), therefore lowering insulin concentrations.

8.2.3. Influence of Baseline Values

This series of studies and many others in literature have repeatedly shown the influence of baseline values on the efficacy of dietary interventions on cardio-metabolic markers. This thesis reasoned the lack of cardio-metabolic dysregulation, often does not provide sufficient scope for an MTC intervention to further regulate cardio-metabolic markers. Consequently, abnormal baseline values are generally required to observe an effect with MTC. This is supported by cherry (Kelley *et al.*, 2006; Ataie-Jafari *et al.*, 2008; Martin *et al.*, 2010; Lynn *et al.*, 2014; Chai *et al.*, 2018) and other dietary anthocyanin-feeding studies in humans (Basu *et al.*, 2009, 2010a, 2011; Stull *et al.*, 2015;

Stote *et al.*, 2017; Willems *et al.*, 2017) and rodents (Seymour *et al.*, 2008, 2009).

Chapter 4 recruited healthy individuals with normal baseline cardio-metabolic values, hence limiting the ability of MTCJ to improve cardio-metabolic parameters and increase fat oxidation. Therefore, **chapters 5** and **6** recruited participants with MetS possessing some cardio-metabolic dysregulation at baseline. Indeed, cardio-metabolic parameters abnormal at baseline were improved with MTC interventions. Lipid profile and lab-based BP changes were particularly influenced by baseline values. In **chapter 4**, total cholesterol, LDL and HDL were within optimal thresholds at baseline; therefore, no effect of the intervention was realised. Likewise, in **chapter 6** the observed reductions for total cholesterol, LDL and TC:HDL ratio after 6 days MTCJ supplementation manifested as baseline values were not within their respective optimal thresholds. As baseline HDL was normal, the change after 6 days MTCJ consumption was not significant, agreeing with previous studies supplementing tart cherries (Ataie-Jafari *et al.*, 2008; Martin *et al.*, 2010; Lynn *et al.*, 2014; Johnson *et al.*, 2017; Chai *et al.*, 2018) and **chapters 4** and **5**. Long-term studies supplementing anthocyanins from tart (Ataie-Jafari *et al.*, 2008; Chai *et al.*, 2018) and sweet (Kent *et al.*, 2015a) cherries lowered SBP, although only in subjects presenting elevated baseline SBP. The lab-based SBP reductions reported in **chapters 5** and **6**, 2-hours post-bolus of MTCJ were similar to Keane *et al.* (2016a) in males with early hypertension, emphasising the requirement of elevated baseline SBP.

Given the influence of baseline values as shown above, results from **chapters 5** and **6** were significantly affected by MetS diagnosis encompassing 5 different criteria. High baseline inter-individual variability amongst participants with MetS was observed, thus diminishing statistical power to detect differences between interventions and controls (Amiot, Riva and Vinet, 2016).

8.2.4. Optimal Supplementation Strategy

A major issue with identification of a recommended supplementation strategy using anthocyanin-rich interventions for individuals with MetS, is the high variation between strategies utilised by previous studies, as shown in *section 2.2.5.2.2.1.1*. High baseline inter-individual variability amongst individuals with MetS along with differences in dosage, matrix, food source, duration, frequency and timing impede the ability to provide a general recommended strategy.

In keeping with the findings from **chapter 4**, where TAS declined to baseline from days 10-20 of supplementation, **chapter 6** supplemented MTCJ for 7 days based on results demonstrating improved cardio-metabolic function in anthocyanin-feeding studies (Stote *et al.*, 2017; Willems *et al.*, 2017; Solverson *et al.*, 2018). Indeed, improvements across various cardio-metabolic markers were observed in **chapter 6**, suggesting the optimal length for MTCJ to enhance cardio-metabolic function may lie around 6-10 days; aligning with elevations in TAS. However, it is important to understand that long-term cardiovascular protection provided by nutritional interventions may not be realised with short-term supplementation (Fairlie-Jones *et al.*, 2017). Hence, the null effects on arterial stiffness in **chapters 5-6** may be due to acute/short-term supplementation, since atherogenesis is a long-term developmental process (Fairlie-Jones *et al.*, 2017).

This thesis has added to the literature by suggesting 270 $mg.day^{-1}$ of anthocyanins from MTC interventions in juice or capsule form improves certain cardio-metabolic markers. Such a daily dose is significantly lower than other tart cherry studies (Ataie-Jafari *et al.*, 2008; Keane, *et al.*, 2016bc; Chai *et al.*, 2018) showing a beneficial effect of MTC on cardio-metabolic markers, providing further support for the low-dose anti-oxidant theorem. Results pertaining to the minimum effective anthocyanin dosage required to improve cardio-metabolic function in individuals with MetS were equivocal (*section 2.2.5.2.2.1.1*), and the data presented in **chapters 5-6** has not fully clarified the issue. *Section 2.2.5.2.2.1* identified components of MetS were improved after supplementation of different anthocyanin dosages in individuals with MetS.

Chapters 5-6 contrasted previous studies (Barona *et al.*, 2012ab) reporting improved SBP with anthocyanin dosages <100 mg.day^{-1}. Moreover, total cholesterol was found to be reduced with anthocyanin dosages ≤154 mg.day^{-1} (Basu *et al.*, 2009; Basu *et al.*, 2010b; Gurrola-Díaz *et al.*, 2010; Jeong *et al.*, 2014), however **chapter 5** indicated a trend towards lower total cholesterol with 540 mg.day^{-1} (60 mL concentrate) and **chapter 6** showed lower concentrations with 270 mg.day^{-1}. However, anthocyanin dosages ≥154 mg.day^{-1} were shown to modulate oxidative stress in individuals with MetS; agreeing with the elevated TAS observed after supplementing 540 mg.day^{-1} **(chapter 4)** and aligning with the anti-oxidant theorem postulated in this thesis. Although statistical analysis was not performed, **chapter 5** showed only 60 mL MTC concentrate improved HDL and TC:HDL ratio. Furthermore, **chapter 5** indicated a beneficial effect of 60 mL concentrate (540 mg.day^{-1}) on central arterial pressure parameters compared to 30 mL concentrate (270 mg.day^{-1}). Concurring with Jennings *et al.* (2012), that a higher consumption of anthocyanins is associated with reductions in aortic blood pressure. Interestingly, twice daily supplementation of 30 mL MTC concentrate (total anthocyanins 540 mg.day^{-1}) in **chapter 4** initially elevated anti-oxidant status but this returned to baseline after 20 days. Split dosages of cherry juice was not as effective at improving cardiovascular function compared to a single bolus (Kent *et al.*, 2016b). Thus, a single bolus of 60 mL MTC concentrate (total anthocyanins 540 mg.day^{-1}) was used in **chapter 5, part B**, showing positive effects on cardiovascular function. Subsequently, single dosages were maintained in **chapter 6**, albeit with 30 mL MTC concentrate to conform to the low-dose hormetic effect.

Chapter 4 highlighted the importance of timing when consuming anthocyanin-rich interventions. Based on the pharmacokinetics, maximising bioavailability of phytochemicals in plasma and target tissues enhances the bioefficacy of anthocyanin-rich interventions. Given the rapid metabolism and elimination rates of anthocyanins (Manach *et al.*, 2005), acute supplementation may be necessary to observe an effect on cardio-metabolic function with anthocyanin-rich interventions.

This was corroborated in **chapter 4** as the lack of acute MTCJ supplementation did not augment fat oxidation during exercise, however acute anthocyanin-rich blackcurrant supplementation 2-hours before exercise increased fat oxidation rate (Cook *et al.,* 2015). Coinciding peak bioavailability and concentrations of anthocyanins, secondary metabolites and phase II conjugates in plasma and target tissues, with the time of measurement of variables greatly improves the ability to observe an effect from anthocyanin-rich interventions. Hence, acute supplementation was incorporated into **chapters 5-6**, which corroborated observations where SBP declined 2-hours post-bolus of MTCJ (Keane *et al.,* 2016bc). This improvement likely coincided with MTC pharmacokinetics where concentrations of plasma parent anthocyanins (cyanidin-3-glucosylrutinoside and cyanidin-3-rutinoside) (Seymour *et al.*, 2014) and secondary metabolites (PCA and VA) (Keane *et al.*, 2016a) were significantly elevated.

Building on the pharmacokinetics of MTC, **chapter 5** directly compared different forms of MTC interventions on health markers for the first time, in any human population. It was postulated MTC capsules would increase phytochemical bioavailability, due to protection afforded by the capsule shell against factors known to influence phytochemical stability such as pH, temperature, light, enzymes (particularly in saliva) and sugars (Robert and Fredes, 2015). Acute cardio-metabolic responses were not different between MTCJ and MTCC, suggesting phytochemical bioavailability was not likely to be augmented with MTCC; however pharmacokinetic data is required to confirm this. Subsequently, **chapter 6** implemented 30 mL MTC concentrate due to superior practicality, feasibility and economic viability as an intervention strategy, compared to MTCC; but also availability of pharmacokinetic data with MTC concentrate (Bell *et al.*, 2014a; Keane *et al.*, 2016a).

Section 2.2.5.2.2.1 reviewed the effects of various dietary sources rich in different anthocyanidin-based anthocyanins on cardio-metabolic function. Bell *et al.* (2014b) identified cyanidin, malvidin and peonidin to be the most abundant anthocyanidins in MTC concentrate; cyanidin accounted for

the overwhelming majority of anthocyanins (Ou *et al.*, 2012; Bell *et al.*, 2014b). Cyanidin was reported to possess strong radical scavenging and anti-inflammatory properties, primarily due to its secondary metabolites PCA and VA (Fang, 2015). The findings in **chapters 4-7** and Keane *et al.* (2016ab) support this notion and again align with the anti-oxidant theorem. The reduction in oxidative stress because of elevated anti-oxidant status, likely improved endothelial function and therefore explains the positive cardiovascular effects observed in **chapters 5-6**. Cyanidin and malvidin-based anthocyanins have been shown to exert improvements in endothelial function, SBP and lipid profile in humans with MetS (*section 2.2.5.2.2.1.1*), supporting results in **chapters 5-6**.

This thesis has highlighted the efficacy of various manipulations of supplementation strategies with MTC interventions, particularly MTCJ. No obvious differences between MTC juice and capsules were seen, however juice seems to be most practical despite capsules appealing to individuals with insulin resistance due to its lower carbohydrate content. Secondly, in line with anti-oxidant status, 6-10 days MTCJ consumption seems to elicit the greatest cardio-metabolic improvements. A recommended minimum effective anthocyanin dosage was not identified, however 270 $mg.day^{-1}$ induced diverse cardio-metabolic improvements in individuals with MetS; and 540 $mg.day^{-1}$ indicated enhanced cardiovascular function (central haemodynamics, arterial stiffness and lipid profile).

Overall, results obtained from **chapters 5-6** agree with previous literature (Basu *et al.*, 2009, 2010a, 2011; Basu and Lyons, 2012; Stull *et al.*, 2015; Amiot, Riva and Vinet, 2016; Vendrame *et al.*, 2016) that MTC as an anthocyanin-rich dietary intervention improves symptoms associated with MetS. However, the synergistic action of other nutrients within MTC should not be disregarded as they may confer greater cardio-metabolic protection compared to the solitary action of anthocyanins alone. Further research is required to confirm findings from this thesis and provide additional novel data, by manipulating strategies in order to facilitate construction of an optimal supplementation regimen for humans with MetS.

8.2.5. Mechanisms of Action

Analysis of mechanisms of action in anthocyanin-feeding studies is rare, therefore complete understanding of their effects on cardio-metabolic responses is lacking. This thesis attempted to decipher mechanisms of action in response to MTCJ, using human **(Chapter 6)** and *C. elegans* **(Chapter 7)** models.

Keane *et al.* (2016b) were unable to confirm nitric oxide or changes in arterial stiffness as mediators of improved blood pressure, after acute MTCJ consumption. Similarly, **chapters 5-6** were also unable to attribute reductions in SBP after MTCJ consumption to changes in arterial stiffness. Moreover, **chapter 6** was unable to demonstrate MTC mediated ACE inhibition to explain SBP responses, despite *in vitro* work suggesting otherwise (Kirakosyan *et al.*, 2018).

Seymour *et al.* (2008, 2009) previously used animal models to examine molecular mechanisms in response to MTC supplementation. Given its comparable homology to the human genome, a *C. elegans* model was used to further explain cardio-metabolic responses to MTC concentrate at the molecular level, in **chapter 7**. In two distinct phyla (Nematoda and Chordate), MTC was shown to operate via the PPAR signalling pathway, strongly indicating a similar mechanism in humans.

Chapter 7 specifically identified modulation of genes in the insulin signalling cascade to be responsible for the metabolic benefits of MTC. Supported by literature (Belwal *et al.*, 2017; Solverson *et al.*, 2018), anthocyanin-mediated AMPK activation, upstream of PPAR, may explain the metabolic benefits of MTC consumption shown in humans **(Chapters 5-6)**; future work should test this hypothesis.

Clearly, more research is required, particularly in humans, to elucidate mechanisms of action pertaining to the cardio-metabolic benefits of MTC. Further work should examine the influence of MTC on gut microbiota given its close relationship with cardio-metabolic health. Lastly, this thesis revealed CRM properties of MTC, thus analysis of epigenetic mechanisms is warranted.

8.3. Clinical Relevance

From the outset, this thesis intended to use MTC as a preventative intervention against cardio-metabolic disease. Hence, human participants recruited in **chapters 4-6** were either healthy **(Chapter 4)** or presented only borderline cardio-metabolic dysfunction **(Chapters 5-6)**. This section summarises clinical applications of MTC interventions, discussed in previous chapters.

Section 2.2 outlined the global burden of cardio-metabolic diseases, but also the efficacy and cost-effectiveness of lifestyle interventions, including physical activity and diet, in combating this issue (Besnier *et al.*, 2015). Recommendations for exercise and diet prescription in public health services are limited compared to pharmacological drugs (Seth, 2014; Nunan, 2016), despite literature showing enhanced cardio-metabolic function after combined exercise and polyphenol supplementation (Venables and Jeukendrup, 2008; Venables *et al.*, 2008; Besnier *et al.*, 2015). Specifically, **chapter 4** showed FATMAX exercise and MTCJ consumption to induce clinically relevant increases in HDL concentrations after 10 days supplementation. In individuals with MetS, clinically relevant improvements in HDL and TC:HDL ratio were observed with acute 60 mL consumption **(Chapter 5)**. Continuous 30 mL MTC concentrate consumption also improved TC:HDL ratio and reduced total and LDL cholesterol **(Chapter 6)**, highlighting lower atherosclerotic and cardiovascular risk (Millán *et al.*, 2009) with MTC concentrate consumption.

Insulin resistance is thought to be central to the underlying pathophysiology of MetS, therefore findings presented in **chapters 5-6** indicating a tendency for increased insulin sensitivity after MTCJ and MTCC consumption require acknowledgement. Fasting insulin was significantly reduced after acute consumption of MTCC, therefore may be advocated for individuals with poor insulin sensitivity or insulin resistance e.g. MetS, diabetics. Short-term improvements in insulin control may develop into long-term adaptations with consistent use. As previously identified, MTC has been shown to operate along the PPAR signalling pathway, therefore they may be used as a safer alternative to pharmacological drugs, such as thiazolidinediones, which improve insulin

resistance through PPAR agonism (Ingram *et al.*, 2006).

Furthermore, **chapter 6** demonstrated 6 days MTCJ consumption tended to reduce insulin resistance, suggesting potential for holistic amelioration of cardio-metabolic dysfunction related to MetS. This was corroborated by concurrent improvements of fasting glucose, lipid profile, 24-hour BP and resting substrate oxidation in **chapter 6**. However, the limitations of HOMA as a surrogate for measuring insulin resistance and sensitivity are recognised. Therefore, future work should consider using tolerance tests or hyperinsulinaemic-euglycaemic clamps (Stull, 2016) to assess the efficacy of MTCJ on post-prandial responses; providing more ecologically valid conclusions.

Arguably, the most clinically relevant finding presented in this thesis was the significant reduction in 24-hour BP **(Chapter 6)**. Previous research (Ataie-Jafari *et al.*, 2008; Keane *et al.*, 2016ab; Chai *et al.*, 2018) and **chapters 5-6**, reported clinically relevant lowering of lab-based BP after tart cherry juice consumption; but never with 24-hour ABPM, regarded as clinically superior than lab-based measurements (Hermida *et al.*, 2011; Hodgkinson *et al.*, 2011). **Chapter 6** also discussed that 24-hour BP responses were supported by the low-dose anti-oxidant hypothesis and indicates that augmentation of anti-oxidant status after 7 days MTCJ supplementation may contribute to the hypotensive effects. The hypotensive properties of other phytonutrients in MTCJ and habitual diet should be considered, since Hassellund *et al.* (2012) reported no effect of purified anthocyanin (640 $mg.day^{-1}$) supplementation on 24-hour BP. The high anthocyanin dosage, designed to augment bioavailability, may have shifted the hormetic balance towards a pro-oxidant state, therefore negating anthocyanin-mediated benefits. Bioavailability may have been limited in Hassellund's *et al.* (2012) study, since other nutrients facilitate absorption of anthocyanins (Bohn, 2014); thus explaining benefits observed with whole-food dietary supplements in **chapter 6** but not with purified anthocyanins.

Lab-based reductions in SBP shown in **chapters 5-6** may have been associated with a 38% and 23% reduced risk of stroke and coronary artery disease, respectively, over a 5-year period (Collins *et al.*, 1990). In **chapter 6**, a clinically significant reduction for mean 24-hour SBP was observed after 7 days MTCJ consumption (-5 mmHg); which would be associated with prevention of all-cause and cardiovascular mortality by 20% (Banegas *et al.*, 2018). To provide further clinical relevance for the hypotensive properties of MTC, future work should decipher the length of time 24-hour BP reductions can be maintained after discontinuing supplementation.

Remarkably, the magnitude of reduction in 24-hour and lab-based BP after MTCJ consumption **(Chapters 5-6)** was equivalent to anti-hypertensive drugs, thus practitioners should query the decision to primarily prescribe pharmacological medication associated with harmful side effects. Clinical decision-making would benefit from future research examining the safety, tolerability and overall interactions between MTC interventions and pharmacological drugs in humans. Subsequently, a clinical application of this may enable practitioners to prescribe hybrid strategies as an adjunctive or replacement therapy to pharmacological drugs (e.g. half dose of MTC and pharmacological drug, as opposed to a full dose of pharmacological drug alone), as a safer method of managing diseases. Moreover, diagnosis and management of hypertension are increasingly based on aortic rather than brachial pressures (McEniery *et al.*, 2014). Physiological reductions of aortic SBP after acute 30 mL **(Chapter 6)** and 60 mL **(Chapter 5)** concentrate consumption, lends further support that MTCJ consumption could be a safe yet effective anti-hypertensive intervention.

Ultimately, the clinically relevant results presented in this thesis suggest MTC interventions can be beneficial for individuals with MetS, to prevent the development of cardio-metabolic diseases that accentuate mortality risk. Although not within the remit of this thesis, given these positive results, future research should explore whether MTC interventions can reverse diagnoses in populations with existing cardio-metabolic diseases e.g. diabetes, atherosclerosis.

8.4. Strengths and Limitations

The following section will identify and discuss overarching strengths and limitations associated with the studies presented in this thesis. High ecological validity is crucial to enable application of research into practice; therefore, the strengths and limitations inextricably relate to the degree of ecological validity in each study.

Due to the pilot nature of the studies conducted within this thesis, the small sample sizes in **chapters 4-6**, compared to other dietary interventional studies (Basu *et al.*, 2009, 2010a, 2011, Stull *et al.*, 2010, 2015; Lynn *et al.*, 2014), may be regarded as a limitation. Despite this, other research (Martin *et al.*, 2010; Udani *et al.*, 2011; Rodriguez-Mateos *et al.*, 2013, 2016; Cook *et al.*, 2015; Kent *et al.*, 2015b) has operated with similar sample sizes as those in **chapters 4-6**. Nevertheless, clinical trials with larger samples sizes would enable more appropriate conclusions to be drawn with greater confidence.

This thesis placed a very strong emphasis on the effect of diet, specifically anthocyanins and their metabolites, on cardio-metabolic function. Naturally, dietary guidelines outlined in **chapters 4-6** likely influenced observed responses. In an attempt to uphold ecological validity this thesis did not take a fully reductionist approach; thus, participants maintained their normal habitual diet including polyphenol consumption primarily through fruits and vegetables. Contrary to other human anthocyanin-feeding studies (Basu *et al.*, 2010ab, 2011; Basu, 2011; Barona *et al.*, 2012ab; Stull *et al.*, 2015; Keane *et al.*, 2016ab) this was a strength of **chapters 4-6**, as the residual synergistic effect of MTC interventions with other dietary micronutrients and polyphenols could be assessed. However, this also limited the ability to completely attribute observed effects to MTC interventions alone.

Additionally, dietary restrictions were enforced before each testing session to limit intra- and inter-individual variability for measured variables. In **chapters 4-6**, participants used food diaries for

dietary recall, however this method is not 100% accurate (Mertz *et al.*, 1991); therefore, absolute adherence to the same diet 3 days before each session could not be guaranteed. The overnight fast was another dietary restriction placed to limit intra- and inter-individual variability, at the expense of upholding ecological validity. Moreover, the overnight fast likely influenced the absorption and bioavailability of MTC polyphenols, as fasting has been shown to augment polyphenol bioavailability due to the absence of absorption inhibitors (Bohn, 2014). The pharmacokinetic profile of MTC suggests an overnight fast may not have limited its bioefficacy, since appearance of polyphenols and their metabolites at nM and µM concentrations (Fernandes *et al.*, 2015; Keane *et al.*, 2016a) were observed up to 48 hours post-consumption and not limited to just a few hours after intake (De Ferrars *et al.*, 2014; Seymour *et al.*, 2014). Consequently, another limitation would be the timeframe that variables were monitored (≤5 hours) was not long enough to study the biological effects of phytochemicals after enterohepatic metabolism. Future work should consider monitoring responses according to the pharmacokinetic profile of secondary metabolites and phase II conjugates and also after discontinuing supplementation.

Section 2.1.4 outlined factors that affect batch-to-batch variation of the polyphenol content within foods. Due to limited resources this thesis was unable to measure polyphenol content in each batch of MTC juice and capsules used.

Another limitation was the theoretical calculation of LDL based on total cholesterol, triglycerides and HDL concentrations using the formulae of Friedewald, Levy and Fredrickson, (1972) and Ahmadi *et al.* (2008). However, due to the resources available direct measurement of LDL was not possible, but future researchers would be advised to follow this approach if possible.

Lastly, double or triple blinding would have made the study design of **chapters 4-6** more robust. However, a major strength of the human trials within this thesis was its research design. **Chapters 4-6** employed crossover, randomised controlled trials; recommended for medical research (Sibbald

and Roland, 1998) and assessing dietary interventions (Lucey, Heneghan and Kiely, 2016). Furthermore, responses in **chapters 4-6** were observed in humans rather than animal models, with measurement of clinically relevant, diagnostic functional and blood-based biomarkers. Application of research into practice was ensured through upholding ecological validity wherever possible; by maintaining habitual diet and using safe, pragmatic, feasible and economically viable supplementation strategies.

Chapter 9

General Conclusion

The global outlook relating to cardio-metabolic disease is bleak and projected to become worse! Therefore, it was important for the research within this thesis to contribute towards alleviating this major problem and ultimately, prevent unnecessary deaths arising from cardio-metabolic disease.

This thesis set out to use lifestyle interventions to prevent the development of cardio-metabolic diseases in healthy and MetS populations, susceptible to greater risk of developing cardio-metabolic diseases. Lifestyle interventions have become increasingly popular amongst policymakers and the public in the fight against disease (Arena *et al.*, 2015). Subsequently, an overarching aim of this thesis was to conduct ecologically valid research with simple application to daily life. This was achieved through provision of a safe, readily available dietary intervention supplementary to habitual diet; used in conjunction with exercise. Specifically, this thesis examined the effects of Montmorency tart cherries, with and without FATMAX exercise on cardio-metabolic biomarkers in healthy and MetS populations. It was envisaged that these interventions would be used as preventative measures to lessen the global burden of cardio-metabolic disease, and thus this thesis aimed to provide evidence for their use.

9.1. Contribution to Knowledge

The discoveries made in this thesis have contributed to knowledge and subsequently filled numerous gaps in literature.

In summation, this thesis has shown MTCJ and FATMAX exercise was not effective in improving cardio-metabolic biomarkers in healthy individuals, however examination of responses within clinical populations is warranted. More significantly, this thesis has highlighted low-dose, short-term anti-oxidant supplementation from MTCJ seems to be effective at improving certain aspects of cardio-metabolic function. Secondly, the potent anti-hypertensive properties of MTCJ were demonstrated in a population of humans with MetS and confirm similar observations from previous literature in other clinical populations. Importantly, the improvement in 24-hour BP,

demonstrated for the first time with a cherry intervention in **chapter 6**, indicates MTCJ should be considered as an anti-hypertensive therapy in future clinical trials. Furthermore, potential for physiological improvements in insulin sensitivity and insulin resistance in humans with MetS and underlying insulin resistance was shown after consuming MTC interventions, which may be of clinical value as it indicates MTC may be able to reverse MetS. Moreover, lipid profile improvements with MTCJ and a shift in substrate oxidation towards augmentation of resting fat oxidation may enhance metabolic flexibility and further contribute to reducing insulin resistance. The implications of such observations are notable, since health practitioners, clinicians and researchers may be able to exploit the benefits of MTC; to prevent and/or slow cardio-metabolic dysregulation and promote healthy ageing through encouraging positive lifestyle changes.

Lastly, identification of the PPAR signalling pathway, in *C. elegans*, as the mechanism through which MTC likely operates, enables mechanism-based strategies using MTC to be formulated to target broader aspects of cardio-metabolic dysregulation, owing to cross-talk between pathways. Due to their specificity in mechanism of action, pharmacological interventions tend to treat only a single component of cardio-metabolic dysregulation; requiring consumption of multiple drugs each with associated side effects. In contrast, as a single, naturally-occurring, safe intervention with currently no reported side effects; this series of studies have shown MTC interventions may clinically improve various cardio-metabolic biomarkers in humans and perhaps be used as an adjuvant to, or replace, pharmacological drugs in the future.

9.2. Future Research

Throughout this thesis, specific recommendations for future work have been detailed. This section indicates directions that future research should pursue to further knowledge in this field.

Given the limited resources, only small-scale studies were conducted within this thesis. Consequently, there is a need to conduct larger, multi-centre, clinical trials in healthy, MetS and

other clinical populations; to fully gauge the efficacy of MTC interventions on cardio-metabolic health. Furthermore, clinical trials with a longer duration (>6 months) of supplementation and monitoring would provide ecologically valid and clinically useful information pertaining to the effectiveness of MTC interventions on cardio-metabolic health. Identification of an optimal supplementation strategy is also required after consideration of the population being examined and the target cardio-metabolic condition to be improved. Overall, comprehensive, large-scale, long-term, examinations of the effect of consuming MTC interventions in conjunction with habitual diet is ultimately required; to draw accurate conclusions on the use of MTC interventions to improve cardio-metabolic function in various populations.

Chapters 4, 5 and **6** monitored habitual dietary consumption through food diaries, to assess compliance to dietary guidelines. Comprehension of the results from these studies would be enhanced by future work conducting detailed statistical analysis of cardio-metabolic responses to MTC interventions after consideration of habitual diet, particularly total polyphenol and anthocyanin intake. This would inform practitioners of the efficacy of MTC on cardio-metabolic function in relation to habitual dietary practices, thus assist in the decision to prescribe MTC interventions on an individual scale.

Across research in the field of polyphenols and health, there is an inherent lack of understanding relating to mechanisms of action, which extends to MTC interventions. **Chapter 6** isolated ACE inhibition to explain the anti-hypertensive properties of MTCJ but was unable to confirm this. **Chapter 7** elucidated molecular mechanisms of action, along the PPAR pathway, with dilutions of MTC concentrate in *C. elegans*, however for clinical benefit a similar study in humans is necessitated.

Recommendations for future mechanistic studies that would enhance current understanding include a comprehensive pharmacokinetic and pharmacodynamic analysis of MTCJ and MTCC in

various populations, including MetS. Analysis of the health effects related to compounds derived from MTC can be correlated if future work can identify compounds and the concentrations they appear in biological samples (Amiot, Riva and Vinet, 2016). Technological advances in recent years have assisted the ability to detect the myriad of parent polyphenolic compounds and their metabolites emanating from MTC. Subsequently, 'omics' technology, particularly nutrigenomics (Ferguson *et al.*, 2016), metabolomics (Brennan, 2008) and epigenomics (Mathers, 2008), would be useful tools for future research to correlate the health benefits of MTC to its polyphenolic compounds. Furthermore, *section 2.1.5.2.2* emphasised the strong influence of the gut microbiome on cardio-metabolic health and the role polyphenols play in that relationship. Given the recent upsurge in gut microbiome research and the revelation that polyphenol metabolism is based on an individuals' enterotype and metabotype (Amiot, Riva and Vinet, 2016; Espín, González-Sarrías and Tomás-Barberán, 2017), future work should profile gut microbiome changes after MTC consumption and correlate this to cardio-metabolic responses. Ultimately, this conforms to the current ideology of 'personalised nutrition' (Perez-Martinez *et al.*, 2012), as it would negate the inter-individual variance observed in **chapters 4-6** and enable prescription of specific dietary strategies involving MTC on an individual case-by-case basis.

Finally, the recommendations for future work outlined above can provide further clarity and understanding to the findings presented within this thesis. Although this thesis presents novel discoveries and corroborates previous literature, more clinical evidence is undoubtedly required to endorse MTC as an intervention against cardio-metabolic disease. Nevertheless, perhaps MTC and exercise are preventative lifestyle interventions to call upon in the battle against cardio-metabolic disease. After all, prevention is better than cure, especially when there is no cure!

Chapter 10

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Chapter 11

Appendices

Appendix 1

Table 11.1. Limits of agreement for FATMAX and MFO for the two protocols assessed during pilot testing.

	Protocol 1 (15 W increments every 2 mins)	Protocol 2 (10 W increments every 3 mins)
FATMAX (% of $\dot{V}O_{2peak}$)	4.50 [-11.50 – 20.50]	4.26 [-6.20 – 10.50]
MFO ($g.min^{-1}$)	-0.04 [-0.23 – 0.14]	0.03 [-0.13 – 0.20]

Table 11.2. Coefficient of Variation for FATMAX and MFO for the two protocols assessed during pilot testing.

	Protocol 1 (15 W increments every 2 mins)	Protocol 2 (10 W increments every 3 mins)
FATMAX	13.74%	6.17%
MFO	22.71%	16.21%

Appendix 2

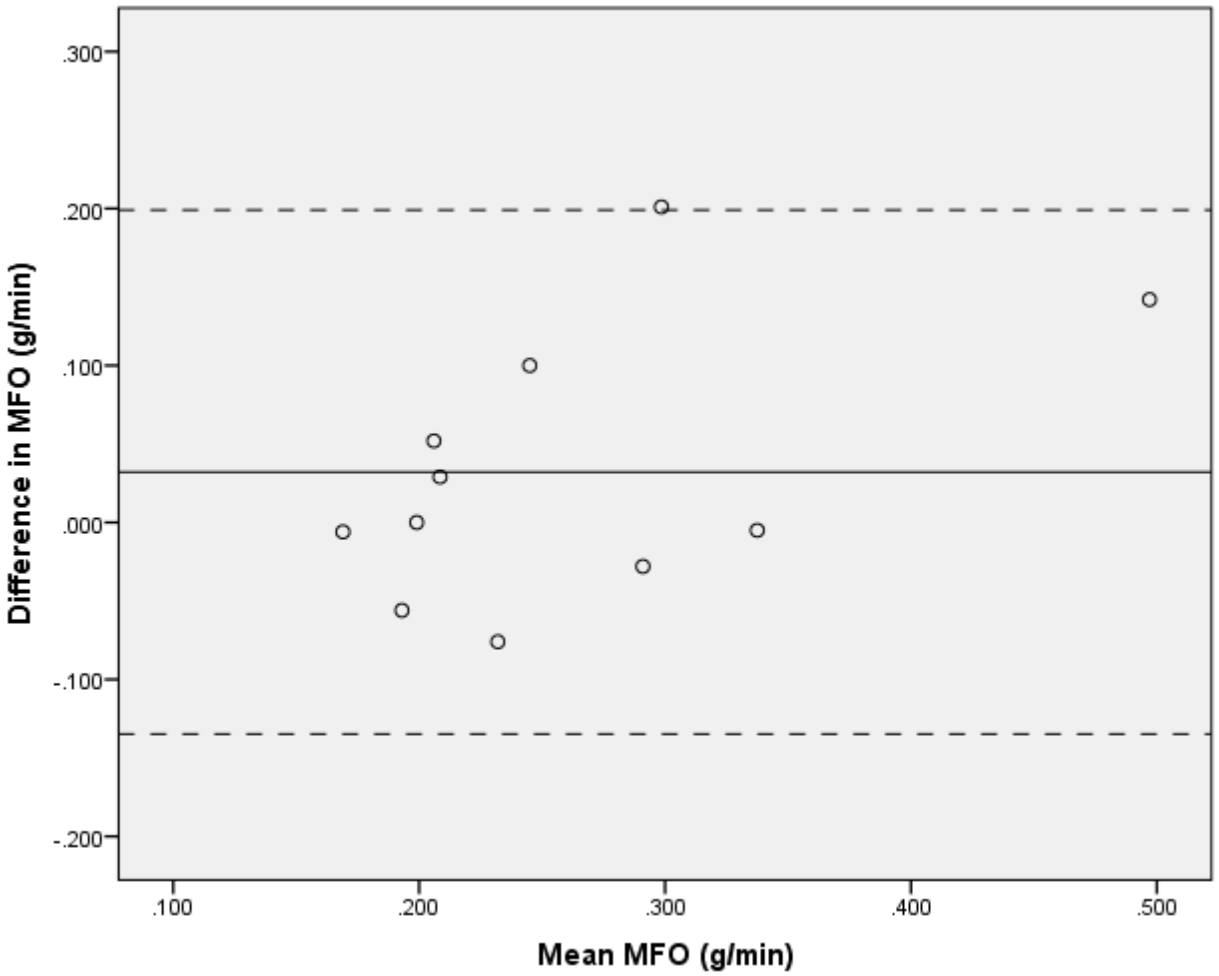

Figure 11.1. Bland-Altman plot of MFO during the final minute of the stage representing FATMAX. Limits of agreement 0.03 [-0.13 – 0.20] g.min^{-1}. Bias (solid line) and 95% limits of agreement (dashed lines).

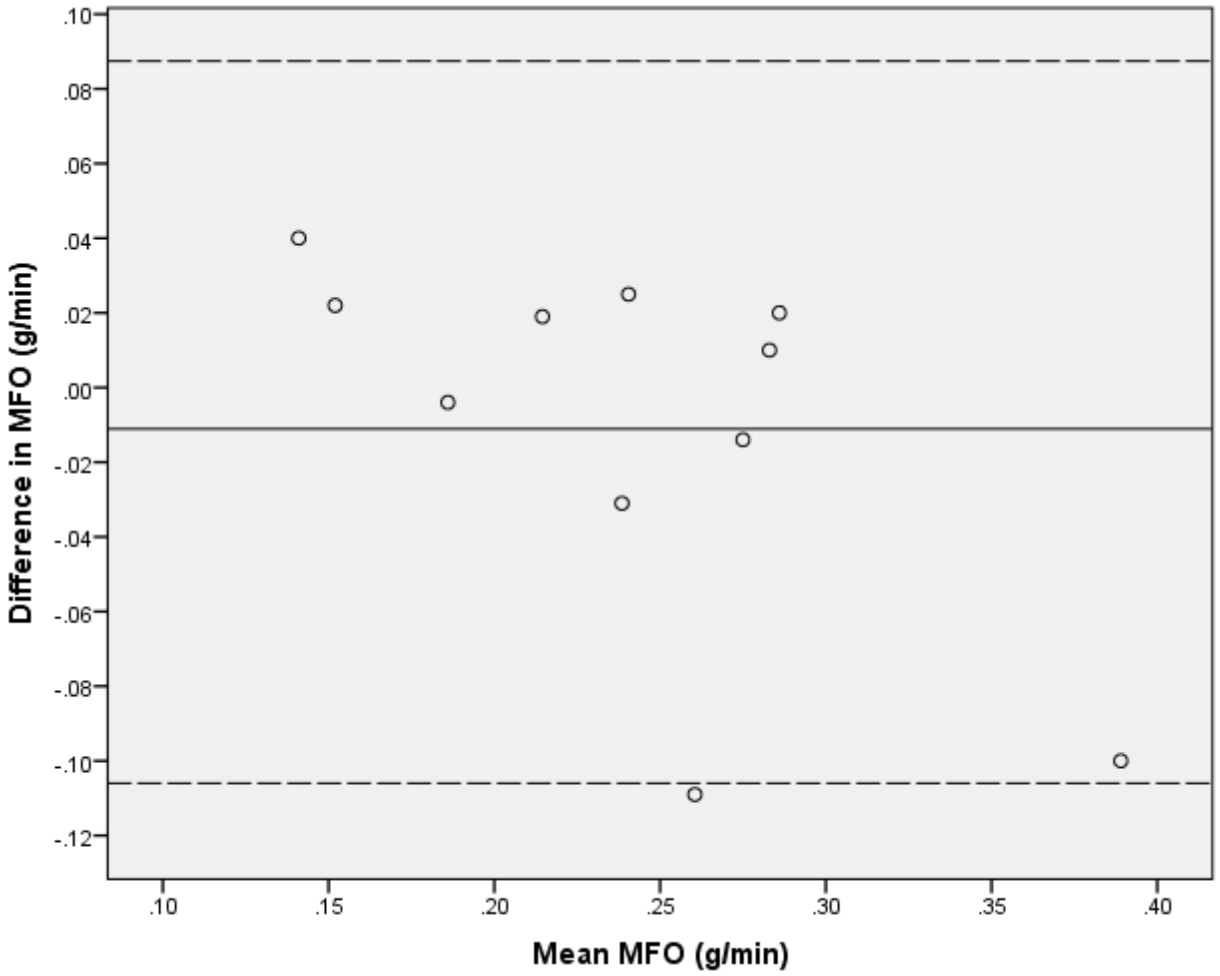

Figure 11.2. Bland-Altman plot of MFO during the final 50 minutes of the one-hour sub-maximal trial at FATMAX. Limits of agreement -0.01 [-0.11 – 0.09] $g.min^{-1}$. Bias (solid line) and 95% limits of agreement (dashed lines).

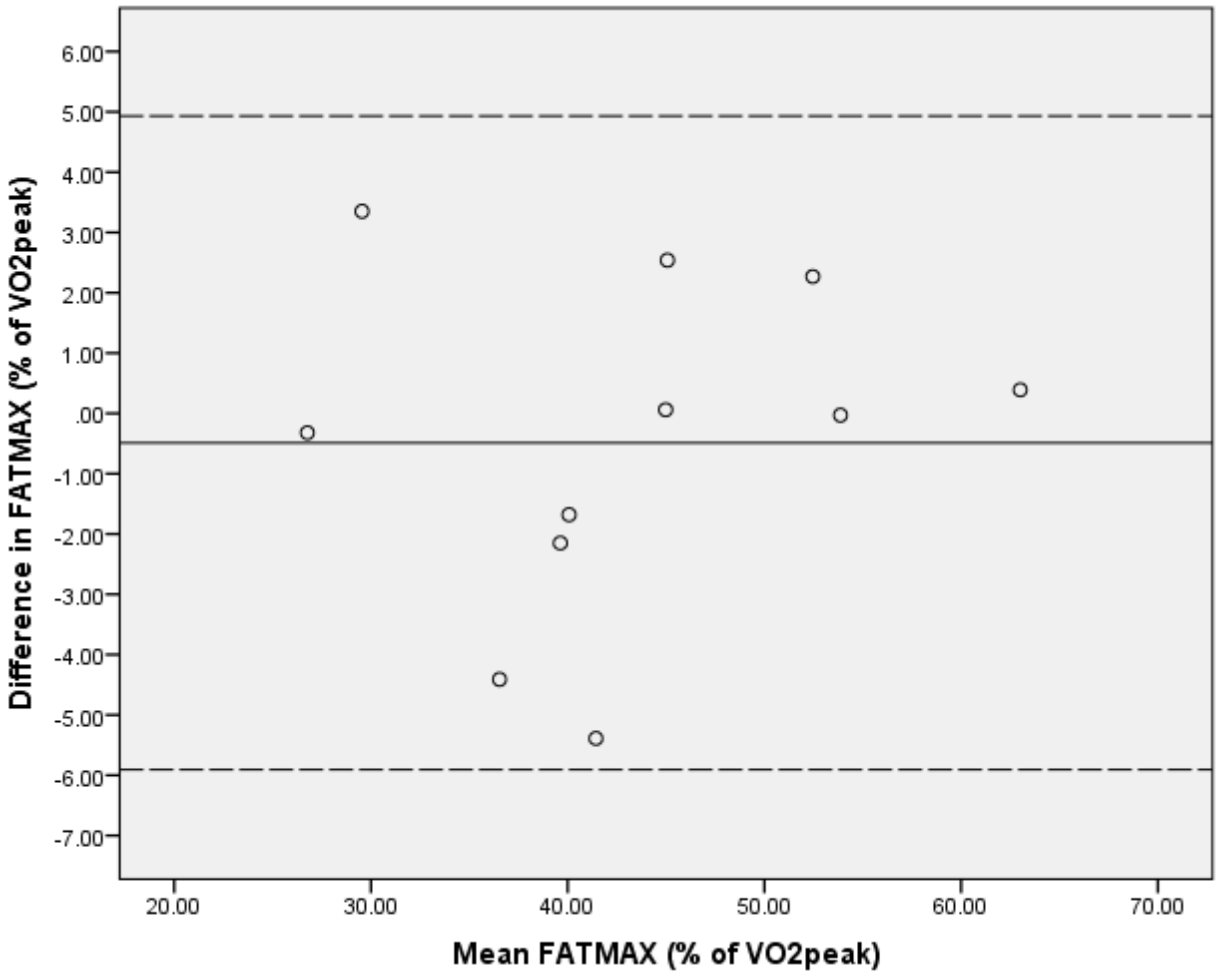

Figure 11.3. Bland-Altman plot of the final minute of the stage representing FATMAX presented in terms of %$\dot{V}O_{2peak}$. Limits of agreement -0.49 [-5.91 – 4.93] %$\dot{V}O_{2peak}$. Bias (solid line) and 95% limits of agreement (dashed lines).

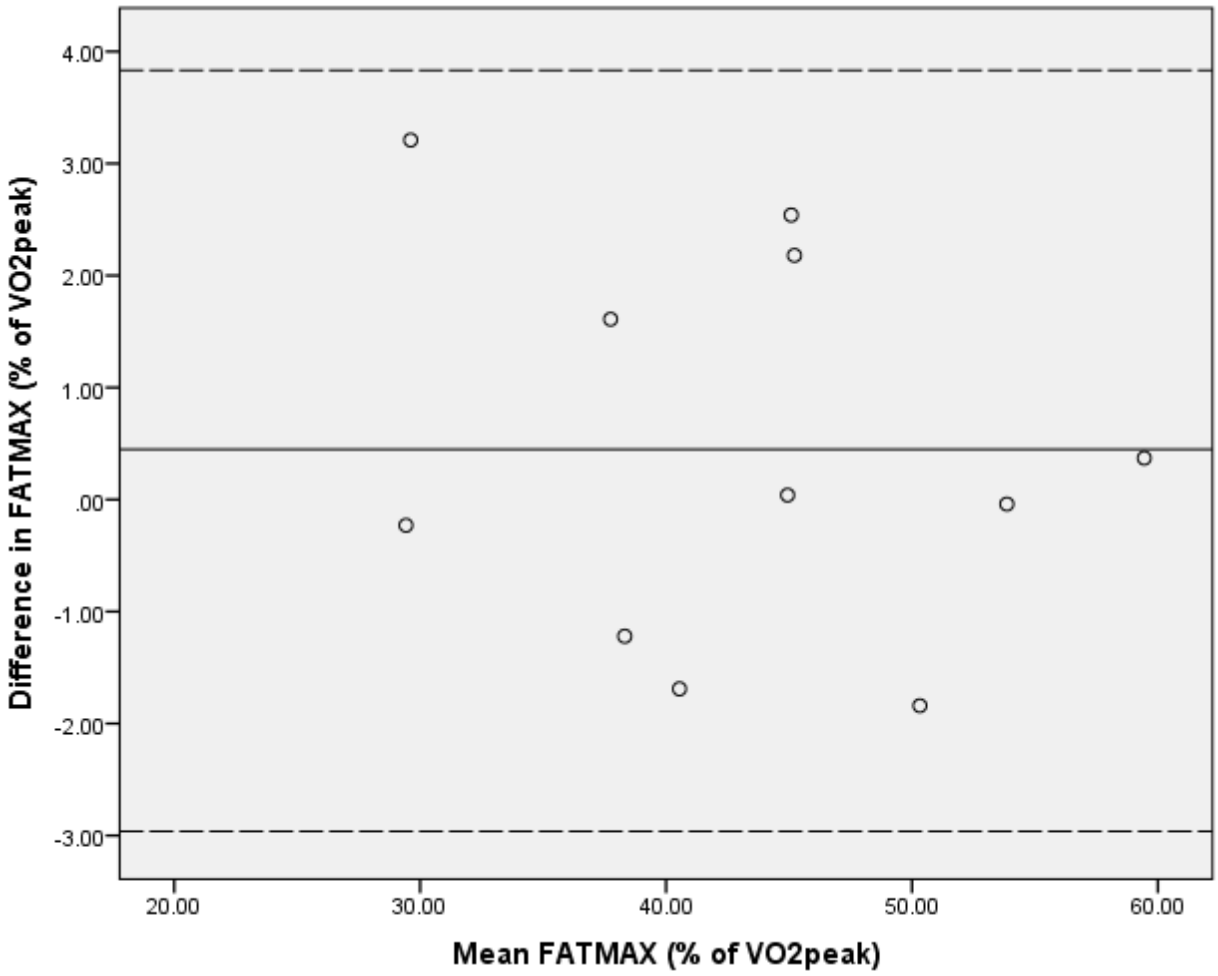

Figure 11.4. Bland-Altman plot of FATMAX presented as $\%\dot{V}O_{2peak}$ during the final 50 minutes of the one-hour sub-maximal trial at FATMAX. Limits of agreement 0.45 [-2.93 – 3.83] $\%\dot{V}O_{2peak}$. Bias (solid line) and 95% limits of agreement (dashed lines).

Appendix 3

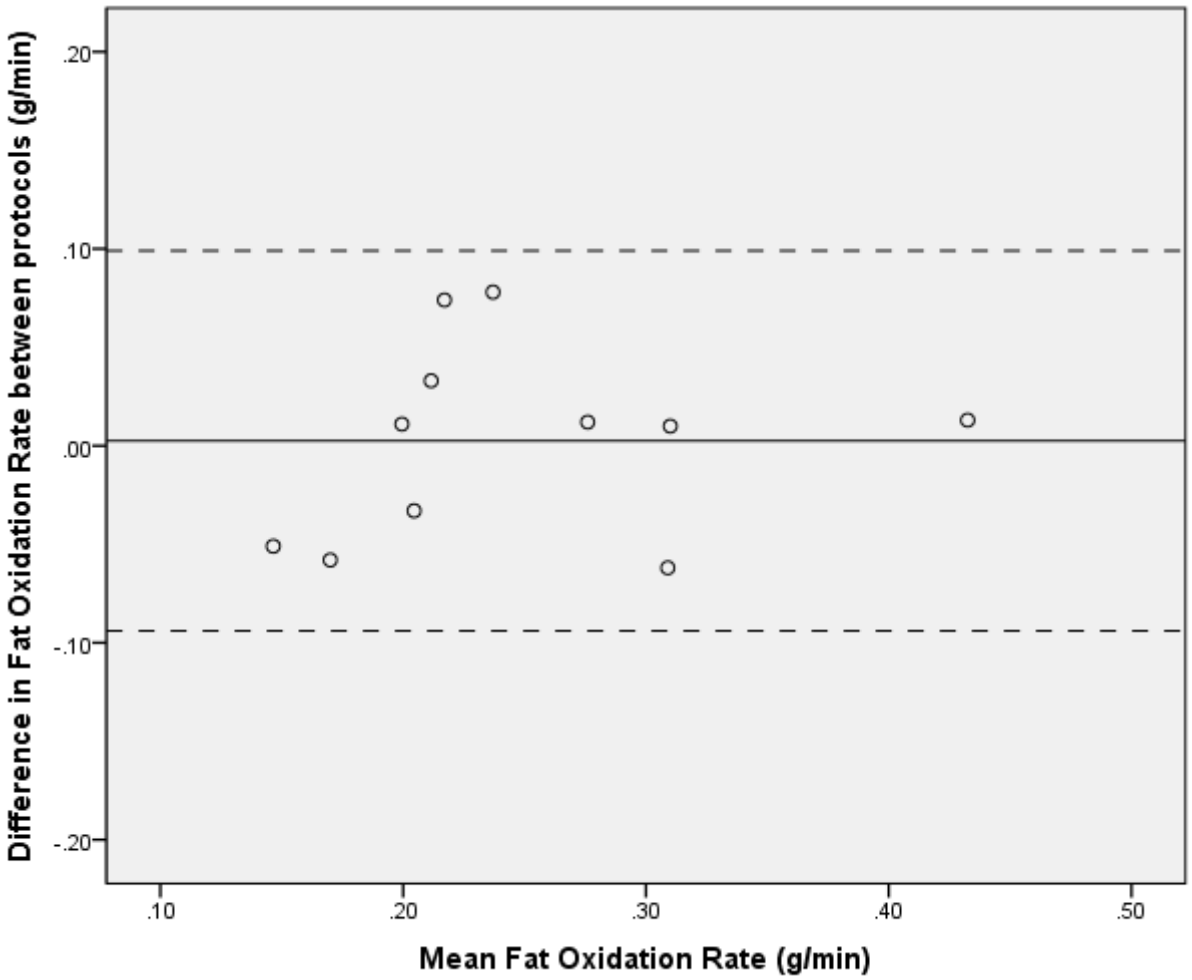

Figure 11.5. Bland-Altman plot comparing fat oxidation rates averaged for the final 50 minutes of the one-hour exercise and mean fat oxidation rates obtained during the final minute of the stage eliciting MFO in FATMAX determination test. Limits of agreement 0.00 [-0.09 – 0.09] $g.min^{-1}$. Bias (solid line) and 95% limits of agreement (dashed lines).

Appendix 4

Table 11.3. Mean ± SD absolute raw data for blood-based biomarkers per treatment condition from **Chapter 5, Part A.**

			Post-bolus Time Points		
		Pre-Bolus	1 hr	3 hr	5 hr
Glucose ($mmol.L^{-1}$)	PLA	5.40 ± 0.11	5.75 ± 0.06	4.82 ± 0.07	4.77 ± 0.09
	MTCC	5.10 ± 0.13	4.99 ± 0.11	4.88 ± 0.14	4.82 ± 0.11
	MTCJ	5.50 ± 0.12	5.28 ± 0.09	4.71 ± 0.18	4.78 ± 0.10
Insulin ($pmol.L^{-1}$)	PLA	115.51 ± 10.39	176.33 ± 7.51	106.52 ± 3.39	99.71 ± 7.00
	MTCC	108.18 ± 4.61	98.33 ± 5.12	77.42 ± 3.04	86.99 ± 1.81
	MTCJ	125.33 ± 10.45	149.15 ± 13.77	76.53 ± 8.59	96.27 ± 4.06
Triglycerides ($mmol.L^{-1}$)	PLA	1.2 ± 0.0	1.3 ± 0.1	1.4 ± 0.1	1.5 ± 0.0
	MTCC	1.4 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.3 ± 0.1
	MTCJ	1.5 ± 0.0	1.4 ± 0.1	1.5 ± 0.1	1.6 ± 0.1
Total Cholesterol ($mmol.L^{-1}$)	PLA	3.51 ± 0.19	3.55 ± 0.21	3.67 ± 0.23	3.80 ± 0.19
	MTCC	3.45 ± 0.12	3.33 ± 0.23	3.33 ± 0.23	3.64 ± 0.07
	MTCJ	3.82 ± 0.09	3.77 ± 0.18	3.85 ± 0.11	3.91 ± 0.12
HDL ($mmol.L^{-1}$)	PLA	1.31 ± 0.08	1.19 ± 0.03	1.21 ± 0.06	1.13 ± 0.02
	MTCC	1.27 ± 0.12	1.22 ± 0.04	1.23 ± 0.09	1.24 ± 0.02
	MTCJ	1.31 ± 0.12	1.24 ± 0.07	1.32 ± 0.05	1.22 ± 0.06
LDL ($mmol.L^{-1}$)	PLA	2.44 ± 0.20	2.69 ± 0.25	2.80 ± 0.23	3.15 ± 1.48
	MTCC	2.53 ± 1.11	2.60 ± 0.23	2.46 ± 0.29	2.59 ± 0.18
	MTCJ	2.78 ± 0.12	2.86 ± 0.22	2.90 ± 0.11	3.02 ± 0.21

Table 11.4. Mean ± SD absolute raw data for cardiac haemodynamic parameters per treatment condition from **Chapter 5, Part A**.

			Post-bolus Time Points					
		Pre-Bolus	30 mins	1 hr	2 hr	3 hr	4 hr	5 hr
Brachial SBP (mmHg)	PLA	130 ± 11	130 ± 12	133 ± 11	133 ± 11	133 ± 10	132 ± 12	134 ± 13
	MTCC	125 ± 11	128 ± 10	128 ± 12	125 ± 10	124 ± 7	124 ± 7	128 ± 10
	MTCJ	129 ± 10	128 ± 10	127 ± 12	121 ± 10	127 ± 7	128 ± 7	126 ± 14
Brachial DBP (mmHg)	PLA	71 ± 4	74 ± 3	76 ± 5	76 ± 4	79 ± 7	76 ± 6	75 ± 6
	MTCC	71 ± 6	72 ± 8	75 ± 10	73 ± 9	71 ± 8	72 ± 9	74 ± 6
	MTCJ	68 ± 4	71 ± 6	74 ± 6	71 ± 7	75 ± 8	74 ± 9	74 ± 8
MAP (mmHg)	PLA	93 ± 6	94 ± 3	98 ± 5	98 ± 5	100 ± 9	98 ± 10	98 ± 9
	MTCC	92 ± 8	95 ± 10	98 ± 11	94 ± 10	94 ± 10	93 ± 11	97 ± 9
	MTCJ	90 ± 5	92 ± 7	92 ± 6	86 ± 8	94 ± 7	94 ± 10	88 ± 10
Cardiac Output ($L.min^{-1}$)	PLA	6.70 ± 1.47	6.81 ± 1.77	6.68 ± 2.61	6.39 ± 2.26	6.12 ± 1.86	6.17 ± 2.23	6.35 ± 1.85
	MTCC	6.68 ± 1.83	6.58 ± 1.76	6.44 ± 2.06	6.55 ± 1.88	6.71 ± 1.77	6.37 ± 2.12	6.47 ± 2.07
	MTCJ	6.73 ± 1.76	6.40 ± 2.32	6.27 ± 2.30	6.69 ± 1.53	6.39 ± 1.90	6.11 ± 2.26	6.12 ± 2.08
Stroke Volume (mL)	PLA	105 ± 19	106 ± 17	104 ± 23	104 ± 22	100 ± 27	100 ± 29	107 ± 21
	MTCC	107 ± 22	113 ± 24	106 ± 32	111 ± 24	115 ± 26	108 ± 27	110 ± 33
	MTCJ	107 ± 23	108 ± 28	103 ± 26	108 ± 21	103 ± 19	108 ± 34	109 ± 28
TPR ($mmHg \cdot min^{-1} \cdot L$)	PLA	0.88 ± 0.15	0.88 ± 0.27	1.03 ± 0.42	1.02 ± 0.37	1.14 ± 0.53	1.04 ± 0.31	0.97 ± 0.28
	MTCC	0.91 ± 0.27	0.92 ± 0.21	1.00 ± 0.33	0.93 ± 0.43	0.99 ± 0.43	1.00 ± 0.41	1.03 ± 0.36
	MTCJ	0.93 ± 0.35	1.01 ± 0.43	1.00 ± 0.32	0.90 ± 0.24	1.03 ± 0.31	1.03 ± 0.34	1.04 ± 0.21
HR ($beats.min^{-1}$)	PLA	65 ± 12	64 ± 11	62 ± 10	60 ± 11	60 ± 9	58 ± 9	58 ± 8
	MTCC	65 ± 10	60 ± 8	61 ± 7	61 ± 8	59 ± 9	60 ± 10	61 ± 9
	MTCJ	63 ± 11	63 ± 11	62 ± 12	58 ± 12	56 ± 10	56 ± 10	55 ± 9

Table 11.5. Mean ± SD absolute raw data for PWA parameters per treatment condition from **Chapter 5, Part A**.

			Post-bolus Time Points					
		Pre-Bolus	30 mins	1 hr	2 hr	3 hr	4 hr	5 hr
Aortic SBP (mmHg)	PLA	120 ± 11	121 ± 12	123 ± 13	124 ± 13	122 ± 13	124 ± 12	125 ± 11
	MTCC	118 ± 11	120 ± 14	122 ± 15	122 ± 15	120 ± 15	120 ± 14	122 ± 13
	MTCJ	120 ± 13	119 ± 13	119 ± 14	118 ± 13	121 ± 13	122 ± 14	123 ± 13
Aortic DBP (mmHg)	PLA	64 ± 5	64 ± 4	64 ± 4	67 ± 6	67 ± 11	68 ± 10	66 ± 6
	MTCC	64 ± 2	69 ± 1	67 ± 10	68 ± 6	64 ± 2	67 ± 8	70 ± 5
	MTCJ	60 ± 6	58 ± 6	58 ± 7	62 ± 7	61 ± 2	61 ± 4	61 ± 4
Pulse Pressure (mmHg)	PLA	56 ± 7	57 ± 8	59 ± 4	57 ± 1	55 ± 8	56 ± 8	59 ± 7
	MTCC	54 ± 4	51 ± 8	55 ± 11	54 ± 4	56 ± 6	53 ± 6	52 ± 11
	MTCJ	60 ± 2	61 ± 5	61 ± 9	56 ± 6	60 ± 8	61 ± 8	62 ± 9
AIx at HR75*^$ (%)	PLA	17 ± 12	17 ± 11	16 ± 11	19 ± 11	19 ± 11	20 ± 10	22 ± 11
	MTCC	17 ± 9	17 ± 13	16 ± 13	15 ± 14	17 ± 12	19 ± 13	20 ± 14
	MTCJ	17 ± 12	17 ± 11	16 ± 12	15 ± 13	15 ± 10	17 ± 12	19 ± 12
SEVR (%)	PLA	177 ± 46	176 ± 40	188 ± 37	192 ± 23	187 ± 36	193 ± 37	180 ± 33
	MTCC	174 ± 45	189 ± 33	183 ± 34	191 ± 39	190 ± 37	193 ± 41	183 ± 35
	MTCJ	185 ± 40	191 ± 37	193 ± 40	216 ± 42	209 ± 38	206 ± 30	197 ± 30

*Denotes significant main effect for time with *post-hoc* identifying differences between 30 minutes and 5-hours post-bolus. ^Denotes significant main effect for time with *post-hoc* identifying differences between 1-hour and 5-hours post-bolus. $^{\$}$Denotes significant main effect for time with *post-hoc* identifying differences between 2-hours and 5-hours post-bolus.

Table 11.6. Mean ± SD absolute raw data for resting metabolic rate parameters per treatment condition from **Chapter 5, Part A**.

			Post-bolus Time Points					
		Pre-Bolus	30 mins	1 hr	2 hr	3 hr	4 hr	5 hr
Resting EE ($kcal.day^{-1}$)	PLA	1685 ± 407	1798 ± 345	1779 ± 351	1720 ± 293	1830 ± 291	1815 ± 285	1810 ± 336
	MTCC	1739 ± 449	1784 ± 372	1791 ± 300	1871 ± 301	1778 ± 299	1882 ± 378	1976 ± 324
	MTCJ	1706 ± 394	1837 ± 415	1795 ± 340	1832 ± 358	1817 ± 363	1873 ± 433	1922 ± 466
Resting RER (AU)	PLA	0.89 ± 0.03	0.92 ± 0.07	0.87 ± 0.07	0.86 ± 0.08	0.82 ± 0.08	0.82 ± 0.07	0.81 ± 0.07
	MTCC	0.90 ± 0.09	0.87 ± 0.09	0.81 ± 0.08	0.80 ± 0.06	0.76 ± 0.06	0.76 ± 0.07	0.77 ± 0.08
	MTCJ	0.92 ± 0.08	0.95 ± 0.07	0.87 ± 0.07	0.84 ± 0.07	0.80 ± 0.07	0.81 ± 0.06	0.79 ± 0.05
Resting Fat Oxidation ($g.min^{-1}$)	PLA	0.03 ± 0.01	0.01 ± 0.04	0.05 ± 0.04	0.05 ± 0.03	0.06 ± 0.06	0.07 ± 0.04	0.09 ± 0.04
	MTCC	0.04 ± 0.04	0.06 ± 0.04	0.07 ± 0.04	0.08 ± 0.03	0.11 ± 0.04	0.10 ± 0.03	0.10 ± 0.04
	MTCJ	0.04 ± 0.03	0.02 ± 0.04	0.06 ± 0.04	0.07 ± 0.04	0.09 ± 0.04	0.09 ± 0.04	0.13 ± 0.10
Resting CHO Oxidation ($g.min^{-1}$)	PLA	0.21 ± 0.07	0.26 ± 0.10	0.20 ± 0.11	0.17 ± 0.11	0.17 ± 0.18	0.15 ± 0.13	0.12 ± 0.10
	MTCC	0.22 ± 0.13	0.18 ± 0.07	0.15 ± 0.09	0.13 ± 0.10	0.05 ± 0.07	0.09 ± 0.11	0.09 ± 0.12
	MTCJ	0.25 ± 0.11	0.27 ± 0.09	0.18 ± 0.08	0.15 ± 0.08	0.11 ± 0.09	0.12 ± 0.06	0.04 ± 0.19

Appendix 5

Table 11.7. Mean ± SD absolute raw data for blood-based biomarkers per treatment condition from **Chapter 6**.

			Post-bolus Time Points		
		Pre-Bolus	1 hr	3 hr	5 hr
Glucose ($mmol.L^{-1}$)	PLA	5.36 ± 0.25	5.83 ± 0.24	5.28 ± 0.13	5.49 ± 0.19
	MTCJ	5.39 ± 0.23	5.88 ± 0.24	5.13 ± 0.22	5.14 ± 0.13
Insulin ($pmol.L^{-1}$)	PLA	136.59 ± 72.18	146.29 ± 76.90	104.26 ± 59.70	97.77 ± 64.19
	MTCJ	125.96 ± 65.57	153.23 ± 87.21	97.43 ± 54.57	93.76 ± 65.29
Triglycerides ($mmol.L^{-1}$)	PLA	1.3 ± 0.1	1.4 ± 0.1	1.5 ± 0.1	1.6 ± 0.1
	MTCJ	1.3 ± 0.1	1.3 ± 0.1	1.5 ± 0.1	1.6 ± 0.1
Total Cholesterol ($mmol.L^{-1}$)	PLA	4.03 ± 0.33	4.17 ± 0.34	4.23 ± 0.24	4.25 ± 0.19
	MTCJ	4.14 ± 0.41	4.17 ± 0.39	4.03 ± 0.45	3.96 ± 0.17
HDL ($mmol.L^{-1}$)	PLA	1.30 ± 0.01	1.30 ± 0.02	1.27 ± 0.02	1.27 ± 0.02
	MTCJ	1.40 ± 0.02	1.40 ± 0.03	1.40 ± 0.01	1.36 ± 0.03
LDL ($mmol.L^{-1}$)	PLA	2.65 ± 0.31	2.99 ± 0.37	3.14 ± 0.30	3.30 ± 0.28
	MTCJ	2.71 ± 0.46	2.77 ± 0.35	2.91 ± 0.36	2.83 ± 0.31

Appendix 6

Table 11.8. Mean ± SD absolute raw data for cardiac haemodynamic and PWA parameters per treatment condition from **Chapter 6**.

				Post-bolus Time Points					
		Pre 6 days Suppl.	Post 6 days Suppl.	30 minutes	1 hr	2 hr	3 hr	4 hr	5 hr
Brachial SBP (mmHg)	PLA	127 ± 16	128 ± 17	133 ± 13	130 ± 14	132 ± 19	132 ± 19	128 ± 10	129 ± 13
	MTCJ	134 ± 17	128 ± 15	127 ± 13	128 ± 13	121 ± 10	127 ± 12	129 ± 18	133 ± 14
Brachial DBP (mmHg)	PLA	75 ± 10	74 ± 7	76 ± 8	72 ± 5	74 ± 5	73 ± 8	74 ± 7	75 ± 5
	MTCJ	75 ± 10	72 ± 7	73 ± 4	74 ± 5	70 ± 7	74 ± 7	73 ± 6	75 ± 4
MAP (mmHg)	PLA	98 ± 12	93 ± 10	99 ± 9	94 ± 8	97 ± 8	96 ± 10	97 ± 10	97 ± 9
	MTCJ	98 ± 12	93 ± 9	95 ± 4	96 ± 7	93 ± 6	95 ± 8	95 ± 8	96 ± 8
HR ($beats.min^{-1}$)	PLA	65 ± 12	67 ± 14	68 ± 16	67 ± 16	66 ± 17	66 ± 16	65 ± 15	65 ± 5
	MTCJ	65 ± 14	63 ± 11	66 ± 11	64 ± 12	62 ± 13	61 ± 11	62 ± 12	60 ± 11
Cardiac Output ($L.min^{-1}$)	PLA	6.85 ± 2.46	6.64 ± 2.37	6.86 ± 2.46	7.05 ± 2.74	7.00 ± 4.07	7.10 ± 3.23	6.02 ± 2.22	6.44 ± 2.03
	MTCJ	6.19 ± 2.81	5.85 ± 2.02	5.69 ± 2.97	5.46 ± 2.64	5.52 ± 2.66	5.70 ± 2.36	5.60 ± 2.47	5.87 ± 2.72
Stroke Volume (mL)	PLA	104 ± 26	98 ± 21	100 ± 22	104 ± 21	101 ± 34	105 ± 23	103 ± 26	98 ± 17
	MTCJ	102 ± 20	110 ± 29	105 ± 25	98 ± 22	101 ± 27	100 ± 26	103 ± 29	109 ± 29
TPR ($mmHg \cdot min^{-1} \cdot L$)	PLA	1.03 ± 0.48	0.91 ± 0.23	0.96 ± 0.33	0.89 ± 0.27	1.04 ± 0.44	0.93 ± 0.32	1.09 ± 0.38	0.99 ± 0.29
	MTCJ	0.93 ± 0.37	0.79 ± 0.28	1.00 ± 0.42	0.98 ± 0.29	0.95 ± 0.29	0.95 ± 0.31	0.97 ± 0.34	0.94 ± 0.38
Aortic SBP (mmHg)	PLA	124 ± 12	119 ± 15	120 ± 12	119 ± 12	121 ± 14	124 ± 13	119 ± 15	120 ± 13
	MTCJ	124 ± 15	120 ± 15	121 ± 14	118 ± 14	116 ± 13	120 ± 14	119 ± 15	120 ± 13

Aortic DBP (mmHg)	PLA	80 ± 7	80 ± 9	82 ± 11	80 ± 11	83 ± 9	84 ± 11	84 ± 8	83 ± 7
	MTCJ	81 ± 9	79 ± 10	79 ± 9	80 ± 9	78 ± 8	83 ± 10	81 ± 7	80 ± 8
AP (mmHg)	PLA	11 ± 6	9 ± 6	8 ± 5	7 ± 5	9 ± 6	11 ± 8	9 ± 6	10 ± 8
	MTCJ	12 ± 7	9 ± 7	9 ± 8	9 ± 8	9 ± 7	10 ± 8	10 ± 8	11 ± 8
Pulse Pressure (mmHg)	PLA	38 ± 9	35 ± 11	38 ± 7	39 ± 7	38 ± 7	39 ± 13	35 ± 8	37 ± 10
	MTCJ	39 ± 11	38 ± 10	42 ± 10	38 ± 11	38 ± 10	37 ± 11	38 ± 11	40 ± 10
AIx (%)	PLA	26 ± 13	23 ± 12	23 ± 12	24 ± 12	25 ± 13	26 ± 15	26 ± 14	27 ± 14
	MTCJ	25 ± 14	24 ± 12	24 ± 14	24 ± 15	22 ± 14	23 ± 14	24 ± 14	26 ± 14
AIx at HR75 (%)	PLA	22 ± 10	19 ± 11	20 ± 11	21 ± 12	21 ± 11	22 ± 12	23 ± 11	23 ± 12
	MTCJ	21 ± 12	20 ± 11	20 ± 10	20 ± 12	19 ± 11	18 ± 12	19 ± 11	19 ± 12
SEVR (%)	PLA	180 ± 30	192 ± 36	187 ± 37	189 ± 35	192 ± 39	194 ± 43	197 ± 39	191 ± 37
	MTCJ	183 ± 39	178 ± 25	175 ± 29	177 ± 25	187 ± 40	189 ± 33	181 ± 29	177 ± 25

Appendix 7

Table 11.9. Mean ± SD absolute raw data for resting metabolic rate parameters per treatment condition from **Chapter 6**.

			Post-bolus Time Points					
		Pre-Bolus	30 mins	1 hr	2 hr	3 hr	4 hr	5 hr
Resting EE ($kcal.day^{-1}$)	PLA	1847 ± 437	1895 ± 397	1890 ± 360	1835 ± 411	1827 ± 394	1785 ± 394	1785 ± 356
	MTCJ	1794 ± 489	1871 ± 467	1892 ± 408	1781 ± 439	1795 ± 459	1790 ± 443	1865 ± 451
Resting RER (AU)	PLA	0.86 ± 0.06	0.96 ± 0.10	0.93 ± 0.08	0.87 ± 0.08$^{\S\$}$	0.84 ± 0.08$^{\S\$*}$	0.82 ± 0.08$^{\S\$*\wedge}$	0.81 ± 0.05$^{\S\$*\wedge}$
	MTCJ	0.83 ± 0.04	0.98 ± 0.08	0.94 ± 0.08	0.89 ± 0.08	0.86 ± 0.08	0.82 ± 0.07	0.81 ± 0.05
Resting Fat Oxidation ($g.min^{-1}$)	PLA	0.06 ± 0.03	0.04 ± 0.07	0.08 ± 0.09	0.06 ± 0.04§	0.08 ± 0.05§	0.08 ± 0.04§*	0.09 ± 0.04§*
	MTCJ	0.06 ± 0.02	0.01 ± 0.03	0.02 ± 0.04	0.05 ± 0.04	0.07 ± 0.05	0.08 ± 0.04	0.09 ± 0.05
Resting CHO Oxidation ($g.min^{-1}$)	PLA	0.20 ± 0.13	0.25 ± 0.15	0.16 ± 0.20	0.18 ± 0.08§	0.14 ± 0.08§*	0.13 ± 0.09§*	0.12 ± 0.09§*
	MTCJ	0.17 ± 0.08	0.33 ± 0.11	0.30 ± 0.14	0.21 ± 0.08	0.18 ± 0.09	0.14 ± 0.08	0.13 ± 0.05

§Denotes significant difference against 30-minutes post-bolus for main effect of time. $^{\$}$Denotes significant difference against 1-hour post-bolus for main effect of time. *Denotes significant difference against 2-hours post-bolus for main effect of time. ^Denotes significant difference against 3-hours post-bolus for main effect of time.

Appendix 8

Table 11.10. Mean ± SD change from pre-bolus values to post-bolus time points for selected blood-based biomarkers based on MTCJ supplementation strategies.

		Post-bolus Time Points		
		1 hr	3 hr	5 hr
Insulin[#] (pmol.L^{-1})	Single Day	23.80 ± 43.26	-48.80 ± 50.01	-29.10 ± 39.65
	6 days Loading	27.26 ± 42.20	-28.53 ± 29.30	-32.20 ± 32.60
Triglycerides* (mmol.L^{-1})	Single Day	-0.1 ± 0.3	0.0 ± 0.2	0.1 ± 0.4
	6 days Loading	0.0 ± 0.2	0.2 ± 0.1	0.3 ± 0.2
Total Cholesterol (mmol.L^{-1})	Single Day	-0.05 ± 0.25	-0.03 ± 0.18	0.13 ± 0.21
	6 days Loading	0.04 ± 0.33	-0.10 ± 0.34	-0.18 ± 0.49
HDL[$] (mmol.L^{-1})	Single Day	-0.07 ± 0.12	0.01 ± 0.11	-0.09 ± 0.17
	6 days Loading	-0.01 ± 0.05	0.00 ± 0.04	-0.04 ± 0.07
LDL (mmol.L^{-1})	Single Day	0.08 ± 0.52	0.12 ± 0.33	0.24 ± 0.66
	6 days Loading	0.06 ± 0.49	0.20 ± 0.29	0.12 ± 0.39
TC:HDL (AU)	Single Day	-0.01 ± 0.10	0.13 ± 0.35	0.17 ± 0.44
	6 days Loading	0.08 ± 0.40	-0.06 ± 0.28	-0.07 ± 0.44

[#]Denotes significant difference between 1-hour and both 3-hours and 5-hours post-bolus time points.
[$]Denotes significant difference between 3-hours and 5-hours post-bolus time points.
*Denotes significant difference between 1-hour and 3-hours post-bolus time points.

Appendix 9

Table 11.11. Mean ± SD change from pre-bolus values to post-bolus time points for selected cardiac haemodynamic, PWA and RMR parameters based on MTCJ supplementation strategies.

		Post-bolus Time Points					
		30 mins	1 hr	2 hr	3 hr	4 hr	5 hr
Brachial SBP[#] (mmHg)	Single Day	-1 ± 6*	-2 ± 9*	-8 ± 6	-2 ± 6*	-1 ± 8*	-3 ± 12*
	6 days Ldg	-1 ± 8	1 ± 9	-7 ± 10	0 ± 10	1 ± 12	5 ± 11
Brachial DBP[#] (mmHg)	Single Day	3 ± 6	6 ± 4*	3 ± 4	7 ± 6	6 ± 8	6 ± 5
	6 days Ldg	1 ± 7	2 ± 9	-2 ± 7	2 ± 8	1 ± 7	3 ± 7
MAP[#] (mmHg)	Single Day	2 ± 3	2 ± 6	-4 ± 5	4 ± 7*	4 ± 8	-2 ± 12
	6 days Ldg	2 ± 9	3 ± 9	0 ± 7	3 ± 10	2 ± 7	3 ± 10
HR[#] ($beats.min^{-1}$)	Single Day	0 ± 5	-1 ± 5	-5 ± 6[§$]	-7 ± 5[§$]	-7 ± 4[§$]	-8 ± 6[§$]
	6 days Ldg	3 ± 3	1 ± 5	0 ± 5	-2 ± 6	-1 ± 5	-3 ± 5
Cardiac Output ($L.min^{-1}$)	Single Day	0 ± 2	0 ± 1	0 ± 2	0 ± 2	1 ± 2	1 ± 2
	6 days Ldg	0 ± 2	0 ± 2	0 ± 1	0 ± 1	0 ± 1	0 ± 1
Stroke Volume (mL)	Single Day	1 ± 27	-4 ± 19	1 ± 27	-4 ± 30	1 ± 24	2 ± 23
	6 days Ldg	-5 ± 29	-20 ± 33	-9 ± 25	-10 ± 32	-7 ± 19	-1 ± 27
TPR ($mmHg \cdot min^{-1} \cdot L$)	Single Day	0.08 ± 0.33	0.07 ± 0.27	-0.03 ± 0.38	0.10 ± 0.34	0.10 ± 0.28	0.11 ± 0.35
	6 days Ldg	0.21 ± 0.44	0.19 ± 0.35	0.16 ± 0.41	0.15 ± 0.33	0.18 ± 0.29	0.15 ± 0.25
Aortic SBP (mmHg)	Single Day	0 ± 8	-1 ± 8	-2 ± 7	1 ± 4	2 ± 7	3 ± 7
	6 days Ldg	1 ± 5	-2 ± 6	-4 ± 8	0 ± 8	-1 ± 8	0 ± 5

Aortic DBP (mmHg)	Single Day	-2 ± 6	-2 ± 6	2 ± 4	1 ± 8	1 ± 7	1 ± 4
	6 days Ldg	0 ± 2	1 ± 5	-1 ± 5	4 ± 6	2 ± 6	1 ± 5
AP[#] (mmHg)	Single Day	0 ± 8	-1 ± 8	-2 ± 7	1 ± 4	2 ± 7	3 ± 7
	6 days Ldg	1 ± 2	0 ± 2	0 ± 2	1 ± 3	1 ± 3	2 ± 3
Pulse Pressure (mmHg)	Single Day	1 ± 6	1 ± 5	-4 ± 4	0 ± 8	1 ± 6	2 ± 5
	6 days Ldg	1 ± 4	-3 ± 7	-1 ± 6	1 ± 5	0 ± 6	2 ± 5
AIx[#] (%)	Single Day	0 ± 5	1 ± 9	-3 ± 6	-1 ± 6	2 ± 9	5 ± 7
	6 days Ldg	0 ± 5	0 ± 7	-1 ± 5	0 ± 5	0 ± 7	2 ± 8
AIx at HR75 (%)	Single Day	0 ± 4	-1 ± 7	-2 ± 7	-2 ± 7	0 ± 8	2 ± 7
	6 days Ldg	0 ± 3	0 ± 3	-1 ± 5	-2 ± 5	-1 ± 5	-1 ± 6
SEVR[#] (%)	Single Day	6 ± 26	8 ± 22	31 ± 32	24 ± 31	21 ± 31	12 ± 20
	6 days Ldg	-4 ± 23	-2 ± 21	8 ± 37	11 ± 33	3 ± 27	-2 ± 17
RER[#^] (AU)	Single Day	0.03 ± 0.04	-0.05 ± 0.07[§]	-0.08 ± 0.08[§$]	-0.12 ± 0.11[§$*]	-0.11 ± 0.09[§$*]	-0.13 ± 0.10[§$*]
	6 days Ldg	0.15 ± 0.09	0.10 ± 0.10	0.05 ± 0.10	0.02 ± 0.10	-0.01 ± 0.10	-0.02 ± 0.08
Resting Fat Oxidation[#^] ($g.min^{-1}$)	Single Day	0.02 ± 0.05	0.03 ± 0.06	0.04 ± 0.06[§]	0.07 ± 0.05[§$*]	0.06 ± 0.05[§$*]	0.06 ± 0.04[§$*]
	6 days Ldg	-0.05 ± 0.04	-0.04 ± 0.05	-0.01 ± 0.04	0.00 ± 0.05	0.02 ± 0.05	0.03 ± 0.05
Resting CHO Oxidation[#^] ($g.min^{-1}$)	Single Day	-0.04 ± 0.13	-0.07 ± 0.13	-0.09 ± 0.15[§]	-0.17 ± 0.12[§$]	-0.13 ± 0.14[§$*]	-0.13 ± 0.11[§$]
	6 days Ldg	0.15 ± 0.10	0.12 ± 0.13	0.04 ± 0.11	0.01 ± 0.12	-0.03 ± 0.13	-0.05 ± 0.11

Ldg (Loading); [#]Denotes significant main effect for time; [^]Denotes significant main effect for supplementation strategy. [§]Denotes significant difference against 30-minutes post-bolus for main effect of time. [$]Denotes significant difference against 1-hour post-bolus for main effect of time. *Denotes significant difference against 2-hours post-bolus for main effect of time.

Appendix 10

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ORIGINAL ARTICLE

The effects of Montmorency tart cherry juice supplementation and FATMAX exercise on fat oxidation rates and cardio-metabolic markers in healthy humans

Terun Desai[1] · Lindsay Bottoms[1] · Michael Roberts[1]

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Abstract
Montmorency tart cherries (*Prunus cerasus* L.) are rich in anthocyanins, compounds capable of augmenting fat oxidation and regulating metabolic dysfunction. The present study examined whether Montmorency tart cherry juice (MTCJ) supplementation could augment fat oxidation rates at rest and during FATMAX exercise, thus improve cardio-metabolic health. Eleven, healthy participants consumed MTCJ or placebo (PLA) twice daily, in a randomised, counterbalanced order for 20 days. Participants cycled at FATMAX for 1-h pre-, mid- (10 days) and post-supplementation whilst substrate oxidation rates were measured. Before exercise anthropometrics and resting metabolic rate were measured. Blood pressure, serum triglycerides, cholesterol, HDL, total antioxidant status (TAS) and glucose were measured immediately before and after exercise. No significant differences between conditions or interactions were observed for any functional and blood-based cardio-metabolic markers or fat oxidation during exercise or rest ($P > 0.05$). Pre-exercise TAS ($P = 0.036$) and HDL ($P = 0.001$) were significantly reduced from mid- to post-supplementation with MTCJ only. Twenty days' MTCJ supplementation had no effect on fat oxidation; therefore, it is unnecessary for individuals in this participant cohort to consume MTCJ with exercise to improve cardio-metabolic biomarkers.

Keywords Anthocyanins · Exercise · Polyphenols · Cardiometabolic Health · Fat oxidation

Abbreviations

BP	Blood pressure
CHO	Carbohydrate
CRM	Calorie restrictive mimetic
DBP	Diastolic blood pressure
EE	Energy expenditure
HR	Heart rate
MFO	Maximal fat oxidation
MTCJ	Montmorency tart cherry juice
NZBE	New Zealand blackcurrant extract
PLA	Placebo
PVC	Plasma volume change
PPAR	Peroxisome proliferator-activated receptor
RER	Respiratory exchange ratio
RMR	Resting metabolic rate
RPE	Ratings of perceived exertion
SBP	Systolic blood pressure
TAS	Total antioxidant status
$\dot{V}O_2$max	Volume of maximal oxygen uptake
$\dot{V}CO_2$	Volume of carbon dioxide production
$\dot{V}O_2$	Volume of oxygen uptake

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✉ Terun Desai
t.desai@herts.ac.uk

[1] Department of Psychology and Sports Science, School of Life and Medical Sciences, University of Hertfordshire, College Lane, Hatfield AL10 9AB, UK

Introduction

Cardiovascular disease, type 2 diabetes mellitus and associated diseases combined are the leading health burden and cause of mortality worldwide (Guo and Ling 2015); therefore, the necessity for an intervention is paramount. Dietary interventions to improve cardio-metabolic health are highly sought after as they possess less risk than pharmacological drugs (Vendrame et al. 2016). Substantial evidence now exists demonstrating the relationship between high consumption of vegetables and fruits, improvements in disease symptoms and the reduced risk of disease development (Li et al. 2017). However, concerns remain over the

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feasibility of maintaining a high fruit and vegetable intake over a prolonged time period; therefore, daily dietary supplementation containing health-promoting phytonutrients is appealing. Subsequently, the present study supplemented small volumes of highly concentrated Montmorency tart cherry juice (MTCJ) to boost consumption of anthocyanins and thus provide sufficient amounts at physiologically relevant concentrations in a more practical and efficient method (Zheng et al. 2017).

Anthocyanins, a major polyphenolic sub-class of flavonoids, are predominantly responsible for the dark red, blue, black and purple pigments found in various fruits and vegetables (Wu et al. 2006). Anthocyanins are powerful antioxidants also capable of ameliorating cardio-metabolic dysfunction (He and Giusti 2010). A recent epidemiological study highlighted a significant correlation with anthocyanin consumption and improved long-term weight management (Bertoia et al. 2016). Tart cherries (*Prunus cerasus* L.) and thus tart cherry juice possess a high phytochemical content particularly rich in specific anthocyanins, flavonols and phenolic acids (Kirakosyan et al. 2009, 2010). These phytochemicals are thought to contribute to effectively combating oxidative stress, inflammation and repairing muscle damage post-exercise (Bell et al. 2014b, 2015). In rodents, tart cherries have shown significant improvements at the molecular, cellular and systemic level predominantly due to modulation of peroxisome proliferator-activated receptor (PPAR) signalling pathways (Seymour et al. 2009, 2008). Consequently, percentage fat mass, hyperinsulinaemia, hyperlipidaemia and inflammation were all reduced (Seymour et al. 2009, 2008). Observations in humans have been more equivocal, with tart cherry supplementation showing no effect on blood pressure and blood-based cardio-metabolic markers in healthy participants (Lynn et al. 2014). More favourable findings have been reported in studies (Ataie-Jafari et al. 2008; Keane et al. 2016a, b; Martin et al. 2010) examining 'at risk' or diseased patients with tart cherry juice, indicating amelioration of cardio-metabolic function.

Skeletal muscle is a critical organ involved in lipid metabolism and dysfunction of cellular and molecular cascades which are implicated in the manifestation of cardiovascular disease, insulin resistance, inflammation and oxidative stress (Stump et al. 2006). During sub-maximal exercise at FATMAX intensities [intensity eliciting maximal fat oxidation rate, conveyed as percentage of maximal oxygen uptake ($\%\dot{V}O_2max$)], the primary source of energy is derived from oxidation of free fatty acids and intramuscular triglycerides in skeletal muscle (Romijn et al. 1993). Therefore, FATMAX exercise, as an intervention, was included in the present study to facilitate fat oxidation and subsequently improve cardio-metabolic function. Perez-Martin et al. (2001) conducted a study comparing substrate utilisation between healthy obese and normal-weight participants during a FATMAX determination test. An earlier shift to CHO oxidation and significantly lower maximal fat oxidation rates (MFO) were observed with obese participants compared to normal-weight, indicating metabolic inflexibility amongst the obese cohort (Perez-Martin et al. 2001). However, in healthy participants Robinson et al. (2015) demonstrated a significant positive correlation between MFO and both 24-h fat oxidation and insulin sensitivity. Furthermore, in obese yet metabolically healthy participants, FATMAX training elicited a 44% increase in fat oxidation rates and 27% increase in insulin sensitivity index (Venables and Jeukendrup 2008). A meta-analysis of FATMAX training in obese, Metabolic Syndrome and type 2 diabetic patients confirmed a shift of fat oxidation to higher exercise intensities and reductions in body weight, fat mass, waist circumference and cholesterol, thus advocating its use to improve health (Romain et al. 2012). Overall, these findings suggest FATMAX exercise encourages enhancements in fat oxidation rate leading to improved body composition, cholesterol and insulin sensitivity (Brun et al. 2011).

Consequently, the purpose of specifically incorporating aerobic FATMAX exercise in the present study was twofold. First, an individualised approach to achieve maximal fat oxidation rates during moderate-intensity exercise has been suggested as the best method to reduce glycated haemoglobin (HbA_{1c}), insulin-dependent glucose, fat mass and total cholesterol (Brun et al. 2011). Second, it is an appropriate methodological test to measure the effects of an intervention on fat oxidation rates during exercise and when combined with a potential calorie restrictive mimetic (CRM), such as MTCJ, may induce additional improvements on cardio-metabolic pathways and, therefore, overall health (Besnier et al. 2015).

Rationale for the present study is based on previous work demonstrating the benefits of anthocyanin supplementation on fat oxidation rates during exercise (Cook et al. 2015, 2017) and the positive results regarding the effects of tart cherry supplementation against cardio-metabolic dysfunction in rodents (Seymour et al. 2009, 2008) and humans (Ataie-Jafari et al. 2008; Keane et al. 2016a, b; Martin et al. 2010). However, to date, no research has explored the cardio-metabolic responses to tart cherry supplementation and exercise in tandem; thus healthy participants were recruited to assess for any adverse effects prior to investigating responses in clinical populations. Subsequently, this study set out to examine the physiological responses of MTCJ supplementation with FATMAX exercise on fat oxidation rates, body composition and cardio-metabolic markers in healthy participants. It was hypothesised that MTCJ supplementation would augment fat oxidation rates at rest and during exercise, thus proving more efficacious at improving body composition, functional and in sera cardio-metabolic markers than previous research conducted with tart (Lynn

et al. 2014; Martin et al. 2010) and sweet (Kelley et al. 2006) cherry supplementation at rest in healthy participants.

Methods

Participants

Eleven (7 males and 4 females) healthy, recreationally active (150 min moderate-intensity aerobic exercise per week), participants (mean ± SD age 30 ± 10 years, height 1.76 ± 0.09 m, body mass 76.42 ± 13.19 kg, BMI 24.43 ± 3.23 kg m^{-2}, $\dot{V}O_2$peak 35.87 ± 4.78 mL kg^{-1} min^{-1}) volunteered for the study. All participants were non-smokers, BMI < 30, injury-free and not diagnosed with any cardio-metabolic or renal diseases at the time of testing but had a family history of cardio-metabolic disease. Participants were instructed to cease consumption of any other supplementation 2 weeks before and for the duration of the study. All participants provided written informed consent to participate in the study and completed health screen questionnaires before the study commenced. Ethical approval was obtained from the University of Hertfordshire Health and Human Sciences Ethics Committee and the study's experimental procedures followed the principles outlined in the 1964 Declaration of Helsinki. The study was registered as a clinical trial on clinicaltrials.gov (NCT02999256).

As this was the first study to examine fat oxidation with cherries, it was difficult to confidently predict a sample size using power analyses. Previous studies that had researched the effects of fat oxidation (Cook et al. 2015; Roberts et al. 2015) or cherry supplementation (Bell et al. 2014a, b; Bowtell et al. 2011) had a total sample size between 10 and 16 participants.

Procedures

Research design

This study utilised a single-blind (blinded to participants), placebo-controlled, randomised, counterbalanced design, where each participant acted as their own control. Participants were required to complete two conditions over 10 weeks, differing only in supplementation, Montmorency tart cherry juice (MTCJ) and placebo (PLA). Participants were randomised to start consumption of either MTCJ or PLA first (6 received PLA first), followed by a 14-day washout period (Cook et al. 2015; Howatson et al. 2012; Keane et al. 2016a, b) and then consumption of the opposite supplement to the first condition.

Both conditions were identical in terms of design and testing procedures and comprised of 5 sessions each, with sessions lasting approximately 2.5 h. Figure 1 shows a timeline for testing sessions and the supplementation period.

Baseline measurements were obtained during the first FATMAX/$\dot{V}O_2$max session in the first condition. Other than blood sampling and the type of exercise itself, all other testing procedures pre- and post-exercise were identical across each session. Blood sampling was conducted pre- and post-exercise to ascertain the acute differences induced by 1 h of FATMAX exercise and pre-, mid- and post-supplementation to assess the longer-term effects of

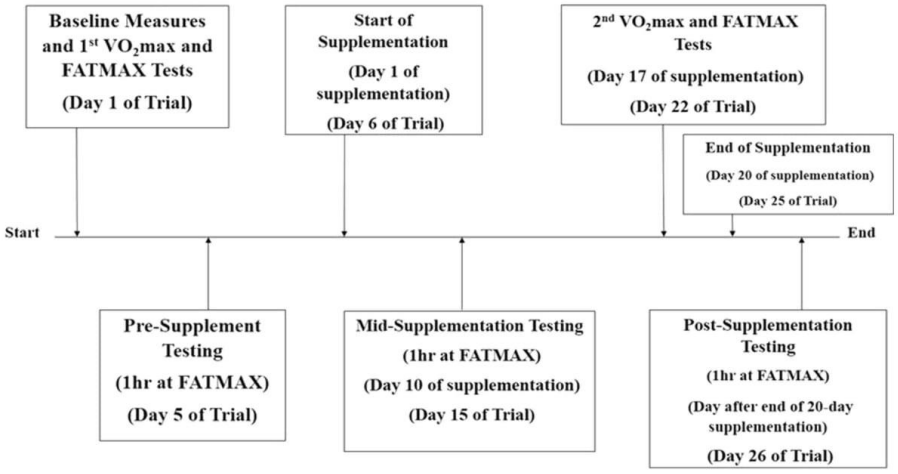

Fig. 1 Schematic of testing protocol for each condition (MTCJ and PLA)

supplementation on cardio-metabolic biomarkers, within and between conditions.

Dietary and exercise guidelines

Participants were instructed to refrain from consuming water and conducting vigorous exercise 3 and 24 h before each testing session, respectively. All participants arrived at the laboratory between 7 and 10am, after an overnight fast of a minimum of 10 h, to account for circadian variation (Bell et al. 2014b). Participants were instructed to drink fluids ad libitum and maintain their habitual polyphenol intake, particularly anthocyanins, as opposed to complete restriction throughout the study. This was to ensure that the polyphenols provided by MTCJ were supplementary to the existing habitual polyphenol intake of each participant representing normal daily activity and, therefore, upholding ecological validity (Meyer et al. 2007).

Total energy, macronutrient and polyphenol intake was assessed through food diaries which were completed 3 days prior to each session. In addition to replicating their 3-day diary before each testing session, a standardised menu for the final 24-h of the 3-day period, was provided to each participant based on their healthy spontaneous habitual diet to reduce day-to-day intra-individual variability in fat oxidation (Roberts et al. 2015). Standardisation of macronutrients was set at 15% protein, 55% carbohydrate (CHO) and 30% fat of energy intake (Ben Ounis et al. 2009; Melanson et al. 2002) with 20% of the 3-day average for habitual energy intake added to account for underreporting dietary consumption with food diaries (Black et al. 1993; Mertz et al. 1991). All participants reported 100% adherence when food diaries were assessed using dietary analysis software (Dietplan 7.0, Forestfield Software Ltd, West Sussex, UK) for percentage contributions of macronutrients to total energy intake, total polyphenols and anthocyanins.

Supplementation

The supplementation period lasted 20 days, similar to Roberts et al. (2015), where participants ceased consumption following the 20th day and during the washout period. The experimental condition included supplementation with MTCJ (Volume: 30 mL, Energy: 102 kcal, Carbohydrates: 24.50 g of which Sugars: 17.90 g, Protein: 1.10 g and Fat: 0 g) whilst the control involved supplementation of an energy matched placebo (Volume: 30 mL, Energy: 102 kcal, Carbohydrates: 25.35 g of which Sugars: 25.32 g, Protein: 0.03 g and Fat: 0 g). The MTCJ was made with 30 mL Montmorency tart cherry concentrate (Cherry Active, Active Edge Ltd, Hanworth, UK) and 100 mL water. Placebo composition consisted of 30 mL commercially available fruit-flavoured cordial (Cherries and Berries, Morrisons, Bradford, UK), with anthocyanins used only for colouring and negligible antioxidant content, mixed with 100 mL water. In order to match placebo for energy, taste and visual appearance, a flavourless carbohydrate (Maltodextrin, My Protein Ltd, Northwich, UK), citric acid (100% Pure Citric Acid, VB and Sons, UK) and black food colouring (Morrisons, Bradford, UK) were added, respectively. Participants consumed two 130 mL servings per day, once in the morning immediately before breakfast, then again in the evening before dinner (Bell et al. 2014b, 2016). Due to fasting restrictions on testing days, participants delayed consumption of the morning serving until lunchtime. Each 30 mL serving of Montmorency tart cherry concentrate provided juice from approximately 90–110 whole Montmorency tart cherries with a total anthocyanin content of 270 mg (9 mg·mL^{-1}) (Howatson et al. 2012).

Testing protocol

All testing sessions were conducted in a temperature-controlled laboratory kept between 21 and 24 °C and 38–45% relative humidity (dry-bulb) (AWS888N, Oregon Scientific, USA).

During all testing sessions, stature (Seca 217 Stadiometer, Seca, Hamburg, Germany), body mass (Seca 799, Germany) and waist circumference (Seca 201, Germany) were measured initially, immediately followed by segmental body composition analysis (Body Composition Analyser BC-418, Tanita, Japan) via bioelectrical impedance. Body composition was measured according to the manufacturer's guidelines and specific pre-measurement conditions to reduce variability between measurements. Pilot work showed test–retest intra-individual variability (measurements taken in the morning from 5 participants according to the timeline of the study) of the body composition analyser to have coefficients of variation of 2.06% for percentage body fat and 1.91% for fat mass. Participants were not on any weight loss or weight gain regimen during this period.

After 10 min rest, resting heart rate (HR) (Polar T31c and FT1, Polar Electro Oy, Finland) and blood pressure (BP) (Omron MX3, Omron, Japan) were obtained followed by resting metabolic rate (RMR). Pre-exercise blood sampling was then performed followed by post-exercise blood sampling during the 1-h sub-maximal sessions only. Heart rate and BP were measured after post-exercise blood sampling, approximately 5–8 min after the cessation of exercise (Fig. 2a, b).

Exercise protocols

During the first and fourth testing sessions of each condition, participants conducted FATMAX and maximal oxygen uptake ($\dot{V}O_2$max) tests.

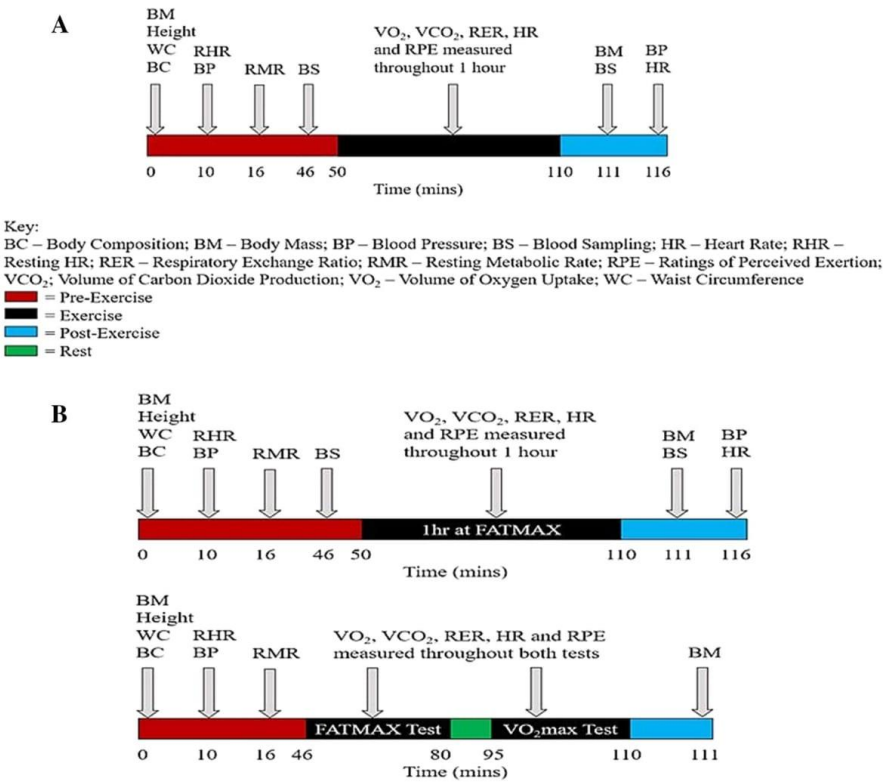

Fig. 2 Schematic of testing procedure during FATMAX and VO_2max testing sessions (**a**) and during 1-h sub-maximal exercise at individually determined FATMAX sessions (**b**)

The FATMAX (expressed as % of $\dot{V}O_2$peak) determination protocol, adapted from Achten et al. (2002) and Alkhatib et al. (2015), required participants to cycle on an electromagnetically braked cycle ergometer (Excalibur, Lode, Groningen, The Netherlands) at 70 rev·min^{-1} at an initial intensity of 30 W with increments of 10 W every 3 min. The test was terminated once respiratory exchange ratio (RER) exceeded 1 for a continuous period of 30 s (Croci et al. 2014).

The $\dot{V}O_2$max test was performed 15 min following the FATMAX test. Due to the low-intensity nature of the FATMAX test, 15 min provided sufficient recovery time before the $\dot{V}O_2$max test. The protocol for determination of $\dot{V}O_2$max consisted of cycling at 70–80 rev·min^{-1}, at an initial intensity of 100 W with wattage increasing by 20 W every minute until volitional exhaustion. The test was terminated according to BASES Sport and Exercise Physiology testing guidelines (Winter 2006).

Heart rate and differentiated (overall and legs) ratings of perceived exertion (RPE) on a 6–20 scale (Borg 1973) were recorded 15 s before the end of each stage for FATMAX and $\dot{V}O_2$max tests. RPE was also measured every 5 min during the 1-h sub-maximal exercise.

FATMAX analysis

FATMAX was determined through visual inspection of the fat oxidation curve generated from the incremental sub-maximal test and confirmed by identifying the highest fat oxidation rate for the final minute of each stage calculated from a 15-s rolling average of each 3 min stage. The corresponding intensity (power output, W) at the peak of this curve was deemed to be FATMAX and was implemented during the 1-h sub-maximal exercise.

The second $\dot{V}O_2$max and FATMAX tests were used to assess changes in fitness status (MacRae and Mefferd 2006) and any differences in FATMAX which may have occurred

due to the previous exercise sessions and supplementation. FATMAX was adjusted to the new exercise intensity for the post-supplementation 1-h sub-maximal exercise if the change in FATMAX was greater than a pre-determined threshold (> 6%).

Measures and equipment

Blood pressure and heart rate Systolic (SBP) and diastolic (DBP) blood pressure was monitored pre- and post-exercise during all testing sessions where four measurements were recorded in an upright seated position, with an average of the final three being taken as BP (Cook et al. 2015). Resting HR was also recorded in the same anatomical position.

Resting metabolic rate RMR, resting energy expenditure, substrate oxidation rates and RER were measured pre-exercise, based on an open-circuit indirect calorimetry system (GEM Nutrition Ltd, Cheshire, UK). Participants lay supine for 30 min with data averaged for the final 20 min only, to achieve steady-state and account for any initial short-term variances in respiration (Kelly et al. 2013). A ventilated hood was placed over the head with a flexible plastic seal around the neck and shoulders to prevent air inside and outside the hood from mixing. Participants remained silent and lay as still as possible, whilst music was played to prevent sleeping.

Respiratory gas analysis Real-time breath-by-breath gaseous exchange data (Metalyzer 3B, Cortex Biophysik, Leipzig, Germany) of $\dot{V}O_2$ (L min^{-1}), $\dot{V}CO_2$ (L min^{-1}) and RER were recorded during all exercise tests. Consequently, indirect calorimetry was used to calculate EE (kJ·min^{-1}) and substrate oxidation rates (g·min^{-1}) using stoichiometric equations, assuming negligible protein oxidation, specifically developed for exercise intensities between 40 and 50% $\dot{V}O_2$peak, as shown below (Jeukendrup and Wallis 2005).

$$\text{Fat oxidation rate} = \left(1.695 \cdot \dot{V}O_2\right) - (1.701 \cdot \dot{V}CO_2)$$

$$\text{CHO oxidation rate} = (4.344 \cdot \dot{V}CO_2) - (3.061 \cdot \dot{V}O_2)$$

$$\text{Energy expenditure} = \left[(0.575 \cdot \dot{V}CO_2) - \left(4.435 \cdot \dot{V}O_2\right)\right]$$

Exported data were analysed only from the final 50 min of all 1-h sub-maximal tests to ensure participants reached steady-state. Data were averaged for every 15 s period during the entire 50 min of exercise.

Blood sampling Venous blood, was sampled pre- and post-exercise, using the butterfly method (BD Vacutainer Safety-Lok Blood Collection Set 21G with Luer Adapter, Becton Dickinson and Co., Oxford, UK) from veins located in the antecubital fossa region, into one lithium heparin tube (4 mL) and one serum separator tube (4 mL) (BD Vacutainer, Becton Dickinson and Co., Oxford, UK). Tubes were centrifuged at 4000 rev·min^{-1}, 4 °C for 10 min (Sorvall ST 8R, Thermo Fisher Scientific, USA). Serum and plasma supernatants were aliquoted and stored at − 80 °C for later analysis. Blood samples were obtained from 10 participants, as one subject was uncomfortable with the venepuncture method.

Biochemical analysis

Plasma volume change Whole blood from lithium heparin tubes was analysed for haemoglobin (HemoCue Hb 201^+ Reader, Sweden) and haematocrit to calculate plasma volume change (PVC). Haematocrit was analysed by pipetting 60 µL of whole blood into micro-haematocrit capillary tubes (Hawksley, Lancing, Sussex, UK). Tubes were then centrifuged (Haematospin 1300, Hawksley, Lancing, Sussex, UK) at 1300 rev·min^{-1} for 3 min. Percentage packed cell volume was determined from a slide reader (Micro Haematocrit Tube Reader, Hawksley, Lancing, Sussex, UK).

Assay results were corrected for PVC as a result of exercise-induced changes in haemoconcentration (Allgrove et al. 2011) by the equation shown below.

$$\text{Corrected assay results} = \%\text{PVC} \times \text{Measured post-exercise assay value.}$$

PVC was determined using the method and equations of Dill and Costill (1974) by means of comparing the pre- and post-exercise haemoglobin and haematocrit values.

Glucose Pre- and post-exercise serum samples were assessed for glucose (range 0.5–50 mmol L^{-1}, CV ≤ 1.5%) (Biosen C-Line, EKF Diagnostics, Cardiff, UK) in duplicates.

Total antioxidant status assay Total antioxidant capacity within serum samples was assessed in duplicates according to the manufacturer's guidelines using the total antioxidant status (Total Antioxidant Status NX2332, Randox Laboratories Ltd, Antrim, UK) colorimetric assay on a semi-automated spectrophotometer (RX Monza, Randox). Intra-assay CV was 3.49%.

Lipid assays Serum lipids were determined in duplicates using commercially available colorimetric assays on a semi-automated spectrophotometer, according to manufacturer's guidelines. Triglyceride (Triglycerides TR210, Randox) values were corrected for free glycerol by subtracting 0.11 mmol·L^{-1} (Stinshoff et al. 1977), according to the manufacturer's guidelines. Intra-assay CV for triglycer-

ides, total cholesterol and HDL were 4.96, 1.99 and 4.66%, respectively. The ratio between total cholesterol and HDL was also determined. LDL was determined indirectly using the formula below (Ahmadi et al. 2008).

$$\text{LDL (mmol L}^{-1}) = \left(\frac{\text{Total Cholesterol}}{1.19}\right) + \left(\frac{\text{Triglycerides}}{0.81}\right) - \left(\frac{\text{HDL}}{1.1}\right) - 0.98$$

Data analysis

Statistical analysis was performed using SPSS v22.0 (IBM, Chicago, USA) where data are reported as means ± SD. Data normality was checked using a Shapiro–Wilk test. Greenhouse-Geisser correction was applied upon violation of Mauchly's test of sphericity for analyses of variance (ANOVA). Statistical significance was set at $P < 0.05$.

A within-group 3-way, 2 × 3 × 2, condition (MTCJ vs PLA), time (pre-, mid-, post-supplementation), exercise (pre- and post-exercise), repeated-measures ANOVA with post-hoc Bonferroni's adjustment, measured differences of body mass, BMI, HR, BP, glucose, TAS, triglycerides, total cholesterol, HDL, total cholesterol:HDL, haematocrit and haemoglobin. Waist circumference, body composition and resting EE, RER, fat and CHO oxidation were analysed using a 2-way, 2 × 3 (condition × time) repeated-measures ANOVA design with post-hoc Bonferroni's adjustment. A paired-samples t-test was used to identify differences between individual data points within conditions and between conditions for the corresponding time point.

Parameters measured during the FATMAX and $\dot{V}O_2$max tests, including MFO (maximal fat oxidation) and power output at FATMAX, FATMAX (% of $\dot{V}O_2$peak), RER at FATMAX, percentage of maximal HR (%HRmax) at FATMAX and $\dot{V}O_2$peak were analysed using a 2 × 2 (condition × time) repeated-measures ANOVA. A paired-samples *t*-test was used to identify differences between conditions for only the first and second FATMAX tests.

Variables measured during the 1-h sub-maximal exercise at FATMAX including, RER, HR, RPE (overall), RPE (legs), EE, CHO oxidation, fat oxidation and percentage contribution of fat and CHO to total EE were analysed using a 2 × 3 (condition × time) repeated-measures ANOVA with post-hoc Bonferroni's adjustment, when averaged for the final 50 min.

Partial Eta-Squared (η^2_{partial}) was used to report effect sizes for ANOVAs where effects were classified as small (0.01–0.08), moderate (0.09–0.25) and large (> 0.25) (Cohen 1988). Cohen's *d* effect size was used for paired-samples *t*-test where effects were classified as no effect (0–0.1), small (0.2–0.4), moderate (0.5–0.7) and high (≥ 0.8) (Cohen 1988).

In order to determine whether independent variables could explain the variance observed for MFO, bivariate correlations were conducted between MFO values obtained during the final minute of the stage corresponding to FATMAX at baseline and independent variables affecting FATMAX, including age, anthropometrics, body composition, dietary intake and $\dot{V}O_2$peak. No significant correlations ($P > 0.05$) were found for any independent variables; thus regression analysis was not performed.

Results

Exercise results

1-h sub-maximal cycling tests

No significant interactions or main effects for condition and time ($P > 0.05$) were observed for mean EE, percentage contributions of fat and CHO to EE, $\dot{V}O_2$ and $\dot{V}CO_2$, HR, overall RPE, legs RPE and serum glucose during 1-h cycling at individual FATMAX suggesting exercise intensities and physiological responses were similar between conditions over the supplementation period (Table 1).

Mean fat oxidation rates during the final 50 min of the 1-h cycling exercise at individual FATMAX were not significantly different between conditions (PLA: 0.25 ± 0.10 g·min^{-1} and MTCJ: 0.26 ± 0.09 g·min^{-1}; $F_{(1, 10)} = 0.35$; $P = 0.567$, $\eta^2_{\text{partial}} = 0.034$), time (Pre-Supplementation: 0.25 ± 0.09 g·min^{-1}, Mid-Supplementation: 0.27 ± 0.08 g·min^{-1}, Post-Supplementation: 0.25 ± 0.11 g·min^{-1}; $F_{(2, 20)} = 1.22$; $P = 0.318$, $\eta^2_{\text{partial}} = 0.108$) or the interaction between condition and time ($F_{(2, 20)} = 0.273$; $P = 0.764$, $\eta^2_{\text{partial}} = 0.027$) (Fig. 3).

There were also no main effects ($P > 0.05$) for condition, time or the interaction for mean CHO oxidation rates.

A significant main effect for time (Pre-Supplementation: 0.88 ± 0.04, Mid-Supplementation: 0.87 ± 0.04, Post-Supplementation: 0.89 ± 0.04; $F_{(2, 20)} = 4.14$; $P = 0.031$, $\eta^2_{\text{partial}} = 0.293$) was detected for mean RER but not between conditions or the interaction ($P > 0.05$). Post-hoc analysis identified a trend towards significance between mid-supplementation and post-supplementation ($P = 0.070$).

FATMAX and $\dot{V}O_2$max determination tests

No significant differences between conditions (PLA: 0.26 ± 0.05 g·min^{-1} and MTCJ: 0.23 ± 0.04 g·min^{-1}; $F_{(1, 10)} = 2.79$; $P = 0.126$, $\eta^2_{\text{partial}} = 0.22$) or time (Test 1: 0.25 ± 0.08 g·min^{-1} and Test 2:

Table 1 Mean (±SD) values of final 50 min for variables measured during 1-h exercise at individual FATMAX throughout the study duration for PLA and MTCJ

	Pre-supplementation	Mid-supplementation	Post-supplementation
CHO oxidation (g min^{-1})			
PLA	0.85 ± 0.33	0.84 ± 0.27	0.89 ± 0.25
MTCJ	0.98 ± 0.25	0.88 ± 0.29	0.99 ± 0.19
EE (kJ min^{-1})			
PLA	4.58 ± 0.99	4.60 ± 1.00	4.68 ± 1.23
MTCJ	4.96 ± 0.93	4.93 ± 1.09	5.00 ± 0.84
Contribution of Fat to EE (%)			
PLA	22.49 ± 17.11	23.33 ± 8.03	21.67 ± 10.34
MTCJ	19.88 ± 8.03	24.06 ± 10.96	20.51 ± 7.37
Contribution of CHO to EE (%)			
PLA	77.51 ± 17.11	76.67 ± 8.03	78.33 ± 10.34
MTCJ	80.12 ± 8.03	75.94 ± 10.96	79.49 ± 7.37
$\dot{V}O_2$ (L min^{-1})			
PLA	1.16 ± 0.26	1.16 ± 0.25	1.18 ± 0.31
MTCJ	1.25 ± 0.23	1.25 ± 0.28	1.26 ± 0.21
$\dot{V}CO_2$ (L min^{-1})			
PLA	1.00 ± 0.25	1.00 ± 0.24	1.003 ± 0.25
MTCJ	1.10 ± 0.21	1.08 ± 0.14	1.12 ± 0.18
RER (AU)			
PLA	0.87 ± 0.05	0.87 ± 0.03	0.88 ± 0.04
MTCJ	0.88 ± 0.03	0.87 ± 0.04	0.89 ± 0.03
HR (beats min^{-1})			
PLA	114 ± 17	112 ± 18	113 ± 17
MTCJ	116 ± 9	116 ± 14	115 ± 15
RPE (Overall) (AU)			
PLA	10 ± 2	10 ± 1	10 ± 2
MTCJ	11 ± 2	10 ± 2	11 ± 1
RPE (Legs) (AU)			
PLA	10 ± 2	10 ± 2	11 ± 1
MTCJ	11 ± 2	11 ± 2	11 ± 1

AU arbitrary units, *CHO* carbohydrate, *EE* energy expenditure, *HR* heart rate, *MTCJ* Montmorency tart cherry juice, *PLA* placebo, *RER* respiratory exchange ratio, *RPE* ratings of perceived exertion, $\dot{V}CO_2$ volume of carbon dioxide production, $\dot{V}O_2$ Volume of oxygen uptake

0.25 ± 0.10 g·min^{-1}; $F_{(1,10)} = 0.06$; $P = 0.807$, $\eta^2_{partial} = 0.01$) were found for MFO during the FATMAX determination tests. A tendency ($t_{(10)} = 2.21$; $P = 0.052$, $d = 0.59$) towards significance was observed between conditions for MFO during the second FATMAX test (PLA: 0.28 ± 0.12 g·min^{-1} and MTCJ: 0.22 ± 0.08 g·min^{-1}).

No main effects ($P > 0.05$) for condition, time or interaction were detected for FATMAX (%$\dot{V}O_2$peak) (Test 1—PLA: 42.77 ± 8.69% and MTCJ: 45.94 ± 9.28%, Test 2—PLA: 45.28 ± 12.07% and MTCJ: 47.91 ± 11.52%), MFO (Test 1—PLA: 0.25 ± 0.08 g·min^{-1} and MTCJ: 0.24 ± 0.08 g·min^{-1}, Test 2—PLA: 0.28 ± 0.12 g·min^{-1} and MTCJ: 0.22 ± 0.08 g·min^{-1}), RER at FATMAX (Test 1—PLA: 0.87 ± 0.04 and MTCJ: 0.88 ± 0.04, Test 2—PLA: 0.87 ± 0.03 and MTCJ: 0.90 ± 0.03), %HR_{max} at FATMAX (Test 1—PLA: 60.27 ± 9.59% and MTCJ: 62.58 ± 7.38%, Test 2— PLA: 59.84 ± 10.43% and MTCJ: 63.04 ± 8.38%), power output at FATMAX (Test 1 – PLA: 66 ± 20 W and MTCJ: 72 ± 18 W, Test 2 – PLA: 70 ± 19 W and MTCJ: 78 ± 18 W) and $\dot{V}O_2$peak (Test 1—PLA: 36.10 ± 5.17 mL kg^{-1} min^{-1} and MTCJ: 36.33 ± 5.34 mL kg^{-1} min^{-1}, Test 2—PLA: 37.07 ± 5.77 mL kg^{-1} min^{-1} and MTCJ: 36.94 ± 5.89 mL kg^{-1} min^{-1}). Values tended to be greater with MTCJ ($F_{(1,10)} = 3.65$; $P = 0.085$, $\eta^2_{partial} = 0.27$) for RER at FATMAX, particularly during the second test (0.90 ± 0.03) compared to the first test (0.88 ± 0.04) ($t_{(10)} = -2.04$; $P = 0.068$, $d = 0.63$). RER at FATMAX was

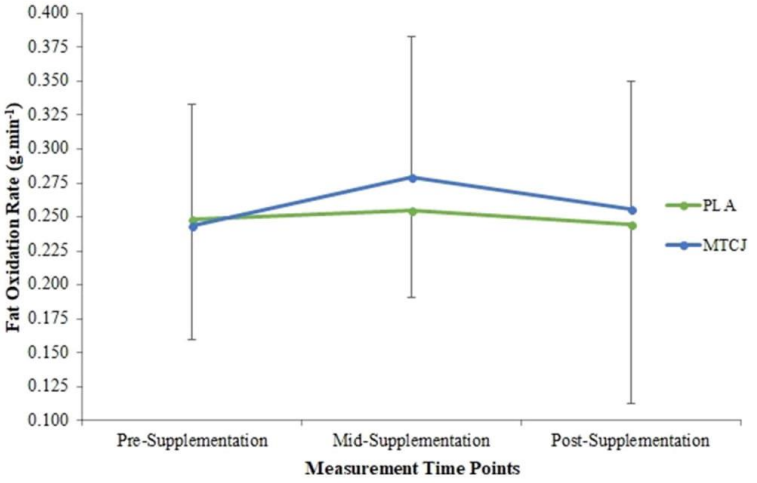

Fig. 3 Mean (±SD) fat oxidation rates measured during final 50 min exercise at individual FATMAX for PLA and MTCJ

significantly greater ($t_{(10)} = -2.77$; $P = 0.020$, $d = 1.1$) during the second test with MTCJ (0.90 ± 0.03) compared to PLA (0.87 ± 0.03).

Paired-samples *t*-test between conditions did not outline any significant differences ($t_{(10)} = -2.04$; $P = 0.256$, $d = 0.63$) for the shift in wattage for FATMAX, where 3 participants increased wattage with PLA whilst 4 participants increased with MTCJ. Thus, the shift in wattage between conditions was considered negligible and not a limitation of the research.

Anthropometric and functional variables

No interactions or main effects for condition and time were detected for anthropometric measurements in Table 2 ($P > 0.05$). However, there was a tendency towards significantly lower percentage body fat ($t_{(10)} = 1.887$; $P = 0.08$, $d = 0.05$) and fat mass ($t_{(10)} = 1.903$; $P = 0.08$, $d = 0.05$) values pre- to post-supplementation with PLA compared to no difference with MTCJ for percentage body fat ($t_{(10)} = -0.841$; $P = 0.42$, $d = 0.02$) and fat mass ($t_{(10)} = -1.386$; $P = 0.196$, $d = 0.04$).

No interactions or main effects for condition, time and exercise ($P > 0.05$) were obtained for functional variables presented in Table 2, apart from a main effect of exercise for HR ($F_{(1,10)} = 25.493$; $P < 0.001$, $\eta^2_{\text{partial}} = 0.718$), as expected.

The change in resting EE from the mid- to post-supplementation trial was significantly different between conditions ($t_{(10)} = -2.602$; $P = 0.026$, $d = 0.86$) suggesting different responses during days 10–20 of supplementation. A tendency towards significance was detected with MTCJ where resting CHO oxidation increased from mid- to post-supplementation but not with PLA ($t_{(10)} = -2.213$; $P = 0.051$, $d = 0.77$).

Glucose and TAS biomarkers

No significant interactions or main effects for condition, time and exercise were detected for glucose ($P > 0.05$).

A main effect for time ($F_{(2,\ 18)} = 11.137$; $P = 0.001$, $\eta^2_{\text{partial}} = 0.55$) and a significant interaction ($F_{(1,\ 9)} = 19.122$; $P = 0.002$, $\eta^2_{\text{partial}} = 0.68$) between condition and exercise were detected for TAS (Fig. 4). Post-hoc analysis revealed significantly lower concentrations post-supplementation compared to mid-supplementation. There was also a tendency towards significance for the interaction between time and exercise ($F_{(2,\ 18)} = 3.466$; $P = 0.053$, $\eta^2_{\text{partial}} = 0.28$).

Pre-exercise TAS with MTCJ was significantly lower post-supplementation (1.18 ± 0.02 mmol·L^{-1}; 98.92% of baseline) compared to mid-supplementation (1.33 ± 0.05 mmol·L^{-1}; 111.93% of baseline) by 11.45%. In comparison, values were statistically similar with PLA ($t_{(9)} = 1.464$; $P = 0.177$, $d = 0.47$) (Table 3). There was a tendency towards significance ($t_{(9)} = -1.998$; $P = 0.077$, $d = 0.71$) for the difference between pre-supplementation (1.17 ± 0.03 mmol·L^{-1}; 101.26% of baseline) and mid-supplementation (1.33 ± 0.05 mmol·L^{-1}; 111.93% of baseline) values prior to exercise with MTCJ, but not for PLA ($t_{(9)} = -0.556$; $P = 0.585$, $d = 0.24$). After the onset of supplementation, the change in TAS during 1-h FATMAX exercise was significantly different ($t_{(19)} = 2.291$; $P = 0.034$, $d = 0.64$), where an average increase of 1.75% was seen with MTCJ and a decrease of 5.17% with PLA.

Table 2 Mean (±SD) for anthropometric, body composition and resting functional variables obtained pre- and/or post-exercise in both conditions at baseline and during pre-, mid- and post-supplementation trials

	Baseline	Pre-supplementation		Mid-supplementation		Post-supplementation	
		Pre-exercise	Post-exercise	Pre-exercise	Post-exercise	Pre-exercise	Post-exercise
Body mass (kg)							
PLA		75.75 ± 13.38	75.37 ± 13.30	75.84 ± 13.33	75.44 ± 13.24	75.48 ± 13.60	75.08 ± 13.70
MTCJ		75.83 ± 13.36	75.47 ± 13.42	75.94 ± 14.15	75.55 ± 14.25	76.05 ± 13.81	75.70 ± 13.85
BMI (kg.m^2)							
PLA		24.48 ± 3.30	24.39 ± 3.30	24.54 ± 3.29	24.41 ± 3.29	24.39 ± 3.33	24.28 ± 3.38
MTCJ		24.53 ± 3.29	24.41 ± 3.29	24.57 ± 3.61	24.41 ± 3.61	24.60 ± 3.48	24.51 ± 3.49
Waist circumference (cm)							
PLA		77.50 ± 11.30		77.30 ± 11.90		78.00 ± 11.30	
MTCJ		75.50 ± 10.80		76.10 ± 11.50		76.60 ± 12.30	
Fat mass (kg)							
PLA		16.43 ± 8.21		15.96 ± 8.46		16.05 ± 7.97	
MTCJ		15.95 ± 7.91		16.04 ± 8.60		16.24 ± 8.34	
Fat free mass (kg)							
PLA		58.75 ± 13.01		59.57 ± 13.00		59.15 ± 13.08	
MTCJ		55.76 ± 20.27		59.64 ± 13.45		54.05 ± 18.72	
Whole-body fat (%)							
PLA		21.91 ± 10.48		21.14 ± 10.45		21.37 ± 10.05	
MTCJ		20.41 ± 10.05		21.23 ± 10.50		21.47 ± 10.43	
Trunk fat (%)							
PLA		22.58 ± 9.66		21.53 ± 9.59		21.98 ± 9.37	
MTCJ		21.85 ± 9.29		21.65 ± 9.58		21.95 ± 9.70	
Resting HR (beats min^{-1})							
PLA	61 ± 9	65 ± 8	74 ± 12	62 ± 6	79 ± 16	65 ± 6	80 ± 10^
MTCJ		65 ± 11	73 ± 8	65 ± 9	72 ± 11	64 ± 7	76 ± 7
SBP (mmHg)							
PLA	117 ± 13	117 ± 16	115 ± 10	115 ± 13	121 ± 18	116 ± 11	114 ± 12
MTCJ		118 ± 12	121 ± 13	119 ± 11	117 ± 12	117 ± 12	116 ± 12
DBP (mmHg)							
PLA	74 ± 9	73 ± 10	73 ± 7	72 ± 10	75 ± 9	72 ± 8	72 ± 10
MTCJ		74 ± 10	75 ± 10	72 ± 9	75 ± 8	72 ± 10	74 ± 7
Resting RER (AU)							
PLA	0.83 ± 0.03	0.83 ± 0.03		0.84 ± 0.03		0.84 ± 0.04	
MTCJ		0.84 ± 0.03		0.83 ± 0.03		0.84 ± 0.04	
Resting EE (kcal day^{-1})							
PLA	1737 ± 285	1718 ± 274		1865 ± 291		1745 ± 279	
MTCJ		1823 ± 233		1825 ± 294		1924 ± 375	
Resting fat oxidation (g min^{-1})							
PLA	0.08 ± 0.03	0.08 ± 0.03		0.07 ± 0.02		0.07 ± 0.03	
MTCJ		0.07 ± 0.02		0.08 ± 0.03		0.08 ± 0.02	
Resting CHO oxidation (g min^{-1})							
PLA	0.14 ± 0.03	0.14 ± 0.03		0.15 ± 0.05		0.15 ± 0.06	
MTCJ		0.16 ± 0.05		0.14 ± 0.05		0.17 ± 0.06	

CHO carbohydrate, *DBP* diastolic blood pressure, *EE* energy expenditure, *HR* heart rate, *MTCJ* Montmorency tart cherry juice, *PLA* placebo, *RER* respiratory exchange ratio, *SBP* systolic blood pressure)

^Denotes significant difference within condition compared to corresponding time point in the pre-supplementation trial

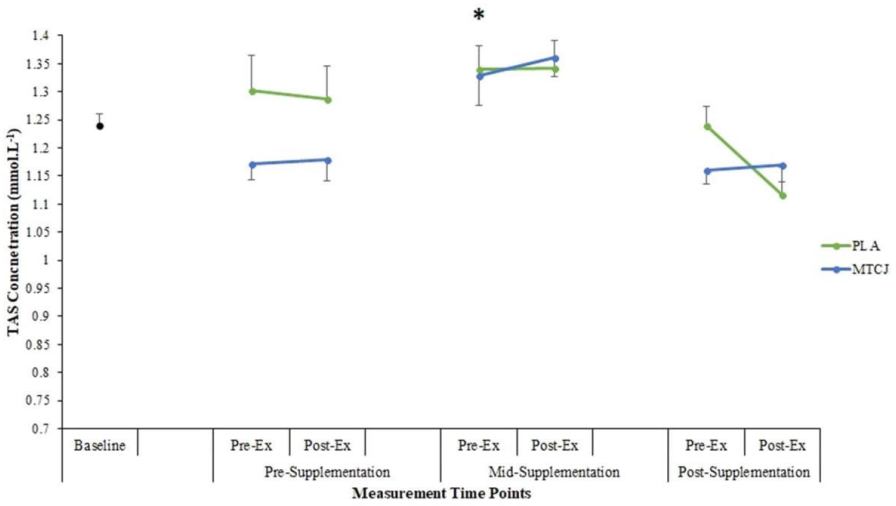

Fig. 4 Mean (±SD) TAS concentrations presented at all measured time points for PLA and MTCJ. *Denotes significant difference between corresponding time point during post-supplementation for MTCJ

Table 3 Mean (±SD) for all blood-based biomarkers measured for PLA and MTCJ during pre-, mid-, post-supplementation, before and after 1-h FATMAX exercise

	Pre-supplementation			Mid-supplementation		Post-supplementation	
	Baseline	Pre-exercise	Post-exercise	Pre-exercise	Post-exercise	Pre-exercise	Post-exercise
Glucose (mmol L^{-1})							
PLA	5.19 ± 0.71	5.15 ± 0.56	5.47 ± 0.77	5.01 ± 0.57	5.33 ± 0.70	5.13 ± 0.86	5.52 ± 0.65
MTCJ		5.22 ± 0.86	5.33 ± 0.52	5.21 ± 0.73	5.39 ± 0.56	5.07 ± 0.68	5.24 ± 0.82
Triglycerides (mmol L^{-1})							
PLA	0.53 ± 0.33	0.48 ± 0.02	0.50 ± 0.02	0.67 ± 0.11	0.65 ± 0.10	0.54 ± 0.01	0.55 ± 0.04
MTCJ		0.60 ± 0.02	0.55 ± 0.05	0.58 ± 0.06	0.61 ± 0.08	0.74 ± 0.02	0.61 ± 0.01
Total cholesterol (mmol L^{-1})							
PLA	3.77 ± 0.67	3.25 ± 0.03	3.20 ± 0.1	2.96 ± 0.11	2.74 ± 0.16	3.29 ± 0.05	3.17 ± 0.06
MTCJ		3.11 ± 0.06	3.01 ± 0.05	2.94 ± 0.2	2.75 ± 0.21	3.40 ± 0.07	2.94 ± 0.2
LDL (mmol L^{-})							
PLA	1.48 ± 0.83	1.30 ± 0.08	1.23 ± 0.13	0.92 ± 0.13	0.63 ± 0.20	1.35 ± 0.07	1.31 ± 0.16
MTCJ		1.09 ± 0.12	1.00 ± 0.13	0.79 ± 0.32	0.66 ± 0.23	1.43 ± 0.12	1.28 ± 0.14
Total cholesterol:HDL (AU)							
PLA	2.28 ± 0.16	1.98 ± 0.08	1.93 ± 0.09	1.83 ± 0.06	1.61 ± 0.18	2.06 ± 0.06	1.98 ± 0.15
MTCJ		2.00 ± 0.14	1.85 ± 0.12	1.69 ± 0.21	1.54 ± 0.12	2.21 ± 0.09	2.05 ± 0.13
Haemoglobin (g L^{-1})							
PLA	143 ± 14.86	141.80 ± 13.55	142.35 ± 15.10	142.75 ± 16.97	143.10 ± 14.00	139.40 ± 16.47	144.80 ± 15.12
MTCJ		144.40 ± 16.17	146.30 ± 16.59	146.30 ± 16.95	147.80 ± 17.91	141.50 ± 16.35	144.00 ± 13.44
Haematocrit (%)							
PLA	44.00 ± 0.05	43.75 ± 0.05	43.85 ± 0.05	44.70 ± 0.05	44.70 ± 0.05	43.50 ± 0.06	45.05 ± 0.05
MTCJ		44.55 ± 0.05	45.00 ± 0.05	45.70 ± 0.05	45.85 ± 0.05	44.20 ± 0.04	44.60 ± 0.04

AU arbitrary units, *LDL* low-density lipoprotein, *TAS* total antioxidant status

Lipid biomarkers

No significant interactions or main effects for condition, time and exercise were detected for triglycerides and LDL ($P > 0.05$).

A main effect for time only was found for HDL ($F_{(1.3,\ 11.703)} = 7.098$; $P = 0.016$, $\eta^2_{\text{partial}} = 0.441$). Post-hoc analysis showed post-supplementation concentrations were significantly lower than mid-supplementation ($P < 0.001$) (Fig. 5). As with TAS, a significant ($t_{(9)} = 3.123$; $P = 0.012$, $d = 0.68$) reduction in pre-exercise HDL concentrations was observed from mid-supplementation (1.84 ± 0.47 mmol.L^{-1}; 114.48% of baseline) to post-supplementation (1.56 ± 0.34 mmol·L^{-1}; 98.62% of baseline) with MTCJ, whereas no difference was found for PLA ($t_{(9)} = 0.719$; $P = 0.490$, $d = 0.15$) (Table 3).

Mean post-exercise values for total cholesterol (main effect for exercise: $F_{(1,\ 9)} = 8.951$; $P = 0.015$, $\eta^2_{\text{partial}} = 0.5$) and total cholesterol:HDL ratio (main effect for exercise: $F_{(1,\ 9)} = 8.951$; $P = 0.015$, $\eta^2_{\text{partial}} = 0.5$) were significantly lower than pre-exercise values. In addition, both total cholesterol ($F_{(1.23,\ 11.154)} = 6.092$; $P = 0.026$, $\eta^2_{\text{partial}} = 0.404$) and total cholesterol:HDL ($F_{(2,\ 18)} = 10.995$; $P = 0.001$, $\eta^2_{\text{partial}} = 0.55$) demonstrated main effects for time; however, no main effect for condition, or significant interactions were detected ($P > 0.05$).

Discussion

This was the first study in any population to combine exercise and tart cherry supplementation whilst examining cardio-metabolic biomarkers. The main findings of this study were that 20 days of MTCJ supplementation did not significantly increase fat oxidation rates at rest or during FATMAX exercise, nor did it alter waist circumference, body composition or cardio-metabolic biomarkers compared to PLA.

The present study did not find significance between conditions for RER, EE, fat and CHO oxidation rates, contrasting previously reported results regarding the effect of dietary anthocyanin supplementation on substrate metabolism with exercise. Cook et al. (2015) observed a 16% increase in fat oxidation with 7 days encapsulated, anthocyanin-rich, New Zealand blackcurrant extract (NZBE) supplementation compared to placebo during cycling exercise at 65% $\dot{V}O_2$max. Crucially, Cook et al. (2015) instructed participants to consume one NZBE capsule, containing 105 mg anthocyanins, 2 h prior to commencing exercise thus coinciding peak bioavailability and concentrations of anthocyanins, secondary metabolites and phase II conjugates in plasma and target tissues with the time of measurement of substrate oxidation rates during exercise. This provides evidence that fat oxidation can be increased with short-term (7 days) and acute (2 h before exercise) supplementation of dietary anthocyanins and highlights the importance of timing of consumption to maximise bioavailability and concentrations in plasma and target tissues. Subsequently, the inclusion of an overnight

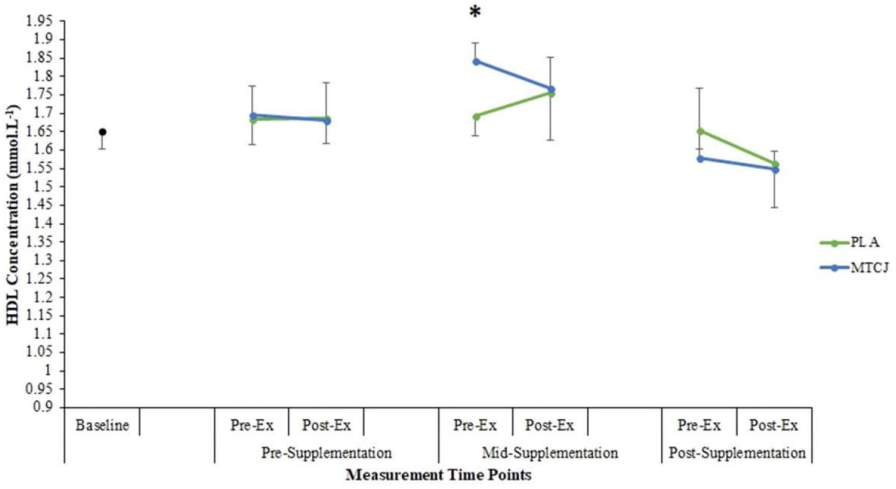

Fig. 5 Mean (±SD) HDL concentrations presented at all measured time points for PLA and MTCJ. Asterisk denotes significant difference between corresponding time point during post-supplementation for MTCJ

fast may explain why Montmorency tart cherry anthocyanins and its metabolites were not able to significantly augment fat oxidation rates.

Differences in fat oxidation responses to anthocyanin supplementation may be explained by the different types of anthocyanins present compared to MTCJ. The main anthocyanins in NZBE (35–50% delphinidin-3-rutinoside and 5–20% delphinidin-3-glucoside) in NZBE are derived from the anthocyanidin, delphinidin (Cook et al. 2017), whereas cyanidin-based anthocyanins (cyanidin-3-glucosylrutinoside and cyanidin-3-rutinoside) comprise 93% of the total anthocyanin content in Montmorency tart cherries (Kirakosyan et al. 2009). It has been previously reported that delphinidin-based anthocyanins provide more effective cardio-metabolic protection than cyanidin-based anthocyanins (Overall et al. 2017). Additionally, cyanidin is less stable in the gastrointestinal environment, although it is not dependent on gut bacteria for metabolism or absorption, whereas delphinidin is dependent on gut bacteria (Overall et al. 2017). Healthy individuals are expected to have greater diversity and concentrations of 'good' gut bacteria, acting to increase the bioavailability of anthocyanins and their metabolites (Fernandes et al. 2014; Hidalgo et al. 2012). Since gut bacteria do not modulate cyanidin-based anthocyanin metabolism and absorption, it is not surprising that cardio-metabolic markers or fat oxidation rates were not improved in the healthy participants from this study.

It is feasible that consumption of a fruit extract (NZBE) in capsule form would not influence substrate oxidation rates to a greater extent than supplements in juice form (MTCJ) when ingested immediately before exercise, given the likely differences in macronutrient content and metabolism such as anthocyanin degradation and absorption rates. Perhaps this explains why Cook et al. (2015) were able to observe greater fat oxidation with NZBE. Cook et al. (2015) reported NZBE anthocyanins contributed 30.70% of fat to total EE during exercise, whilst MTCJ peaked during the mid-supplementation test at 24.06% and averaged 21.48% throughout the present study. Similar results were reported with the placebo, with the most likely explanation being the high carbohydrate content of the MTCJ and placebo drinks, compared to the capsules provided in the NZBE study which were completely devoid of carbohydrate. The significant increase in resting EE was facilitated by upregulation of CHO oxidation signifying that the higher CHO content of MTCJ did induce greater glycolytic flux from days 10–20 of supplementation. This provides an explanation for the lower MFO rates and percentage contributions of fat to total EE in the present study compared to Cook et al. (2015). The absorption of maltodextrin, present in the placebo, from the small intestine into systemic circulation, has not been proven to be different from pure glucose; therefore, the magnitude and time course of the glycaemic and insulinaemic response are expected to be similar either at rest or during exercise (Hofman et al. 2016). However, as MTCJ contained fructose and glucose the significant elevation in CHO oxidation, at rest with MTCJ during the final 10 days of supplementation is likely due to fructose accumulation. In support of these findings, co-ingestion of glucose and fructose as opposed to either alone has been shown to significantly increase CHO oxidation rates in healthy participants (Jentjens and Jeukendrup 2005).

The tendency towards significance for the 13.35% increase in TAS from pre-supplementation to mid-supplementation with MTCJ may explain the tendency for greater fat oxidation with MTCJ during 1-h FATMAX exercise between these time points. The increase in oxygen flux and, therefore, generation of ROS during exercise (Radak et al. 2013), as a by-product of fatty acid oxidation at FATMAX, may have been mitigated by MTCJ antioxidants. These antioxidants may have retarded mitophagy and preserved mitochondrial function, thus enabling the continuation of fat oxidation (Montgomery and Turner 2015). The 12.76% decrease in TAS from mid-supplementation to post-supplementation prior to exercise suggests TAS does not increase linearly with further ingestion of MTCJ after 10 days but returns to baseline. As far as the authors are aware, this is the first study to demonstrate such an effect. Consequently, this may have contributed to the different responses observed between conditions for HDL and resting EE during days 10–20 of supplementation. Endogenously derived antioxidants are the primary contributors to the antioxidant balance; thus, supplementation of exogenous antioxidants may inhibit synthesis of endogenous antioxidants in order to maintain a homeostatic balance (Poljsak et al. 2013). Thus, it is plausible that the synthesis of endogenous antioxidants was reduced during the final 10 days of exogenous antioxidant supplementation with MTCJ resulting in a net reduction of TAS. A potential increase in oxidative stress from mid- to post-supplementation may have contributed to inefficient oxidative metabolism and thus greater resting EE (Frisard and Ravussin 2006). These findings are supported by Timmers et al. (2011) who reported significantly lower basal EE due to improved mitochondrial function and, therefore, metabolic efficiency after 30 days' resveratrol supplementation, a potent antioxidant, in healthy, obese humans. Furthermore, the reduced synthesis/uptake of antioxidants may be a defensive mechanism against an excessively elevated antioxidant balance which may affect hormetic responses. Such is the complexity of the interactions of free radicals, pro-oxidant species, antioxidants and the cellular mechanisms involved that no one reason is liable to be responsible for this potential antioxidant effect.

In relation to lipid responses, Ataie-Jafari et al. (2008) reported tart cherry juice significantly reduced total cholesterol and LDL, whilst Martin et al. (2010) reported a

significant improvement in triglycerides and VLDL. In both studies, participants were found to be hyperlipidaemic for the specific variables in which a treatment effect was observed. Likewise, studies (Kelley et al. 2006; Lynn et al. 2014) reporting non-significant differences with the cherry treatment recruited healthy participants; therefore, it is reasoned the lack of cardio-metabolic dysregulation does not provide sufficient scope for a cherry intervention to further regulate cardio-metabolic function at rest. Despite the addition of an exercise intervention, a similar reasoning can be applied to the present study, given the healthy baseline lipid concentrations presented by the participants in this study. This observation is further supported by findings presented in rodents susceptible to dyslipidaemia responding significantly better to cherry consumption than those without dyslipidaemia (Seymour et al. 2008, 2009; Wu et al. 2006). The molecular mechanisms associated with these responses were suggested to be via increased mRNA transcription of peroxisome proliferator-activated receptor alpha (PPARα), peroxisome proliferator-activated receptor gamma (PPARγ) and hepatic activation of these isoforms by cherry anthocyanins (Seymour et al. 2008, 2009). Subsequently, enhancing skeletal muscle insulin sensitisation due to increased fat oxidation through sirtuin-1 mediated PPARα and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) activation (Huffman 2010).

It is apparent accumulation of anthocyanins and their metabolites with long-term supplementation likely did not occur in the present study. This is likely due to the poor bioavailability, intestinal absorption and rapid elimination rates of anthocyanins (Manach et al. 2004). Alternatively, it may be that the anthocyanin and secondary metabolite concentrations in target tissues reached a ceiling level below a physiologically relevant threshold concentration (Krga et al. 2016) to affect serum lipids, thus explaining pre-exercise HDL responses to MTCJ consumption. A biphasic response was observed where a non-significant, yet clinically relevant, increase in HDL from pre- to mid-supplementation was followed by a significant decline from mid- to post-supplementation with MTCJ, but not PLA. Based on data from epidemiological studies with middle-age and high-risk subjects, suggesting an increase of 0.026 mmol·L^{-1} could reduce the risk of coronary heart disease by 2% in males (Gordon et al. 1989), the change in HDL from pre- to mid-supplementation would result in a risk reduction of 12% with MTCJ. However, this finding should be interpreted with caution as the translation across to healthy subjects of both sexes and the lack of statistical and clinically significant improvements for other lipid profile markers with MTCJ render the increase in HDL less important. The decline from mid- to post-supplementation suggests that administering cherry interventions longer than 10 days does not maintain elevated HDL concentrations, but rather a return to baseline as supported by findings from Kelley et al. (2006), Ataie-Jafari et al. (2008), Martin et al. (2010) and Lynn et al. (2014).

Given the variability in study design and supplementation strategy in the present and previous studies (Ataie-Jafari et al. 2008; Kelley et al. 2006; Lynn et al. 2014; Martin et al. 2010) it is possible to conclude that the length of supplementation, volume and concentration of juice are unlikely to be factors responsible for the equivocal results regarding lipids after cherry consumption. Rather, the initial physiological status of participants is thought to be more influential regarding the efficacy of a cherry intervention.

Finally, limitations associated with this study include the overnight fast dietary restriction put in place to reduce intra- and inter-individual variability, but at the expense of upholding ecological validity. Second, previous research has shown improvements in body mass, fat mass and metabolic function required participants to exercise at FATMAX 2–4 times per week for 2–12 months (Brun et al. 2011). Thus, Montmorency tart cherry anthocyanins were not able to compound upon any cellular and molecular adaptations promoting fat oxidation which may have occurred with FATMAX training. Third, the small sample size did not assist the ability to find significant differences with MTCJ as post-hoc power analysis suggested the study was underpowered. Additionally, due to the differences in viscosity of the placebo and MTCJ, 9 out of 11 participants correctly identified which supplement they were provided when asked at the end of the study. Consequently, this may have contributed to the lack of significance between conditions as participants could manipulate their activity during the study. This highlights a limitation of juice concentrate as a form of supplementation and perhaps alternative forms such as capsules are preferable to uphold anonymity between conditions.

Future work should attempt to elucidate the cellular and molecular mechanisms, including epigenetic changes, in humans to provide a basis upon which theories explaining the obtained responses can be either accepted or refuted. This would provide crucial information by which supplementation strategies can be altered to maximise the efficacy of MTCJ. Based on Cook et al. (2015) findings, it would be appropriate to suggest that any effect of anthocyanin supplementation on fat oxidation is short-term and provides a rationale to acutely supplement Montmorency tart cherry anthocyanins in future studies. The augmentation of fat oxidation with acute supplementation may then mitigate the development of cardio-metabolic symptoms in clinical populations such as those with Metabolic Syndrome.

Conclusion

This was the first study to examine the effect of cherry supplementation on fat oxidation rates at rest and during exercise. Findings showed that MTCJ did not significantly increase fat oxidation rates at rest or during FATMAX exercise. Additionally, secondary cardio-metabolic markers were also not significantly different with MTCJ supplementation, the primary reason being that this intervention does not indicate CRM properties in healthy participants. Consequently, it is unnecessary for healthy participants to supplement MTCJ to reduce body fat percentage and improve both functional and blood-based cardio-metabolic markers. Previous studies to report a significant response to cherry supplementation in animal (Seymour et al. 2008, 2009; Wu et al. 2006) and human (Ataie-Jafari et al. 2008; Keane et al. 2016a, b; Martin et al. 2010) studies occurred when initial values were abnormal; thus further research is warranted in clinical populations.

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Author contributions TD, LB and MR conceived and designed the experiments; TD performed the experiments; TD, LB and MR analysed the data; TD, LB and MR wrote the paper.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Appendix 11

Effects of Montmorency Tart Cherry Supplementation on Cardio-Metabolic Markers in Metabolic Syndrome Participants: a pilot study

Terun Desai, Michael Roberts and Lindsay Bottoms

School of Life and Medical Sciences, University of Hertfordshire, Hatfield, UK.

Abstract

This study compared acute supplementation of Montmorency tart cherries in capsule (MTCC) and juice (MTCJ) forms in MetS humans, as potential prophylactic interventions against cardio-metabolic diseases. In an acute, single-blind, placebo-controlled, randomised, crossover trial, eleven participants with MetS (49 ±12 years; 6M/5F), consumed one bolus of MTCC, MTCJ or placebo (PLA) on different occasions. Blood-based and functional cardio-metabolic biomarkers were measured pre-bolus and up to 5 hours post-bolus. MTCJ significantly reduced systolic blood pressure compared to PLA at 2-hours post-bolus (P = 0.018). Insulin was significantly lower with MTCC (P = 0.016) and MTCJ (P = 0.028) than PLA at 1-hour and 3-hours post-bolus, respectively. No significant differences between MTCC and MTCJ were seen. This study demonstrated for the first time that MTCC could reduce insulin concentrations in humans. Importantly, MTCJ induced a clinically relevant reduction in systolic blood pressure and also lowered insulin compared to PLA, in MetS humans.

Keywords: polyphenols; anthocyanins; cardiometabolic health; functional foods; hypertension; diabetes

Corresponding Author:
Terun Desai
Department of Psychology and Sports Science,
School of Life and Medical Sciences,
University of Hertfordshire,
College Lane,
Hatfield,
AL10 9AB.
Tel: +44 (0) 1707 277657
Email: t.desai@herts.ac.uk

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1. Introduction

Often accompanied by endothelial dysfunction, a pro-inflammatory, pro-oxidant and pro-thrombotic state (Srikanthan, Feyh, Visweshwar, Shapiro, & Sodhi, 2016), Metabolic Syndrome (MetS) encompasses a cluster of cardio-metabolic conditions including insulin resistance, central adiposity, dyslipidaemia and hypertension (Kaur, 2014). The presence of three or more of these symptoms combined, augments the incidence of chronic diseases such as cardiovascular disease (CVD) by 2-fold and type 2 diabetes (T2D) by 5-fold (Falkner & Cossrow, 2014). Ultimately, this highlights the strong association between MetS and higher rates of cardiovascular and total mortality (Falkner & Cossrow, 2014). Despite the difficulty in measuring MetS prevalence rates, it is accepted that global rates are on the rise (O'Neill & O'Driscoll, 2015). In the United States [~35% MetS prevalence rate (Aguilar, Bhuket, Torres, Liu, & Wong, 2015)], low fruit and vegetable intake accounted for 15.1% of all diet-related cardio-metabolic deaths (Micha *et al.,* 2017). Therefore, fruit and vegetables rich in polyphenols, especially anthocyanins, are attractive interventions against cardio-metabolic disease risk factors (Vendrame, Del Bo, Ciappellano, Riso, & Klimis-Zacas, 2016); as supported by epidemiological evidence (Arts & Hollman, 2005; Cassidy *et al.*, 2013; Wallace, 2011).

Substantial literature (Amiot, Riva, & Vinet, 2016; Basu *et al.,* 2011; Basu *et al.,* 2010; Basu & Lyons, 2012; Basu *et al.,* 2009; Stull *et al.,* 2015; Vendrame *et al.,* 2016) exists suggesting consumption of anthocyanin-rich dietary interventions are beneficial for mitigating the development of symptoms associated with MetS. Montmorency tart cherries (MTC) is one such intervention not only abundant in anthocyanins, but also other phenolics, resulting in being one of the most potent dietary antioxidant interventions when consumed in habitual portion sizes (Ou, Bosak, Brickner, Iezzoni, & Seymour, 2012). As far as the authors are aware, no study has been published assessing the responses of human participants with MetS, to MTC

supplementation; however individual aspects of the MetS cluster have been explored in pathological populations. Keane *et al.* (2016b) showed significant reductions in systolic blood pressure (SBP) after acute ingestion of Montmorency Tart Cherry Juice (MTCJ) (60 mL concentrate) in hypertensive males. Additionally, Martin, Bopp, Neupane, and Vega-Lopez (2010) demonstrated significant reductions in serum triglycerides, triglyceride/HDL ratio and VLDL (very low-density lipoprotein) concentrations after 4 weeks tart cherry juice (230 $mL.day^{-1}$) supplementation in participants with central adiposity and dyslipidaemia. Moreover, Ataie-Jafari, Hosseini, Karimi, and Pajouhi (2008) reported 6 weeks concentrated sour cherry juice (40 $g.day^{-1}$) supplementation improved glycated haemoglobin ($HbA1_c$), in T2D females, along with total cholesterol and LDL (low-density lipoprotein) in those participants with hyperlipidaemia.

Anthocyanin pharmacokinetics after consuming whole Montmorency tart cherries indicated parent anthocyanin concentrations in plasma peaked 2-4 hours post-ingestion, and excretion rates in urine peaked 6-8 hours post-ingestion (Seymour *et al.,* 2014). Similarly, Bell *et al.* (2014a) reported increased uptake of cyandin-3-O-glucoside in plasma, compared to baseline at 3- and 5-hours post-consumption of 30 mL MTC concentrate in healthy humans. Correspondingly, Keane *et al.* (2016a) reported the secondary metabolites of cyandin-3-O-glucoside, protocatechuic and vanillic acids were also elevated at 1-hour and 2-hours post-consumption of 30 mL MTC concentrate, respectively, in healthy humans. The pharmacokinetics of powdered freeze-dried MTC anthocyanins and metabolites are currently unknown when administered in encapsulated form, but bioavailability is anticipated to be enhanced. Anthocyanin bioavailability is notoriously poor (Kay, 2006), hence capsules as a delivery vehicle of anthocyanins to target tissues via systemic circulation may be useful as they selectively degrade once in the gastrointestinal tract, therefore potentially maximising bioavailability (Oidtmann *et al.,* 2012). This is achieved due to protection of anthocyanins by

the exterior capsule shell against factors known to influence anthocyanin stability such as pH, temperature, light, enzymes (particularly in saliva) and sugars (Robert & Fredes, 2015). Hydroxypropyl methylcellulose (HPMC) is a material used to form capsule shells and has been shown to delay the release of green tea catechins (Glube, Moos, & Duchateau, 2013) and mulberry extract anthocyanins (Lown *et al.,* 2017). Consequently, this delayed glucose and insulin responses until 30 minutes after ingestion, coinciding with the disintegration and dissolution of the capsule shell (Lown *et al.,* 2017). Subsequently, Montmorency Tart Cherry Capsules (MTCC) will be provided to assess whether encapsulation, and thus protection of MTC anthocyanins from premature degradation, potentially maximises bioavailability and therefore their beneficial effects on cardio-metabolic function. Furthermore, the high sugar content in polyphenol-rich juices (Vendrame *et al.,* 2016), including MTCJ (Desai, Bottoms, & Roberts, 2018), are purported to nullify and mask some of the beneficial cardio-metabolic effects induced by the polyphenols. Thus, MTCC, with a significantly lower sugar content, may be more efficacious at improving cardio-metabolic markers compared to MTCJ.

Centred around the pharmacokinetics of MTC parent anthocyanins and their metabolites, this study aimed to explore the short-term responses to acute supplementation of MTCJ and MTCC on cardiac haemodynamics, arterial stiffness by pulse wave analysis (PWA), substrate metabolism and various blood-based cardio-metabolic biomarkers in humans with MetS. Given that these subjects present at least three or more risk factors resulting in a holistic state of cardio-metabolic dysregulation, it was hypothesised that both MTC interventions would induce favourable responses on diagnostic cardio-metabolic markers, in particular glucose, lipids and SBP, based on previous human studies with tart cherry supplementation (Ataie-Jafari *et al.,* 2008; Keane *et al.,* 2016b; Martin *et al.,* 2010). Furthermore, it was hypothesised that MTCC would be more effective than MTCJ or placebo in mediating these benefits, as the bioavailability of anthocyanins and their metabolites were anticipated to be superior.

2. Methods

2.1. Participants

Eleven (6 males and 5 females) participants (mean ±SD, age 49 ± 12 years, stature 1.72 ± 0.11 m, body mass 99.53 ± 20.49 kg) with Metabolic Syndrome (Table 1) volunteered for this pilot study. All participants were screened for MetS prior to formal inclusion onto the study according to the harmonised criteria outlined by Alberti *et al.* (2009), where 3 of the 5 qualifying criteria [waist circumference: ethnicity and sex specific criteria; fasting triglycerides: ≥1.69 mmol.L^{-1}; fasting high-density lipoprotein: <1.03 mmol.L^{-1} (men), <1.29 mmol.L^{-1} (women); brachial blood pressure: ≥130 mmHg SBP or ≥85 mmHg DBP and/or fasting glucose: ≥6.1 mmol.L^{-1}] had to be met. Recruitment (Figure 1) was conducted via word of mouth, flyers and email advertisements. Participants were excluded from the study if they did not meet the criteria for MetS at screening, were smokers, pregnant, heavy alcohol consumers (>14 units per week), current or previous diagnosis of chronic disease (gastrointestinal, cardiovascular, hepatic or renal), or were on statins, hyperlipidaemic, anti-hypertensive, anti-diabetic, anti-inflammatory or steroidal medication. All participants were instructed to abstain from consuming any other multivitamin, antioxidant, fish oil, dietary or herbal supplementation two weeks prior to and for the duration of the study. Verbal and written information was provided to all participants regarding the study purpose and procedures. Ethical approval was obtained from the University of Hertfordshire HSET Ethics Committee (LMS/PGT/UH/02843) and the study's experimental procedures followed the principles outlined in the Declaration of Helsinki. Written informed consent was provided by all participants prior to enrolment. This study was registered prospectively as a clinical trial on clinicaltrials.gov (NCT03615885).

Table 1. Baseline characteristics obtained during screening of all participants (n = 11).

Characteristics	Mean (95% CI)	Participant										
		1	2	3	4	5	6	7	8	9	10	11
BMI ($kg.m^{-2}$)	33 (30 – 37)	30	39	28	29	44	36	28	36	32	36	29
Waist Circumference (cm)	106 (97 – 115)	102.2*	133*	93	90.8	119*	119*	95	104.1*	94	112*	104*
Fasting Glucose ($mmol.L^{-1}$)	5.63 (5.12 – 6.14)	6.20*	5.33	6.72*	4.48	5.93	6.50*	6.21*	5.12	5.78	4.85	4.79
Fasting Triglycerides ($mmol.L^{-1}$)	1.9 (1.57 – 2.23)	1.9*	2.0*	2.6*	1.7*	0.9	1.5	2.6*	2.1*	2.0*	2.1*	1.5
Fasting HDL ($mmol.L^{-1}$)	1.23 (0.99 – 1.48)	2.23	1.25	1.22*	0.94*	1.23*	0.83*	1.19*	1.30	1.06*	1.28*	1.01*
bSBP (mmHg)	132 (127 – 138)	122	146*	130*	130*	136*	143*	129	132*	142*	120	126
bDBP (mmHg)	80 (74 – 87)	67	90*	86*	87*	79	91*	72	72	88*	67	85*
Fasting Insulin ($pmol.L^{-1}$)	115.85 (64.37 – 167.34)	53.10	81.65	182.85	72.37	216.88	280.16	116.33	56.81	91.83	62.09	60.29
HOMA2-IR (AU)	2.2 (1.2 – 3.2)	1.0	1.5	3.5	1.3	4.0	5.3	2.3	1.1	1.8	1.1	1.1
HOMA2-β (%)	121.3 (97.4 – 145.2)	61.8	111.7	126.2	145.2	179.2	181.9	106.1	94.5	103.4	111.8	112.4
HOMA2-%S (%)	61.8 (42.3 – 81.4)	95.5	64.9	28.2	76.5	24.7	19.0	44.3	93.6	56.7	87.0	89.8

*Denotes value met threshold for MetS inclusion criteria. AU (Arbitrary Units); BMI (Body Mass Index); bDBP (Brachial Diastolic Blood Pressure); bSBP (Brachial Systolic Blood Pressure); HDL (High-density Lipoprotein); HOMA2-IR (Homeostatic Model Assessment of Insulin Resistance); HOMA2-β (Homeostatic Model Assessment of pancreatic β-cell function); HOMA2-%S (Homeostatic Model Assessment of Insulin Sensitivity).

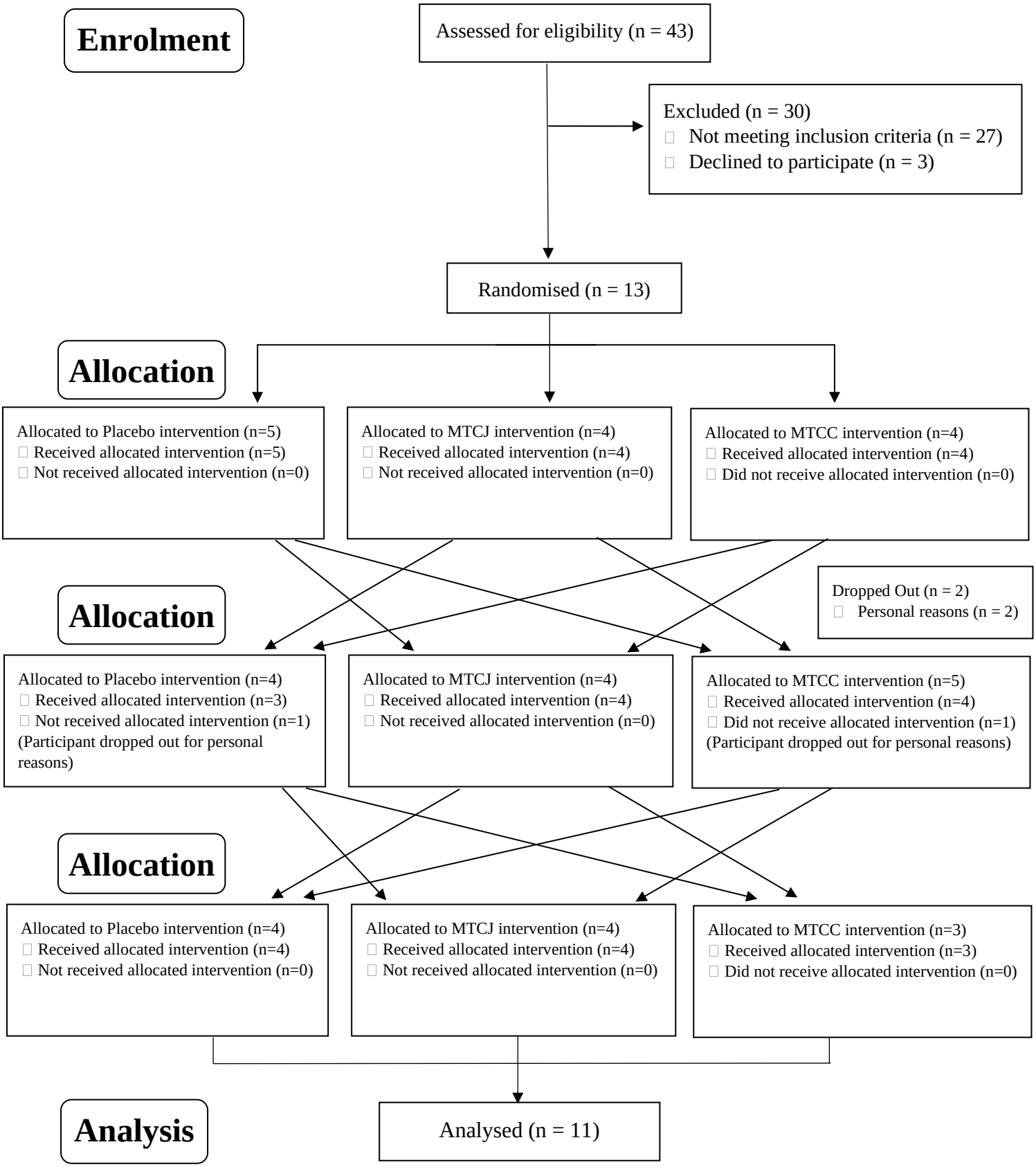


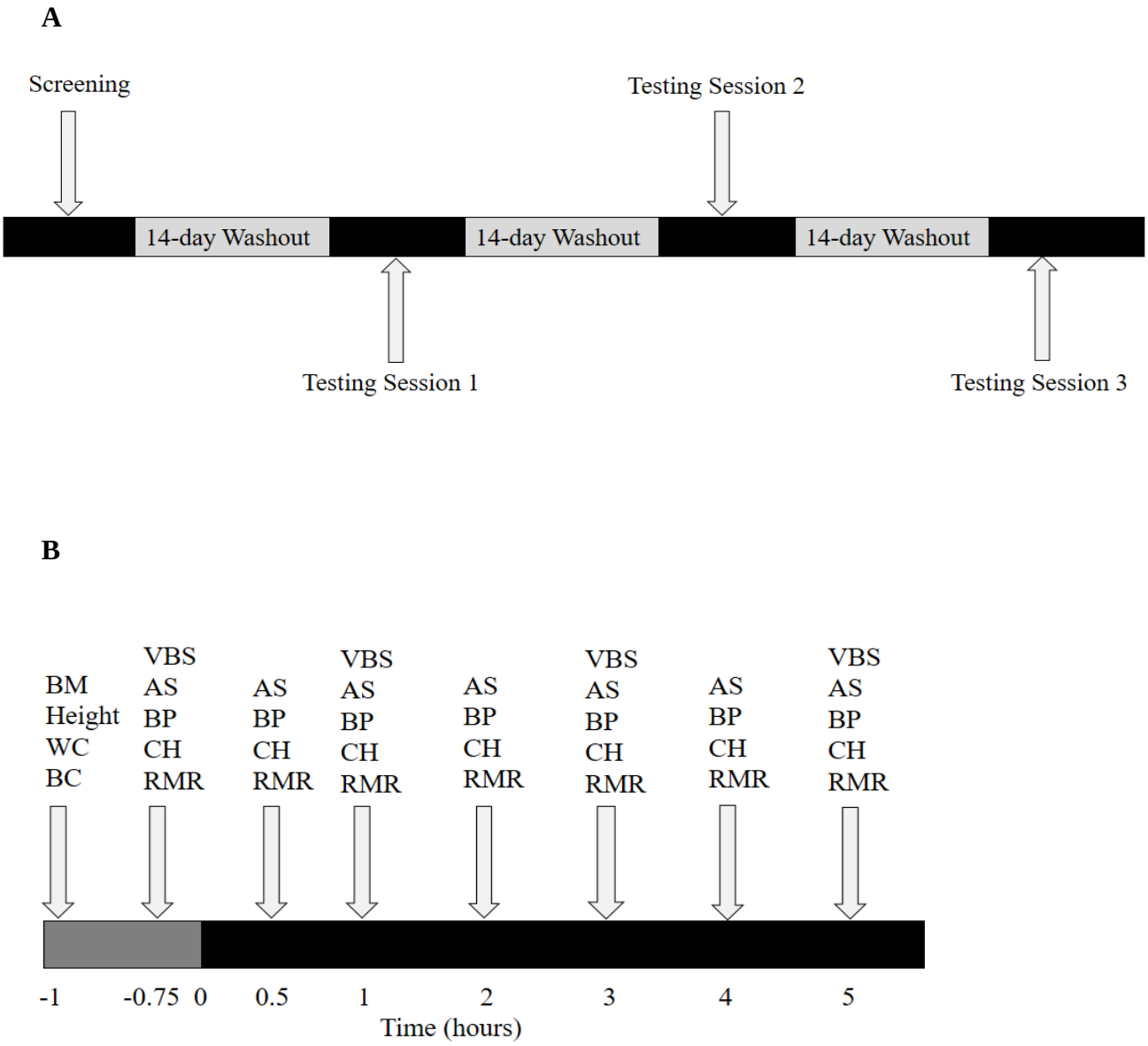
Figure 1. CONSORT flow diagram of the participants recruited, screened, tested, analysed and excluded during the course of the study.

2.2. Procedures

2.2.1. Research Design

A single-blind (blinded to participant), placebo-controlled, randomised, crossover design was implemented; each participant acted as their own control. During the 6-week study duration, all participants completed three testing sessions during which one of three [placebo (PLA) or MTCJ or MTCC] different supplements were provided each time. GraphPad software (GraphPad, QuickCalcs, San Diego, California, USA) was used to randomise supplementation order. A 14-day washout period (Cook, Myers, Blacker, & Willems, 2015; Howatson *et al.*, 2012; Keane *et al.*, 2016c) was incorporated between testing sessions and between the screening and first testing sessions.

Testing sessions lasted 6 hours and were identical in terms of design and testing procedures. Figure 2 depicts schematics of the overall study design and specific procedures during a testing session. Baseline anthropometric (stature, body mass, waist circumference) and functional (PWA, cardiac haemodynamics, RMR) measurements were obtained prior to consumption of the supplement. Functional measurements were recorded at 30 minutes, 1, 2, 3, 4- and 5-hours post-bolus. Venous blood sampling was performed at baseline, 1, 3- and 5-hours post-bolus only.



Key:
AS – Arterial Stiffness; BC – Body Composition; BM – Body Mass; BP – Blood Pressure; CH – Cardiac Haemodynamics; RMR – Resting Metabolic Rate; VBS – Venous Blood Sampling; WC – Waist Circumference

= Pre-Bolus
= Post-Bolus

Figure 2. (A) Schematic representation of the overall study design. (B) Schematic representation of the specific procedures during each testing session.

2.2.2. Dietary Guidelines

All participants arrived at the laboratory between 7 – 10am, after an overnight fast of a minimum of 10 hours, to account for circadian variation (Bell, Walshe, Davison, Stevenson, & Howatson, 2014c). Participants were instructed to maintain their habitual polyphenol intake, including anthocyanins, as opposed to complete restriction throughout the study. This was to ensure that the polyphenols provided by MTCJ were supplementary to the existing habitual polyphenol intake of each participant representing normal daily activity and therefore upholding ecological validity. Fluids or food were not provided during testing sessions, but participants were able to drink water *ad libitum*, where volume of consumption was monitored during the first session and repeated in subsequent sessions (Keane *et al.*, 2016b).

Total energy, macronutrient and polyphenol intake of participants' 'Western' habitual diet was analysed through food diaries. This was to assess compliance of replicating dietary intake for the 3 days prior to each testing session (Alkhatib, Seijo, Larumbe, & Naclerio, 2015; Nordby, Saltin, & Helge, 2006; Roberts, Roberts, Tarpey, Weekes, & Thomas, 2015). Participants were given instructions on how to complete the food diaries, including portion sizes. Dietary analysis software (Dietplan 7.0, Forestfield Software, UK) was used to monitor compliance of the 3-day food diaries before each session. All participants complied with dietary guidelines upon analysis for percentage contributions of macronutrients to total energy intake [mean ± SD (protein 16 ± 23%), (CHO 45 ± 56%), (fat 39 ± 38%)], total polyphenols (70 ± 83 mg) and anthocyanins (26 ± 22 mg).

2.2.3. Supplementation

This study acutely administered three different supplements including a placebo which acted as the control condition and two experimental conditions, MTCJ and MTCC. The placebo was composed of 30 mL commercially available fruit-flavoured cordial (Cherries and Berries, Morrisons, Bradford, UK) mixed with 100 mL water. The placebo drink was also matched

against MTCJ for energy content, taste and visual appearance by adding a flavourless carbohydrate (Maltodextrin, My Protein Ltd, Northwich, UK), citric acid (100% Pure Citric Acid, VB and Sons, UK), red and black food colouring (Morrisons, Bradford, UK), respectively. MTCJ consisted of 30 mL Montmorency tart cherry concentrate (Cherry Active, Active Edge Ltd, Hanworth, UK) and 100 mL water. Each 30 mL serving of MTC concentrate provided a total anthocyanin content of 270 mg (9 $mg.mL^{-1}$) (Howatson *et al.*, 2010). In MTC concentrate, the most abundant anthocyanins include, cyanidin-3-glucosylrutinoside, cyanidin-3-rutinoside and peonidin-3-rutinoside (Bell *et al.*, 2014a). In order to match the anthocyanin content of MTCJ, participants consumed 10 Montmorency tart cherry capsules (Cherry Active Capsules, Active Edge, Ltd, Hanworth, UK) with 130 mL water. Each capsule contained 435 mg of freeze-dried Montmorency tart cherry skin powder (Table 2). Independent analysis (AtlasBiosciences, 2018) showed total anthocyanin content in each capsule was 27 mg. The 30 mL serving of Montmorency tart cherry concentrate contained ~90-110 whole Montmorency tart cherries whilst 10 capsules were made from ~100 whole cherries. Anonymity of supplementation was ensured by blinding the participants to the source of anthocyanins. This was achieved by explaining that an ‘anthocyanin-rich supplement’ would be provided rather than disclosing Montmorency tart cherries as the specific source.

Table 2. Nutritional information of each supplement provided.

	Montmorency tart cherry concentrate (per 30 mL)	Montmorency tart cherry capsules (per capsule – 435 mg)	Placebo (per 30 mL)
Energy (kcal)	102	1.3	102
Carbohydrate (g)	24.5	0.3	25.35
of which sugars (g)	17.9	0.1	25.32
Protein (g)	1.1	Trace	0.03
Fat (g)	0	Trace	0
of which saturates (g)	0	0	0
Fibre (g)	2.6	Trace	Trace
Total Anthocyanins (mg)	270	27	0

2.2.4. Measures and Equipment

2.2.4.1. Blood Pressure

Brachial systolic (bSBP) and diastolic (bDBP) blood pressure (Omron MX3, Omron, Japan) were measured four times in an upright seated position, with an average of the final three being taken as BP (Cook *et al.*, 2015).

2.2.4.2. Cardiac Haemodynamics

Beat-to-beat resting cardiac haemodynamic parameters including heart rate (HR), cardiac output (CO), stroke volume (SV), mean arterial pressure (MAP) and total peripheral resistance (TPR) were measured non-invasively (Finometer MIDI Model-2, Finapres Medical Systems BV, Amsterdam, The Netherlands) at all time points, using the arterial volume clamp method (Penaz, 1992). To avoid data selection, recordings were averaged over 10 consecutive beats

with the lowest values for each parameter taken for statistical analysis (Cook, Myers, Gault, Edwards, & Willems, 2017).

2.2.4.3. Pulse Wave Analysis

Pulse wave analysis (PWA) was used to monitor arterial stiffness non-invasively using applanation tonometry (SphygmoCor, ScanMed Medical, UK), as there is a strong association between PWA and cardiovascular disease incidents, independent of traditional risk factors (Choi *et al.,* 2004; Keane *et al.,* 2016b). A pencil-type probe was placed over the radial artery on the right wrist which was slightly extended and rested on a pillow (Doupis, Papanas, Cohen, McFarlan, & Horton, 2016). Aortic pulse waveforms, aortic SBP, aortic DBP, pulse pressure (PP), augmentation pressure (AP), augmentation index (AIx), AIx normalised to 75 beats.min^{-1} (AIx at HR75) and subendocardial viability ratio (SEVR) were determined by the in-built software (SphygmoCor version 7.0, ScanMed, UK) using generalised transfer functions. Recordings with a quality-index ≥80%, as determined by the manufacturer, were selected for statistical analysis.

2.2.4.4. Resting Metabolic Rate (RMR)

RMR, resting energy expenditure, substrate oxidation rates and respiratory quotient (RQ) were measured using an open-circuit indirect calorimetry system (GEM Nutrition Ltd, Cheshire, UK). Participants lay supine for 20 minutes with data averaged for the final 17 minutes only, to achieve steady-state and account for any initial short-term variances in respiration (Kelly, King, Goerlach, & Nimmo, 2013). A ventilated hood was placed over the head with a flexible plastic seal around the neck and shoulders to prevent air inside and outside the hood from mixing.

Resting fat and carbohydrate oxidation rates were calculated using stoichiometric equations, assuming negligible protein oxidation (Jeukendrup & Wallis, 2005).

$$\text{Fat Oxidation Rate } (g.min^{-1}) = (1.695 \cdot \dot{V}O_2) - (1.701 \cdot \dot{V}CO_2) \quad (1)$$

$$\text{CHO Oxidation Rate } (g.min^{-1}) = (4.344 \cdot \dot{V}CO_2) - (3.061 \cdot \dot{V}O_2) \quad (2)$$

2.2.5. Blood Sampling and Analysis

2.2.5.1. Blood Sampling

Venous blood was sampled through 4 individual venepunctures (one at each time point: pre-bolus and 1, 3, 5 hours post-bolus), using the butterfly method (BD Vacutainer Safety-Lok Blood Collection Set 21G with Luer Adapter, Becton Dickinson and Co., Oxford, UK). A total of 5 mL venous blood was collected into serum-separation tubes (BD Vacutainer, Becton Dickinson and Co., Oxford, UK) for each sample. Tubes were centrifuged at 4000 $rev.min^{-1}$, 4°C for 10 minutes (Sorvall ST 8R, Thermo Fisher Scientific, USA). Serum supernatants were aliquoted and stored at -80°C for later analysis.

2.2.5.2. Glucose

Serum samples were assessed for glucose (range 0.5-50 $mmol.L^{-1}$, CV ≤ 1.5%) (Biosen C-Line, EKF Diagnostics, Cardiff, UK) in duplicates.

2.2.5.3. Insulin Assay

Serum insulin samples were measured in duplicates using a human 96-well colorimetric insulin enzyme-linked immunosorbent assay (ELISA) (Insulin Human ELISA KAQ1251, Invitrogen, Thermo Fisher Scientific, USA), with a common sample measured on each plate for determining inter-plate CV. A four-parameter algorithm standard curve was generated using standard curve fitting software (SigmaPlot, Systat Software Inc, San Jose, USA), where values were converted from $\mu IU.mL^{-1}$ to $pmol.L^{-1}$ by multiplying by 6.945 (Bos *et al.*, 2010). Inter- and intra-plate precision were 8.8% and 7.69%, respectively.

2.2.5.4. Insulin Resistance and Sensitivity Indexes

Homeostatic Model Assessment (HOMA) was used to estimate fasting steady-state pancreatic β-cell function (HOMA2-β) and insulin resistance (HOMA2-IR index) through the HOMA2 spreadsheet model (HOMA2-IR, available from https://www.dtu.ox.ac.uk/homacalculator/) (Levy, Matthews, & Hermans, 1998). *Equations 1, 2 and 3* (Matthews et al., 1985), outline the determination of pancreatic β-cell function, insulin sensitivity (HOMA2-%S) and insulin resistance, respectively. As recommended by Wallace, Levy and Matthews (2004), HOMA2-%S was calculated to assist in the correct interpretation of HOMA2-β.

$$\text{HOMA2-}\beta\ (\%) = \frac{\left(20 \cdot \text{Fasting Insulin } (\mu\text{IU.mL}^{-1})\right)}{(\text{Fasting Glucose } (\text{mmol.L}^{-1})\text{-}3.5)} \cdot 100 \qquad (3)$$

$$\text{HOMA2-\%S } (\%) = \left(\frac{1}{\text{HOMA-IR}}\right) \cdot 100 \qquad (4)$$

$$\text{HOMA2-IR} = \frac{[\text{Fasting Glucose } (\text{mmol.L}^{-1}) \cdot \text{Fasting Insulin } (\mu\text{IU.mL}^{-1})]}{22.5} \qquad (5)$$

2.2.5.5. Lipid Assays

Serum lipids were determined in duplicates using commercially available colorimetric assays on a semi-automated spectrophotometer (RX Monza, Randox Laboratories Ltd, Antrim, UK), according to manufacturer's guidelines. Triglyceride (Triglycerides TR210, Randox) values were corrected for free glycerol by subtracting 0.11 mmol.L^{-1} (Stinshoff *et al.,* 1977), according to the manufacturer's guidelines. Intra-assay CV for triglycerides, total cholesterol and HDL were 2.33%, 4.45% and 3.63%, respectively. LDL was determined indirectly using the formula below (Ahmadi, Boroumand, Gohari-Moghaddam, Tajik, & Dibaj, 2008).

$$\text{LDL } (\text{mmol.L}^{-1}) = \left(\frac{\text{Total Cholesterol}}{1.19}\right) + \left(\frac{\text{Triglycerides}}{0.81}\right) - \left(\frac{\text{HDL}}{1.1}\right) - 0.98 \qquad (6)$$

2.3. Data Analysis

Statistical analysis was performed using SPSS v22.0 (IBM, Chicago, USA) where data are reported as means [95% Confidence Intervals] (CI). Data normality was checked using a Shapiro-Wilk test. Greenhouse-Geisser correction was applied upon violation of Mauchly's test of sphericity for ANOVAs. Statistical significance was set at $P < 0.05$. Due to this being a pilot study and the lack of prior data on the effects of Montmorency tart cherry products in humans with MetS from which to conduct power calculations, a minimum sample size of 10 was established (Udani, Singh, Singh, & Barrett, 2011). Subsequently, the statistics performed are exploratory given the small sample size.

To account for day-to-day physiological variances at pre-bolus between conditions for each variable, data was analysed as change from pre-bolus for each time point measured post-bolus. This enabled a fair assessment of the post-bolus responses to each condition from pre-bolus across all variables. The pre-bolus time point was not included as a covariate, as one-way ANOVA analysis indicated no significant differences between conditions for all variables at the pre-bolus time point, hence two-way repeated-measures ANOVA was performed.

A within-group two-way, 3 x 6, condition (PLA vs MTCC vs MTCJ) x time (30 minutes, 1, 2, 3, 4- and 5-hours post-bolus), repeated-measures ANOVA design with *post-hoc* Bonferroni's adjustment, measured differences for bSBP, bDBP, RMR, cardiac haemodynamic and PWA parameters on change from pre-bolus values for each condition.

Blood-based biomarkers were analysed using the same model but with a 3 x 3, condition (PLA vs MTCJ vs MTCC) by time (1, 3- and 5-hours post-bolus) design on change from pre-bolus values for each condition. A paired-samples *t*-test was used to identify differences between individual data points within conditions and between conditions for the corresponding time point.

Partial Eta-Squared ($\eta^2_{partial}$) was used to report effect sizes for ANOVA where effects were classified as small (0.01-0.08), moderate (0.09-0.25) and large (>0.25) (Cohen, 1998). Cohen's *d* effect size was used for paired-samples *t*-test and *post-hoc* interaction comparisons where effects were classified as no effect (0-0.1), small (0.2-0.4), moderate (0.5-0.7) and high (≥0.8) (Cohen, 1998).

3. Results

The present study measured variables relevant to MetS, and as the defining aspect is insulin resistance, serum insulin and glucose were regarded as primary variables. Other variables focused on the other two main aspects of MetS, cardiovascular dysfunction and lipidaemia. Raw data for all variables is provided in Supplemental Tables 1-4.

3.1. Blood Biomarkers

Analysis of change from pre-bolus responses for serum insulin (Figure 3) showed a significant main effect for condition ($F_{(2, 20)} = 3.653$; $P = 0.044$, $\eta^2_{partial} = 0.27$), time ($F_{(1.07, 10.67)} = 13.411$; $P = 0.000$, $\eta^2_{partial} = 0.57$) and interaction ($F_{(4, 40)} = 5.837$; $P = 0.001$, $\eta^2_{partial} = 0.37$). The change from pre-bolus to 1-hour post-bolus between PLA (60.84 [24.33 – 97.34] pmol.L^{-1}) and MTCC (-9.86 [-25.16 – 5.46] pmol.L^{-1}) was significantly different ($P = 0.016$, $d = 1.70$); 7/11 participants lowered insulin with MTCC compared to PLA. The change from pre-bolus to 3-hours post-bolus between PLA (-8.99 [-35.55 – 17.57] pmol.L^{-1}) and MTCJ (-48.80 [-82.40 – -15.20 pmol.L^{-1}) was significantly different ($P = 0.028$, $d = 0.88$); 9/11 participants had lower insulin with MTCJ compared to PLA. Mean post-bolus responses showed PLA (12.01 [-4.42 – 28.44] pmol.L^{-1}) increased more than MTCJ (-18.01 [-40.33 – 4.31] pmol.L^{-1}) ($P = 0.039$). The change from 1- to 3-hours post-bolus was significant between PLA and MTCC ($t_{(10)} = -2.295$; $P = 0.045$, $d = 0.85$) and between MTCJ and MTCC ($t_{(10)} = 3.361$; $P = 0.007$, $d = 1.06$).

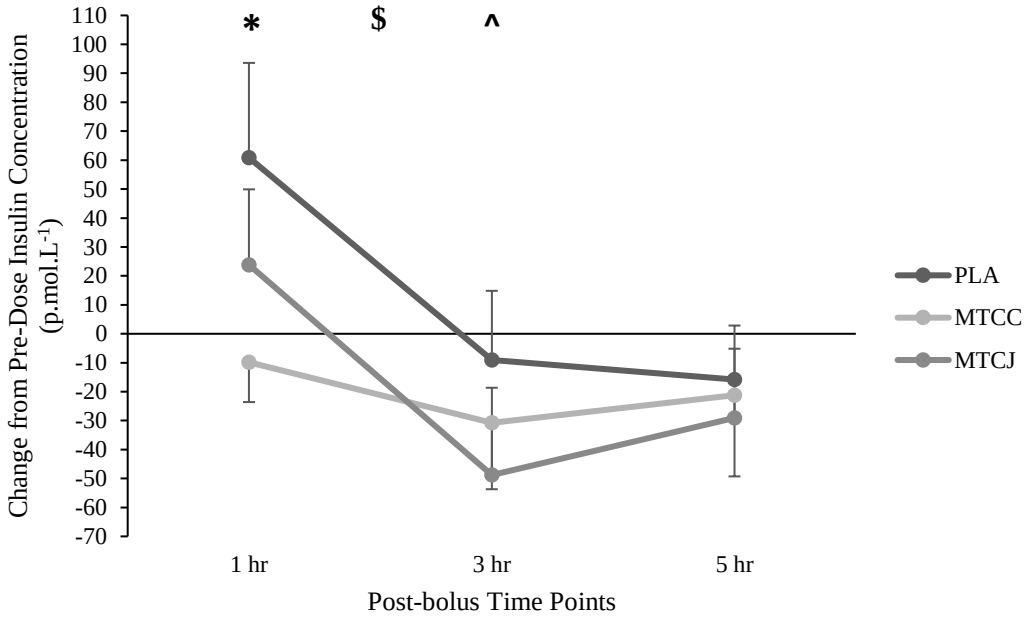


Figure 3. Response curves for the mean (error bars indicate 95% CI) change in insulin concentrations from pre-bolus values to time points post-bolus for each condition. *Denotes significant difference between PLA and MTCC at 1-hour time point. ^Denotes significant difference between PLA and MTCJ at 3-hour time point. $^{\$}$Denotes significant difference for change in insulin from 1-3 hours post-bolus between PLA and MTCJ against MTCC.

Individual responses showed 10/11 participants responded with an increase in LDL concentrations at 5-hours post-bolus (P = 0.057, d = 0.77) with PLA (0.71 [0.33 – 1.09] mmol.L^{-1}) compared to MTCC (0.10 [-0.27 – 0.47] mmol.L^{-1}). Change from pre-bolus responses for serum glucose did not show significant main effects for condition, time or interaction (P > 0.05). Statistical analysis for total cholesterol (TC) ($F_{(1.159,\ 11.587)}$ = 6.470; P = 0.023, $\eta^2_{partial}$ = 0.39), indicated a main effect for time only (Table 3). No main effects for condition, time or interaction were detected for triglycerides and HDL (P > 0.05) (Table 3).

Table 3. Mean [95% CI] change from pre-bolus values to post-bolus time points for selected blood-based biomarkers per treatment condition.

		Post-bolus Time Points		
		1 hr	3 hr	5 hr
Glucose ($mmol.L^{-1}$)	PLA	0.35 [-0.77 – 1.46]	-0.58 [-1.16 – -0.01]	-0.63 [-1.07 – -0.18]
	MTCC	-0.11 [-0.37 – 0.12]	-0.22 [-0.59 – 0.19]	-0.28 [-0.80 – 0.25]
	MTCJ	-0.22 [-0.66 – 0.24]	-0.79 [-1.42 – -0.13]	-0.72 [-1.55 – 0.10]
Triglycerides ($mmol.L^{-1}$)	PLA	0.09 [-0.06 – 0.26]	0.11 [-0.15 – 0.38]	0.21 [-0.08 – 0.49]
	MTCC	-0.05 [-0.09 – 0.08]	-0.06 [-0.31 – 0.20]	-0.05 [-0.29 – 0.18]
	MTCJ	-0.05 [-0.10 – 0.15]	0.03 [-0.10 – 0.19]	0.09 [-0.17 – 0.33]
Total Cholesterol*$ ($mmol.L^{-1}$)	PLA	0.04 [-0.16 – 0.23]	0.16 [-0.15 – 0.48]	0.29 [0.03 – 0.55]
	MTCC	-0.16 [-0.50 – 0.19]	-0.16 [-0.55 – 0.26]	0.15 [-0.31 – 0.57]
	MTCJ	-0.05 [-0.21 – 0.11]	-0.03 [-0.16 – 0.10]	0.13 [-0.02 – 0.27]
HDL ($mmol.L^{-1}$)	PLA	-0.12 [-0.15 – 0.06]	-0.10 [-0.12 – 0.02]	-0.18 [-0.26 – 0.09]
	MTCC	-0.05 [-0.16 – 0.05]	-0.04 [-0.11 – 0.03]	-0.03 [-0.15 – 0.09]
	MTCJ	-0.07 [-0.15 – 0.01]	0.01 [-0.06 – 0.09]	-0.09 [-0.21 – 0.03]
LDL ($mmol.L^{-1}$)	PLA	0.25 [-0.08 – 0.57]	0.36 [-0.04 – 0.76]	0.71 [0.33 – 1.09]
	MTCC	0.30 [-0.44 – 0.51]	-0.07 [-0.64 – 0.51]	0.06 [-0.38 – 0.51]
	MTCJ	0.08 [-0.27 – 0.43]	0.12 [-0.10 – 0.34]	0.24 [-0.20 – 0.68]

AU (Arbitrary Units); HDL (High-density Lipoprotein); LDL (Low-density Lipoprotein); TC (Total Cholesterol). *Denotes significant main effect for time with *post-hoc* identifying differences between 1-hour and 3-hours post-bolus. $Denotes significant main effect for time with *post-hoc* identifying differences between 3-hour and 5-hour post-bolus.

3.2. Cardiac Haemodynamics

No significant main effects for condition, time or the condition by time interaction were detected for bDBP, MAP, SV, CO, TPR ($P > 0.05$) (Table 4).

The change from pre-bolus to 2-hours post-bolus between PLA and MTCJ tended to be significantly different ($P = 0.051$, $d = 1.48$); 9/11 participants had lower MAP with MTCJ compared to PLA.

Despite there being no main effects for condition, time or interaction, pairwise comparisons revealed a significant difference ($P = 0.018$, $d = 1.75$) between PLA (3 [-1 – 8] mmHg) and

MTCJ (-8 [-13 – -4] mmHg) at 2-hours post-bolus for bSBP (Figure 4). Individual responses showed 10/11 participants responded with a lower bSBP after MTCJ consumption compared to PLA. Moreover, the mean difference (-11 [-19 – -4] mmHg) in responses between PLA and MTCJ at 2-hours post-bolus was greater than the clinically relevant threshold of 5 mmHg in 8/11 participants.

Heart rate responses revealed a significant main effect for time ($F_{(1.992, 19.917)} = 6.854$; $P = 0.005$, $\eta^2_{partial} = 0.41$) and the condition by time interaction ($F_{(10, 100)} = 5.301$; $P = 0.000$, $\eta^2_{partial} = 0.35$). *Post-hoc* analysis displayed a tendency towards significance for HR at 30 minutes post-bolus between PLA (-1 [-3 – 1] beats.min^{-1}) and MTCC (-5 [-9 – -2] beats.min^{-1}) ($P = 0.080$, $d = 0.27$), where all 11 participants responded with lower HR at 30 minutes post-bolus with MTCC compared to PLA. Individual responses at 30 minutes post-bolus showed SV increased in 8/11 participants with MTCC compared to PLA.

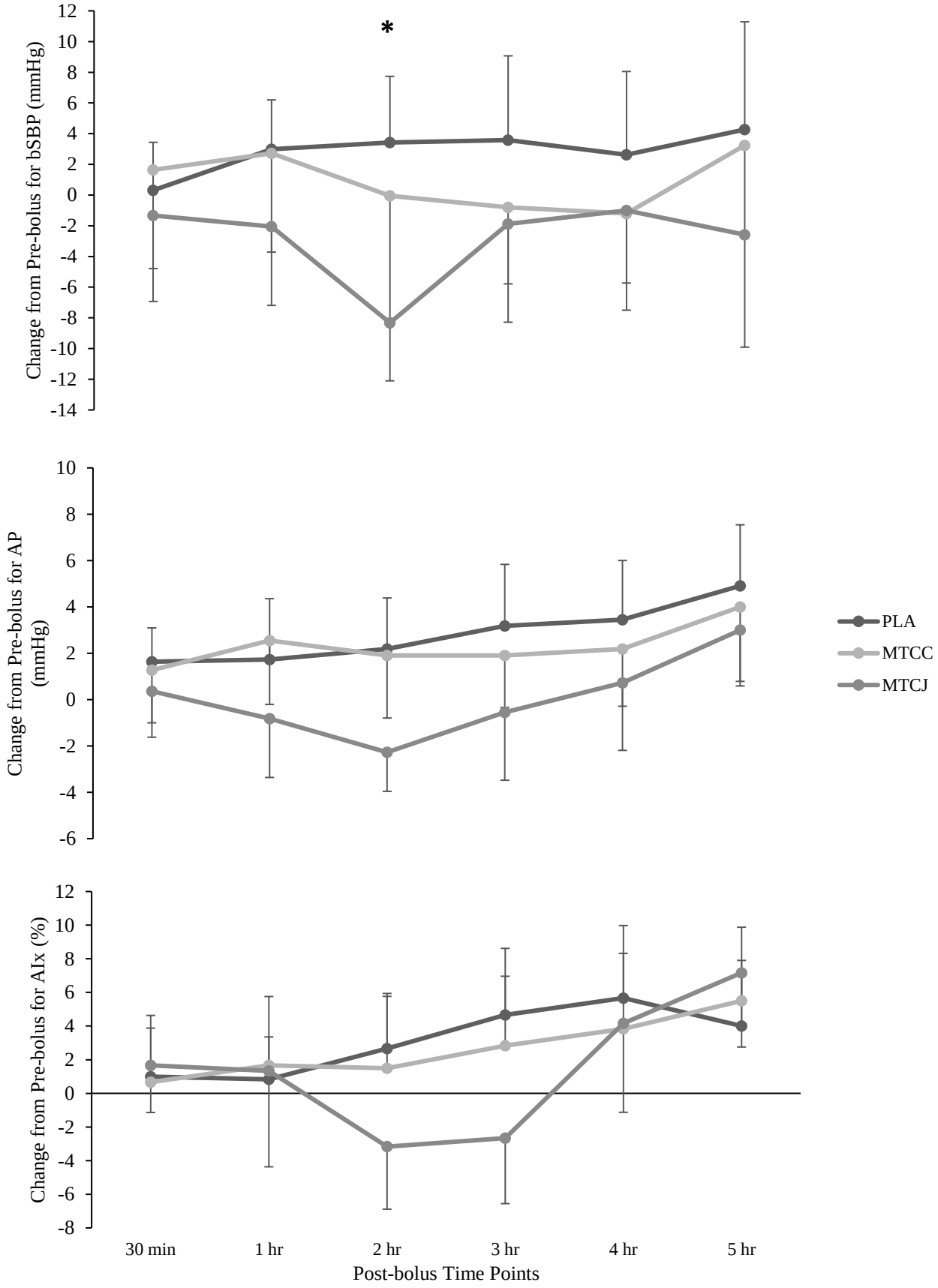


Figure 4. Mean (error bars indicate 95% CI) bSBP, augmentation pressure (AP) and augmentation index (AIx) response curves from respective pre-bolus time points for each condition. *Denotes significant difference between PLA and MTCJ at 2-hours post-bolus.

Table 4. Mean [95% CI] change from pre-bolus values to post-bolus time points for selected cardiac haemodynamic and PWA parameters per condition.

		Post-bolus Time Points					
		30 minutes	1 hr	2 hr	3 hr	4 hr	5 hr
Brachial DBP (mmHg)	PLA	3 [0 – 5]	5 [0- 10]	5 [2 – 9]	8 [3 – 13]	5 [0 – 9]	4 [0 – 8]
	MTCC	1 [-5 – 7]	4 [-2 – 11]	2 [-4 – 9]	0 [-4 – 4]	1 [-5 – 6]	3 [-2 – 9]
	MTCJ	3 [-1 – 7]	6 [3 – 9]	3 [0 – 6]	7 [3 – 11]	6 [1 – 11]	6 [2 – 10]
MAP (mmHg)	PLA	1 [-2 – 5]	5 [0 – 10]	5 [0 – 9]	7 [1 – 13]	5 [-1 – 11]	5 [-1 – 11]
	MTCC	3 [-4 – 11]	6 [0 – 13]	2 [-5 – 9]	2 [-2 – 7]	1 [-5 – 7]	5 [0 – 11]
	MTCJ	2 [0 – 4]	2 [-2 – 6]	-4 [-7 – 1]	4 [0- 8]	4 [-2 – 9]	-2 [-10 – 6]
Cardiac Output ($L.min^{-1}$)	PLA	0.11 [-0.53 – 0.75]	-0.02 [-1.08 – 1.03]	-0.31 [-1.20 – 0.58]	-0.58 [-1.27 – 0.11]	-0.53 [-1.64 – 0.57]	-0.35 [-1.32 – 0.61]
	MTCC	-0.10 [-0.56 – 0.35]	-0.24 [-0.91 – 0.43]	-0.13 [-0.90 – 0.64]	0.03 [-0.69 – 0.74]	-0.31 [-1.31 – 0.68]	-0.21 [-1.13 – 0.71]
	MTCJ	-0.33 [-1.54 – 0.87]	-0.46 [-1.45 – 0.52]	-0.04 [-1.37 – 1.28]	-0.34 [-1.64 – 0.97]	-0.62 [-1.62 – 0.39]	-0.61 [-1.66 – 0.43]
Stroke Volume (L)	PLA	1 [-9 – 12]	-1 [-14 – 11]	-1 [-11 – 9]	-5 [-17 – 8]	-5 [-21 – 12]	2 [-15 – 18]
	MTCC	6 [-1 – 13]	-1 [-12 – 11]	4 [-1 – 10]	6 [-1 – 17]	1 [-12 – 13]	3 [-10 – 17]
	MTCJ	1 [-17 – 19]	-4 [-16 – 9]	1 [-17 – 19]	-4 [-24 – 16]	1 [-14 – 17]	2 [-14 – 17]
TPR ($mmHg.min^{-1}.L$)	PLA	0.00 [-0.14 – 0.14]	0.15 [-0.10 – 0.40]	0.14 [-0.06 – 0.35]	0.26 [-0.05 – 0.57]	0.16 [-0.02 – 0.34]	0.09 [-0.09 – 0.26]
	MTCC	0.01 [-0.10 – 0.13]	0.09 [-0.10 – 0.28]	0.02 [-0.23 – 0.26]	0.08 [-0.14 – 0.29]	0.09 [-0.17 – 0.35]	0.12 [-0.11 – 0.35]
	MTCJ	0.08 [-0.14 – 0.31]	0.07 [-0.11 – 0.25]	-0.03 [-0.29 – 0.23]	0.10 [-0.13 – 0.32]	0.10 [-0.09 – 0.28]	0.11 [-0.13 – 0.34]
HR ($beats.min^{-1}$)	PLA	-1 [-3 – 1]	-3 [-6 – -1]	-5 [-8 – -1]	-5 [-10 – -1]	-7 [-12 – -1]	-7 [-11 – -2]
	MTCC	-5 [-9 – -2]	-4 [-7 – 0]	-4 [-9 – 1]	-6 [-11 – -2]	-5 [-12 – 1]	-4 [-10 – 3]
	MTCJ	0 [-3 – 4]	-1 [-5 – 2]	-5 [-9 – -1]	-7 [-11 – -4]	-7 [-10 – -4]	-8 [-11 – -4]
Aortic SBP (mmHg)	PLA	1 [-4 – 6]	3 [-3 – 8]	4 [-2 – 10]	2 [-5 – 10]	4 [-1 – 10]	5 [2 – 8]
	MTCC	2 [-2 – 5]	4 [-3 – 10]	4 [-6 – 13]	2 [-5 – 8]	2 [-4 – 7]	4 [0 – 7]
	MTCJ	-1 [-5 – 5]	-1 [-6 – 4]	-2 [-7 – 2]	1 [-2 – 3]	2 [-3 – 6]	3 [-2 – 8]

Aortic DBP (mmHg)	PLA	0 [-4 – 4]	0 [-5 – 5]	3 [-6 – 12]	3 [0 – 6]	4 [-1 – 8]	2 [-3 – 6]
	MTCC	5 [1 – 9]	3 [-1 – 6]	4 [-2 – 9]	0 [-6 – 6]	3 [-2 – 8]	6 [1 – 10]
	MTCJ	-2 [-6 – 2]	-2 [-6 – 2]	2 [-1 – 4]	1 [-4 – 6]	1 [-4 – 6]	1 [-2 – 4]
Pulse Pressure (mmHg)	PLA	1 [-2 – 9]	3 [-2 – 9]	1 [-5 – 7]	-1 [-4 – 7]	0 [-1 – 8]	3 [1 – 8]
	MTCC	-3 [-6 – 4]	1 [-5 – 7]	0 [-3 – 6]	2 [-3 – 3]	-1 [-5 – 4]	-2 [-4 – 6]
	MTCJ	1 [-6 – 2]	1 [-5 – 2]	-4 [-7 – 2]	0 [-3 – 7]	1 [-4 – 4]	2 [-1 – 6]
AIx at HR75*^$ (%)	PLA	0 [-2 – 2]	-1 [-3 – 1]	2 [-2 – 6]	2 [-1 – 5]	3 [-1 – 6]	5 [2 – 8]
	MTCC	0 [-4 – 4]	-1 [-5 – 3]	-2 [-6 – 2]	0 [-3 – 4]	2 [-2 – 5]	3 [-2 – 8]
	MTCJ	0 [-2 – 3]	-1 [-6 – 4]	-2 [-7 – 2]	-2 [-7 – 2]	0 [-5 – 5]	2 [-2 – 7]
SEVR (%)	PLA	-1 [-24 – 21]	11 [-3 – 24]	15 [-3 – 33]	10 [-4 – 24]	16 [-1 – 34]	3 [-11 – 17]
	MTCC	15 [0 – 30]	9 [-1 – 20]	17 [3 – 31]	16 [1 – 31]	19 [3 – 35]	9 [-7 – 26]
	MTCJ	6 [-11 – 24]	8 [-7 – 23]	31 [10 – 52]	24 [3 – 46]	21 [0 – 41]	12 [-3 – 27]

AIx at HR75 (Augmentation Index at Heart Rate 75 bpm); DBP (Diastolic Blood Pressure); HR (Heart Rate); MAP (Mean Arterial Pressure); mmHg (millimetres of Mercury); mmHg (millimetres of Mercury); MTCC (Montmorency Tart Cherry Capsules); MTCJ (Montmorency Tart Cherry Juice); PLA (Placebo); SEVR (Subendocardial Viability Ratio); TPR (Total Peripheral Resistance). *Denotes significant main effect for time with *post-hoc* identifying differences between 1-hour and 3-hours post-bolus. ^Denotes significant main effect for time with *post-hoc* identifying differences between 1-hour and 5-hours post-bolus. $Denotes significant main effect for time with *post-hoc* identifying differences between 3-hour and 5-hours post-bolus.

3.3. Pulse Wave Analysis (PWA)

A main effect for time ($F_{(5, 50)}$ = 7.839; *P* = 0.000, $\eta^2_{partial}$ = 0.44) was observed for the change in AP from pre-bolus. No main effects for condition or interaction (*P* > 0.05) were found, yet *post-hoc* revealed a difference (*P* = 0.001, *d* = 1.37) between PLA (2 [0 – 5] mmHg) and MTCJ (-2 [-4 – 0] mmHg) at 2-hours post-bolus (Figure 4). Individual responses showed 10/11 participants responded with a lower AP, 2-hours after MTCJ consumption compared to PLA.

A main effect for time ($F_{(5, 50)}$ = 10.090; *P* = 0.000, $\eta^2_{partial}$ = 0.50) was detected for AIx, but not for condition or interaction (*P* > 0.05). However as with AP, *post-hoc* comparisons identified a significant difference (*P* = 0.016, *d* = 1.22) at 2-hours post-bolus between PLA (4 [1 – 8] %) and MTCJ (-3 [-7 – 2] %). This indicates more compliant arteries with MTCJ at this time point whereby 9/11 participants reduced arterial stiffness compared to PLA. There were no significant correlations between bSBP and AIx (ρ = 0.105; *P* = 0.401) or AP (ρ = 0.145; *P* = 0.247) at 2-hours post-bolus for MTCJ.

No significant main effects for condition, time or the condition by time interaction were detected for aortic SBP, aortic DBP, and pulse pressure (*P* > 0.05). A main effect for time was detected for AIx at HR75 ($F_{(5, 50)}$ = 7.747; *P* = 0.000, $\eta^2_{partial}$ = 0.44) and SEVR ($F_{(5, 50)}$ = 3.903; *P* = 0.005, $\eta^2_{partial}$ = 0.28), however no other main effects for condition or interaction were found (*P* > 0.05) (Table 4).

3.4. Resting Metabolic Rate

Resting RQ did not show a significant main effect for condition or interaction (*P* > 0.05). However, a main effect for time ($F_{(1.938, 19.376)}$ = 28.261; *P* = 0.000, $\eta^2_{partial}$ = 0.74) was found, *post-hoc*

analysis for the main effect of time showed all post-bolus time points to be lower than 30 minutes post-bolus ($P < 0.05$).

No main effects for condition or interaction ($P > 0.05$) were seen, however significant main effects for time were observed for resting fat ($F_{(5, 50)} = 18.096$; $P = 0.000$, $\eta^2_{partial} = 0.64$) and carbohydrate oxidation ($F_{(5, 50)} = 16.750$; $P = 0.000$, $\eta^2_{partial} = 0.63$). Specifically, significant differences were observed between all post-bolus time points against 30 minutes post-bolus for fat and carbohydrate oxidation ($P < 0.05$). Finally, no significant main effects for condition, time or interaction were seen for resting energy expenditure ($P > 0.05$).

4. Discussion

The present study examined cardio-metabolic responses of humans with MetS to an acute bolus of MTCC and MTCJ in a randomised, placebo-controlled trial, for the first time. The hypotheses were partially accepted as the main findings from this research demonstrated significantly lower post-prandial responses to serum insulin with MTCJ compared to PLA and a blunted insulin response with MTCC compared to PLA, 1-hour post-bolus. In accordance with previous research (Keane *et al.,* 2016b), bSBP was significantly lower with MTCJ compared to PLA, 2-hours post-bolus. Finally, reported for the first time, responses between MTCC and MTCJ were not statistically different, thus refuting the hypothesis that MTCC may be superior than MTCJ; although physiological differences were apparent at particular time points for certain markers.

A novel finding from the present research was mean post-bolus insulin responses were significantly lower with MTCJ than PLA, and also significantly lower at 3-hours post-bolus. Although not statistically significant, insulin responses between PLA and MTCC were also physiologically different. The mean change from pre-bolus across all post-bolus time points

increased with PLA, but decreased by 20.61 pmol.L^{-1} with MTCC. However, this was likely due to the carbohydrate content in the placebo beverage (99.88% of which sugars), where maltodextrin was added to match the energy content of the placebo to MTCJ. Consequently, this provoked a contrived response of the placebo on insulin, making comparisons against MTCC and MTCJ difficult. Nevertheless, as this was the first study to assess acute glycaemic and insulinaemic tolerability to MTCC and MTCJ consumption, it is possible to discern novel findings. A blunted insulin secretion response throughout the 5-hour post-bolus period with MTCC was observed, as participants only consumed 1 gram of carbohydrate from the 10 capsules ingested. Additionally, in alignment with MTC pharmacokinetics (Keane *et al.*, 2016a), absolute insulin concentrations at 3-hours post-bolus were similar between MTCC (77.42 [54.44 – 100.39] pmol.L^{-1}) and MTCJ (76.53 [49.07 – 103.99] pmol.L^{-1}). Despite differences in glycaemic load, this highlights a potential action of MTC anthocyanins and their metabolites, given the only similarity between MTCC and MTCJ was the total anthocyanin content. Interestingly, the similar insulin concentrations suggest no difference between the delivery of MTC anthocyanins in capsule or juice form, indicating enhanced bioavailability of anthocyanins with capsules likely did not occur. The data indicates both MTC interventions effectively blunted insulin responses when parent anthocyanin and secondary metabolite plasma concentrations were likely elevated.

The high glucose load in the placebo elicited a significantly higher insulin secretion response 1-hour post-bolus compared to MTCC. Although not statistically different, a similar physiological response was found with MTCJ compared to MTCC (7/11 participants had increased insulin with MTCJ, 3/11 increased with MTCC). This may potentially be due to the lower sugar content in MTCJ than PLA, along with the effect of anthocyanins inhibiting glucose absorption across the apical membrane of enterocytes into systemic circulation via suppression of sodium-dependent

glucose transporter-1 (SGLT-1) (Alzaid, Cheung, Preedy, & Sharp, 2013). Likewise, insulinaemic and glycaemic responses to 7 days of New Zealand blackcurrant powder (NZBP) supplementation suggested a 14.3% reduction in fasting insulin compared to control (Willems, Silva, Cook, & Blacker, 2017). Crucially, the authors measured fasting insulin 1-hour post-consumption of the final bolus, implying an acute effect of NZBP anthocyanins and metabolites on insulin. Willems *et al.* (2017) explained that an increased glycaemic and insulinaemic response was expected, however the carbohydrate load was not sufficient to induce a glycaemic or insulinaemic response and ascribed the decline in insulin to improved insulin sensitivity. A similar mechanism may be cited to explain the smaller changes from baseline for insulin with MTCJ and MTCC in the present study, given baseline HOMA2-IR (2.2 [1.2 – 3.2]) and HOMA2-%S (61.8 [42.3 – 81.4] %) values indicated insulin resistance (Geloneze *et al.,* 2009) and low insulin sensitivity respectively.

The effect of lower insulin concentrations with normal glucose suggests MTC improved insulin sensitivity (Willems *et al.,* 2017) which may prevent pancreatic β-cell glucotoxicity (Amiot *et al.,* 2016), reducing the burden on β-cells by averting excess insulin secretion (Alvarado *et al.,* 2016). Additionally, the acute nature of this study limits the mechanisms through which MTC phytochemicals can operate. Hence, improved insulin sensitivity within 5-hours of ingesting MTC may have been mediated through enzyme-interactions involving adenosine monophosphate-activated protein kinase (AMPK) (Tsuda, 2016) and/or receptor-interactions along the insulin signalling cascade. Specifically, binding of insulin to insulin-receptor substrate 1 (IRS-1) prompting glucose transporter 4 (GLUT-4) translocation to the cell membranes of adipocytes and myocytes (Belwal, Nabavi, Nabavi, & Habtemariam, 2017). Subsequently, adaptations may include greater expression of AMPK and GLUT-4 mRNA; more efficient insulin receptor activity through greater insulin receptors on hepatocytes, myocytes and adipocytes; and peroxisome

proliferator-activated receptor agonism (Seymour *et al.,* 2009). Through the physiological mechanisms hypothesised above, improvements in insulin sensitivity may manifest to lower insulin resistance and theoretically reverse MetS; in the long-term. The direction of the insulinaemic response and its low carbohydrate content, provides evidence that MTCC may be a more tolerable intervention for individuals with dysfunctional glycaemic control and insulin resistance, such as type 2 diabetics. Moreover, the findings even hint at short-term improvements in insulin control with both MTC interventions, which may develop into long-term adaptations with consistent use; however, a direct investigation of this is required.

The most noteworthy cardiovascular response observed in the present study was the significant reduction in bSBP at 2-hours post-bolus with MTCJ compared to PLA. This finding was consistent with previous literature assessing bSBP with cherry interventions (Chai, Davis, Wright, Kuczmarski, & Zhang, 2018; Keane *et al.,* 2016b; Keane *et al.,* 2016c; Kent, Charlton, Jenner, & Roodenrys, 2016). Keane *et al.* (2016bc) observed significantly lower bSBP with MTCJ compared to PLA for the first 3-hours after ingestion, with peak reductions of 7 mmHg occurring at 2-hours post-bolus (Keane *et al.,* 2016b) and 6 mmHg at 1-hour post-bolus (Keane *et al.,* 2016c). Similarly, the present study observed the greatest reduction in bSBP (-8 mmHg) also at 2-hours post-bolus, thus it can be hypothesised that MTCJ modulates bSBP during this time period as it likely coincides with parent anthocyanin and metabolite pharmacokinetics (Keane *et al.,* 2016a). Likewise, sweet cherry juice also had its greatest effect on bSBP (-5.50 mmHg) 2-hours post-prandially, in old and young adults (Kent *et al.,* 2016), with the authors attributing the improvement to the parent anthocyanins but not phenolic metabolites, as peak metabolite concentrations did not coincide with the time course of the observed effect. Interestingly, modulation of bSBP with MTCC did not occur, despite administering the same total anthocyanin

content. Based on Keane *et al.* (2016b) explaining that phenolic metabolites (protocatechuic and vanillic acids) from MTCJ, likely exerted the dampened bSBP response, it may be that these metabolites were not present at physiologically relevant concentrations within systemic circulation with MTCC. This could be due to the variable mechanics of capsule shell disintegration impeding anthocyanin metabolism.

Hypertension and isolated systolic hypertension increases risk of CVD, the leading cause of morbidity and mortality worldwide (Feresin *et al.,* 2017), however mean bSBP reductions of at least 5-6 mmHg over 5 years has been linked with a lower risk of coronary heart disease and stroke by 20-25% and 35-40%, respectively (Collins *et al.,* 1990). Bundy *et al.* (2017) reported that a reduction in bSBP of greater than 5 mmHg was clinically relevant and associated with lower CVD/mortality risk. Thus, the magnitude of change with MTCJ indicates clinically relevant reductions in bSBP; mean difference between MTCJ and PLA at 2-hours post-bolus was -11 mmHg. Consequently, this emphasises the highly potent properties of MTCJ acutely improving bSBP and the effect of a lower MTCJ dosage compared to Keane *et al.* (2016b). This finding is of high importance in the context that approved anti-hypertensive drugs, with associated harmful side effects, comparably lower bSBP by a similar magnitude (Bramlage & Hasford, 2009) as MTCJ, which currently has no known side effects. A limitation of examining acute cardio-metabolic responses is low ecological validity and biological significance of the results (Rodriguez-Mateos *et al.,* 2013). However, long-term studies supplementing anthocyanins from tart (Keane *et al.,* 2016c) and sweet (Kent *et al.,* 2017) cherries have also been shown to lower bSBP, although only in subjects presenting elevated baseline bSBP. Furthermore, the significance of short-term decrements of bSBP in a MetS population may mean subsequent long-term improvements in arterial compliance and endothelial function. Consequently, this may reverse the

systemic pro-inflammatory and pro-oxidative state of MetS and reduce the risk of developing cardiovascular disease (Yanai *et al.,* 2008); although this requires direct investigation.

Arterial stiffness, particularly of the aorta, is an independent predictor of cardiovascular morbidity and mortality (Cecelja & Chowienczyk, 2012). Evidence exists that a higher consumption of anthocyanins is associated with reductions in aortic blood pressure and arterial stiffness (Jennings *et al.,* 2012). Previous research pertaining to supplementation of MTCJ in healthy (Lynn *et al.,* 2014), early-hypertensive (Keane *et al.,* 2016b) and MetS (Johnson *et al.,* 2017) participants had not demonstrated improvements in arterial stiffness. Individual responses and statistical analysis from the present study demonstrated an improvement in arterial stiffness (AIx) only at 2-hours post-bolus with MTCJ compared to PLA. However, this finding remains dubious given that PP, aortic (AP) and peripheral (TPR) indicators of vascular stiffness were not improved with MTCJ. Furthermore, AIx and AP were not significantly correlated to bSBP at 2-hours post-bolus with MTCJ; suggesting modulation of arterial stiffness does not explain the reduction in bSBP at that time point.

As the results from this research seem to not support a mechanical mechanism of action through improved arterial compliance, the findings may be explained by a chemical mechanism of action involving nitric oxide (NO) and/or inhibition of angiotensin-converting enzyme (ACE). However, Keane *et al.* (2016b) were unable to demonstrate augmented plasma nitrate/nitrite concentrations after 60 mL MTCJ consumption, suggesting NO mediated vasodilation may not be the responsible pathway. Conversely, Kirakosyan, Gutierrez, Ramos Solano, Seymour, and Bolling (2018) demonstrated MTC extract to inhibit ACE, potentially explaining reductions in bSBP from the present study; future work should assess ACE inhibition in humans following MTC consumption.

However, concomitant stimulation of endothelial nitric oxide synthase and ACE inhibition should not be ruled out.

MTCC induced vasodilation may have facilitated greater venous return thus explaining HR, SV and CO responses at 30 minutes post-bolus. A similar mechanism was provided by Willems *et al.* (2017) in response to acute, encapsulated NZBP supplementation. The observed response with MTCC was remarkable, as disintegration of the capsule shell requires 30 minutes (Lown *et al.*, 2017), indicating that responses on HR and SV were immediate after dissolution. Subsequently, it was thought that provision of MTC in liquid form (MTCJ) would facilitate faster absorption of parent anthocyanins and metabolites (Bohn, 2014; McGhie & Walton, 2007) than MTCC. However, the lack of response with MTCJ would suggest either extensive degradation by salivary amylase (Kamonpatana *et al.*, 2012) and/or a delayed uptake of secondary metabolites and phase II conjugates, due to gut metabolism of anthocyanins (Fernandes, Faria, Calhau, de Freitas, & Mateus, 2014). This delayed uptake of metabolites may be the rate-limiting step.

In agreement with responses observed in participants with MetS during the present investigation, Johnson *et al.* (2017) reported 12 weeks MTCJ consumption had no significant effect on lipids. However, Johnson *et al.* (2017) observed a trend for lower total cholesterol with MTCJ compared to PLA. This suggests lipids may be more sensitive to improvement at higher anthocyanin, secondary metabolite and phase II conjugate concentrations; through higher dosages and/or longer supplementation length.

As with all research, certain limitations are apparent with the present study. Firstly, the artificial effect of the placebo on glycaemic and insulinaemic responses influenced the data and nullified comparisons against MTCC and MTCJ. Subsequent studies should ensure the placebo is matched for relative percentage of each macronutrient to total energy or alternatively use a water placebo

if aiming to evaluate the intervention as a whole, including detrimental effects of juices such as sugars (Peluso & Palmery, 2014). Furthermore, the parent anthocyanin and secondary metabolite pharmacokinetics of MTCC in humans was and remains unknown. Thus, there is a necessity to address this gap in the literature, to enable better understanding of the data from this study and future work incorporating MTCC. Additionally, any advantageous responses observed with MTC interventions may be due to a residual synergistic effect of habitually consumed polyphenols, as this study aimed to uphold ecological validity by permitting habitual polyphenol intake. The beneficial responses observed with MTCJ compared to MTCC and PLA, may be a consequence of other nutrients, such as fibre which was shown to reduce bSBP, total cholesterol and post-prandial glucose and insulin (Hodgson, 2004); aligning with findings from the present study. Although much attention has been given to the beneficial effects of anthocyanins and their metabolites, the synergistic influence of other phytonutrients within MTC require consideration. Finally, acute cardio-metabolic responses were monitored during this study, however to enhance ecological validity and clinical relevance, monitoring data over a longer time frame is required.

5. Conclusion

This research is the first to present data that acute administration of MTC interventions in capsule and juice form can modulate certain cardio-metabolic markers in humans with MetS. Further evidence is provided that MTCJ is an effective, low-risk intervention for reducing bSBP in various populations and should be considered for individuals with isolated systolic hypertension. Despite recruiting humans with MetS, the effects of MTC on cardio-metabolic markers seemed to only apply to certain variables which were abnormal at baseline. The physiological responses to either MTC intervention differed depending on the cardio-metabolic marker examined, although there were no statistically significant differences between MTCC and MTCJ. Consequently,

recommendations for which MTC intervention is more suitable should be based on the cardio-metabolic marker that requires most attention, with consideration of the individuals' overall cardio-metabolic health.

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Author Contributions

Conceptualization, TD, LB and MR.; methodology, TD; writing—original draft preparation, TD.; writing—review and editing, TD, LB and MR; supervision, LB and MR. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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