Effects of Tea on Peripheral and Cerebral Micro- and Macrovascular Function in Humans

Kirsty Anne Roberts

A thesis submitted in partial fulfilment of the requirements of Liverpool John Moores University for the Degree of Doctor of Philosophy

October 2017

Abstract

Cardiovascular disease (CVD) is the leading cause of global mortality, with the incidence of cardiovascular related pathologies remaining a public health burden. CVD encompasses pathologies of the vascular tree and heart, including, for example, peripheral artery disease, coronary heart disease and ischaemic stroke. Atherosclerosis is the primary pathological process leading to CVD and is characterised by a multifactorial pathophysiology that first manifests in the vascular endothelium. Termed endothelial dysfunction, this early marker of atherosclerosis has become a focus of interest for identifying individuals at risk of a profound cardiovascular insult, particularly arising from lifestyle choices such as physical inactivity and calorie-rich diets. Dietary interventions have received increasing attention in recent years as inexpensive strategies to potentially combat the ever-increasing global burden of CVD. A high dietary flavonoid intake is associated with a reduction in CVD risk and several studies have revealed a strong, inverse relation between the regular intake of tea, a major source of dietary flavonoids, and CVD risk. Tea has demonstrated improved conduit artery endothelial function and glucose handling in both healthy individuals and in those with overt CVD. However, the effects of tea on the microvasculature and cerebrovasculature are not yet understood, particularly in relation to lifestyle factors. The primary aim of this thesis was to explore the impact of tea ingestion on peripheral and cerebral micro- and macrovascular function in humans.

In an initial methodological study, the day-to-day reproducibility of thermally stimulated cutaneous microvascular function was assessed. Fifteen, healthy males (28 ± 5 yrs, BMI 25 ± 2 kg/m^2) attended two experimental trials 2-7 days apart. During each trial, baseline and maximal thermally stimulated forearm skin responses were examined simultaneously at four sites on the dominant forearm using laser Doppler flowmetry (LDF). The following heating protocols were adopted: 1. *Rapid 39°C* (0.5°C/5-s), 2. *Rapid 42°C* (0.5°C/5-s) 3. *Gradual 42°C* (0.5°C/2-min 30-s) and 4. *Slow 42°C* (0.5°C/5-min). The coefficient of variation (CV) was calculated for absolute flux, cutaneous vascular conductance (CVC; flux/mean arterial pressure, MAP) and CVC expressed as a percentage of maximal CVC at 44°C (%CVC$_{max}$) at three different time points; baseline (33°C), plateau (39/42°C) and maximal (44°C). Reproducibility of baseline flux, CVC and %CVC$_{max}$ was 17-29% across all protocols. During the plateau, *Rapid*, *Gradual* and *Slow 42°C* demonstrated a reproducibility of 13-18% for flux and CVC and 5-11% for %CVC$_{max}$. However, *Rapid 39°C* demonstrated a lower reproducibility for flux, CVC and %CVC$_{max}$ (21%). Reproducibility at 44°C was 12-15% for flux and CVC across all protocols. The good-to-moderate reproducibility of the *Rapid, Gradual and Slow 42°C* protocols supported their (simultaneous) use to assess peripheral microvascular function.

The aim of Chapter 5 was to examine the acute (2-hour) cutaneous vascular responses to local skin heating following ingestion of black tea in a healthy adult population. Twenty healthy participants (58 ± 5 yrs, BMI 26 ± 4 kg/m^2, 9 men) attended two experimental trials (tea, placebo), 7-days apart in a randomised, controlled, double-blind, cross-over design. Participants ingested a single dose of 200 ml black tea or placebo, followed by assessment of forearm cutaneous microvascular function using LDF and three distinct local skin heating protocols to distinguish between axon- and endothelium-dependent vasodilation: 1. *Rapid 42°C*, 2. *Rapid 39°*C and 3. *Gradual 42°C*. On the contralateral arm, full-field laser perfusion imaging (FLPI) was used to assess forearm cutaneous microvascular function

during *Gradual 42°C*. Data were analysed as CVC and %CVC_{max}. Rapid local heating to 39°C or 42°C demonstrated no effect of tea for flux, CVC or %CVC_{max} (all P>0.05). Gradual local heating to 42°C, however, produced a higher skin blood flow following black tea ingestion for absolute CVC (P=0.04) when measured by LDF, and higher absolute flux (P<0.001) and CVC (P<0.001) measured with FLPI. No effect of tea was found for %CVC_{max} when assessed by either LDF or FLPI.

The aim of the study outlined in Chapter 6 was to examine the effect of daily green tea consumption (equivalent to 6 cups/day) on changes in peripheral vascular function and glucose handling after a 7-day 'unhealthy' lifestyle in healthy males. Twelve healthy males (29 ± 6 yrs, BMI 25 ± 2 kg/m^2) underwent two periods of 7-days 'unhealthy' lifestyle (UL) comprising of combined physical activity reduction (-50% steps per day) and high fat, high carbohydrate overfeeding (+50% kcal per day, comprising 65% fat) in a randomised, controlled, double-blind, cross-over design. Each intervention period was separated by a 2-week washout. During each 7-day UL-period, participants ingested three doses of an active green tea drink (UL-Tea) or a placebo drink (UL-Placebo) per day at regular intervals. Participants attended the laboratory before and after each 7-day intervention (a total of 4 visits). During each visit the following were examined: mean arterial blood pressure (MAP), dominant forearm cutaneous microvascular function using LDF and local heating protocols 1. *Rapid 42°C*, 2. *Rapid 39*℃ and 3. *Gradual 42°C*, macrovascular function using brachial artery and femoral artery endothelium-dependent function via flow-mediated dilation (FMD), carotid artery vasoreactivity to the cold pressor test (CAR%), cerebrovascular function via CO_2 reactivity and dynamic cerebral autoregulation, and insulin sensitivity and glucose handling through a mixed-meal (1200kcal, comprising 60% carbohydrates, 33% fat and 7% protein) tolerance test. Linear mixed models (main effects of intervention and time) were used to examine the impact of the lifestyle intervention (pre vs post) and green tea ingestion (UL-Tea vs UL-Placebo). Body mass demonstrated a slight increase following both UL-Tea and UL-Placebo (P>0.05). MAP was increased after UL-Placebo, whereas it was reduced after UL-Tea (P=0.06). LDF responses to rapid local heating demonstrated non-significant reductions in CVC following UL-Placebo but no difference following UL-Tea (P>0.05), with a significant interaction of time*condition*temperature observed following *Gradual 42°C* (P=0.02). Brachial artery FMD was not different pre vs post or between UL-Placebo and UL-Tea (P>0.05), whereas femoral artery FMD decreased after UL-Placebo, which was prevented during UL-Tea (P<0.001). CAR% decreased following UL-Placebo, which was prevented during UL-Tea (P=0.04). CO_2 reactivity and dynamic cerebral autoregulation demonstrated no differences between UL-Placebo and UL-Tea or over time. Postprandial glucose was increased after UL-Placebo, whereas a reduction in postprandial glucose occurred after UL-Tea (P=0.03). Postprandial insulin levels were higher after UL-Placebo, consistent with insulin resistance, whereas following UL-Tea the insulin response was reduced and demonstrated an interaction of time*condition (P<0.001).

The aim of Chapter 7 was to examine the effect of acute oral (-)-epicatechin ingestion on cerebrovascular function in healthy adults. Seven healthy males (32 ± 13 yrs, BMI 25 ± 1 kg/m^2) attended two experimental trials ((-)-epicatechin and placebo) 7-days apart in a randomised, controlled, double-blind, cross-over design. Participants underwent baseline assessment of cerebrovascular function using transcranial Doppler ultrasound (TCD), comprising CO_2 reactivity to hypercapnia and dynamic cerebral autoregulation via squat-stand manoeuvres at 0.10 Hz and 0.05 Hz.

Following completion of the baseline measures, participants immediately consumed an oral dose of the test product (2 x 50 mg capsules of (-)-epicatechin or 2 capsules of colour-matched placebo) together with a glass of water, following which participants relaxed in the laboratory. 2-hours post-ingestion repeat measures of cerebrovascular function were performed. Linear mixed models (main effects of condition and time) examined the differences between (-)-epicatechin and placebo interventions (pre vs post) on cerebrovascular function. No differences were observed at pre vs post baseline for middle cerebral artery velocity (MCAv) or MAP (all $P>0.05$). There were no differences in the cerebrovascular responses to CO_2 or dynamic autoregulation between (-)-epicatechin and placebo.

The findings from this thesis suggest that, firstly, use of simultaneous skin local heating protocols provides a valuable means of interrogating the cutaneous microvessels for mechanistic insight in intervention studies. Secondly, current findings evidence improved cutaneous microvascular function following acute black tea consumption. Furthermore, the research work undertaken in this thesis provides important insight into the effects of tea consumption on peripheral (micro- and macro-) vascular function and insulin sensitivity, particularly its abrogative effects on lifestyle-induced vascular impairments. However, the effects of tea consumption on the cerebrovasculature remain uncertain. Overall, based on the current findings, tea consumption presents a simple, inexpensive, non-pharmacological cardioprotective strategy to help combat the ever-increasing global burden of CVD.

Acknowledgements

I wish to express my sincere gratitude to my Director of Studies, Dr David Low, for his considerable support, guidance, knowledge and expertise throughout the last three years. Having first encountered Dave when he posed a challenging question during my presentation as an undergraduate at ECSS in Barcelona in 2013, I am now very grateful both for the opportunity to work with such an accomplished scientist and for having a better understanding of the relevant physiology so that I might be able to pre-empt and answer similarly challenging questions in the future!

Secondly, I am forever grateful to Dr Nicola Hopkins, without whom I would likely not have been fortunate to undertake a studentship at the Research Institute for Sport and Exercise Sciences at LJMU. As ever, Niki's knowledge and guidance have been invaluable throughout this PhD journey. I would also like to sincerely thank Professor Dick Thijssen, whose immense knowledge and passion for research have been an inspiration.

I would like to express my gratitude to Unilever R & D for giving me the opportunity to undertake this doctorate and for supporting me over the last three years. I am also hugely indebted to my primary collaborator, Dr Richard Draijer, for his direction, support and for sharing his wealth of knowledge. My thanks also to Dr Young de Graaf for his guidance and methodological assistance and to Dr Peter Hollman for his advice and collaboration regarding the final study of this PhD.

For their help over the last three years, I wish to thank the department's exceptional technicians, Gemma, Dean and George. Likewise, my thanks also to Louise, Rachel and Zoe for their assistance with various matters. RISES really does have the best support staff!

I would also like to thank my postgraduate colleagues for their friendship and support, with particular thanks to Sophie Holder and Sophie Carter for their help with *Chapter 6* of this thesis and to Áine Brislane for assisting with *Chapter 7*. It has truly been a privilege to be part of RISES, particularly the exceptional cardiovascular team.

My thanks also to the individuals who gave up their valuable time to participate in my research and for their enthusiasm throughout.

To my wonderful parents and sister, Pip, I wish to take this opportunity to thank them all for their unwavering support and belief in me. I would also like to thank my Gran for her faith in me over the years and together with my dearly departed grandparents, I wish to thank them all for instilling a work ethic in me that has been invaluable over recent years. Finally, I wish to thank Richard for his support and encouragement throughout the completion of this doctorate; the last twelve months have been hectic to say the least, but definitely worth it! All my love, as always.

If I become half the scientist of those that I have been fortunate to work with, and half the person of the family that I am blessed with, then I shall have accomplished a great deal.

This thesis is dedicated to all of those who have had the belief in me to see it through to completion.

Declaration

I declare that the work contained within this thesis is entirely my own.

Submitted manuscripts directly based on the work contained within this thesis

Woodward KA, Hopkins ND, Draijer R, de Graaf Y, Low DA & Thijssen DHJ (2016). Acute black tea consumption improves cutaneous vascular function in healthy middle-aged humans. *Clinical Nutrition.* Advance online publication https://doi.org/10.1016/j.clnu.2016.12.013.

Roberts KA, van Gent T, Hopkins ND, Jones H, Dawson EA, Draijer R, Carter HH, Atkinson CL, Green D, Thijssen DHJ & Low DA (2017). Reproducibility of four frequently used local heating protocols to assess cutaneous microvascular function. *Microvascular Research* ***112***, 65-71.

Woodward KA, Draijer R, Thijssen DHJ & Low DA (2017). Polyphenols and microvascular function: A systematic review. *Current Pharmaceutical Design* ***23****,* 1-24.

Other publications completed by the candidate during PhD tenure

Woodward KA, Unnithan VB & Hopkins ND (2015). Forearm skin blood flow after kinesiology taping in healthy soccer players: an exploratory investigation. *Journal of Athletic Training,* ***50****(10),* 1069-1075.

Oral communications

Woodward KA, Hopkins ND, Draijer R, de Graaf Y, Low DA & Thijssen DHJ. Acute black tea consumption improves cutaneous vascular function in healthy middle-aged humans. Faculty of Science Postgraduate Research Conference. Liverpool, UK, June 2016.

Roberts, KA, Draijer R, Thijssen DHJ, Hopkins ND, de Graaf Y, Holder SM, Carter SE, Low DA. Effects of green tea consumption on micro- and macrovascular function under metabolically challenging conditions in healthy men. The 8th International Conference on Polyphenols and Health 2017. Québec, Canada, October 2017.

Roberts, KA, Draijer R, Thijssen DHJ, Hopkins ND, de Graaf Y, Holder SM, Carter SE, Low DA. Tea time: a British solution to improve cardiovascular health? Northern Vascular Biology Forum 2017. Liverpool, UK, December 2017.

Poster communications

Woodward KA, Hopkins ND, Thijssen DHJ, van Gent T & Low DA. The reproducibility of four local heating protocols for assessing skin microcirculation in healthy young individuals. Faculty of Science Postgraduate Research Conference. Liverpool, UK, June 2015.

Woodward KA, Hopkins ND, Draijer R, de Graaf Y, Low DA & Thijssen DHJ. Acute black tea consumption improves cutaneous vascular function in healthy middle-aged humans. Physiology 2016, a joint meeting of the American Physiological Society and The Physiological Society. Dublin, Ireland, July 2016.

Woodward KA, Hopkins ND, Draijer R, de Graaf Y, Low DA & Thijssen DHJ. Acute black tea consumption improves cutaneous vascular function in healthy middle-aged humans. Life Science Showcase. Liverpool, UK, May 2017.

Contents

List of abbreviations

Abbreviation	Title
ACh	Acetylcholine
ANOVA	Analysis of variance
ASL	Arterial spin labelling
AVAs	Arteriovenous anastomoses
BMI	Body mass index
BOLD	Blood oxygen level dependent
BP	Blood pressure
CAR%	Carotid artery reactivity
CarVC	Carotid artery vascular conductance
CBF	Cerebral blood flow
CBVC	Cerebrovascular conductance
CHD	Coronary heart disease
cIMT	Carotid intima-media thickness
CO_2	Carbon dioxide
CPT	Cold pressor test
CVC	Cutaneous vascular conductance
$\%CVC_{max}$	CVC normalised to the maximal flux achieved during 44°C
CV	Coefficient of variation
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DICOM	Digital imaging and communications in medicine
EC	Epicatechin
ECG	Epicatechin-3-gallate
EGC	Epigallocatechin
EGCG	Epigallocatechin-3-gallate
EDHF	Endothelial derived hyperpolarising factor
eNOS	Endothelial nitric oxide synthase
FBF	Forearm blood flow
FLPI	Full-field laser perfusion imaging
FMD	Flow-mediated dilation
HR	Heart rate
ICA	Internal carotid artery
IPAQ	International physical activity questionnaire
KCAL	Kilocalorie
LDF	Laser Doppler flowmetry
LMM	Linear mixed model
L-NAME	N-nitro-L-arginine methyl ester
L-NMMA	NG-monomethyl-L-arginine
MAP	Mean arterial pressure
MCA	Middle cerebral artery
MCAv	Middle cerebral artery velocity

MRI	Magnetic resonance imaging
NIRS	Near infra-red spectroscopy
NPY	Neuropeptide-Y
NO	Nitric oxide
NOS	Nitric oxide synthase
$PaCO_2$	Partial pressure of arterial carbon dioxide
PCA	Posterior cerebral artery
$P_{ET}CO_2$	End-tidal carbon dioxide
PU	Perfusion unit
RBCF	Red blood cell flux
RMANOVA	Repeated measures analysis of variance
ROI	Region of interest
SBP	Systolic blood pressure
SD	Standard deviation
SkBF	Skin blood flow
SR_{AUC}	Shear rate area under the curve
TCD	Transcranial Doppler
TRVP-1	Transient receptor vanilloid
WHO	World Health Organisation

List of Figures

List of Tables

CHAPTER 1: INTRODUCTION

Chapter 1. Introduction

Cardiovascular disease (CVD) remains the leading cause of global mortality, representing ~30% of all deaths annually (WHO, 2016). Despite a decline in CVD mortality in the United Kingdom during the last 25 years, the incidence of cardiovascular related pathologies remains a public health burden, as CVD morbidity has shown little change (Bhatnagar *et al.*, 2016). CVD encompasses pathologies of the vascular tree and heart, including, for example, peripheral artery disease, coronary heart disease (CHD) and ischaemic stroke. Atherosclerosis is the primary pathological process leading to CVD and is characterised by a multifactorial pathophysiology that first manifests in the vascular endothelium, prior to any detectable structural changes to the vessel wall being apparent on angiography or ultrasound imaging (Davignon & Ganz, 2004). Termed endothelial dysfunction, this early marker of atherosclerosis has become a focus of interest for identifying individuals at risk of a profound cardiovascular insult, as well as investigating therapeutic strategies targeting a reversal of this pathological process.

Ongoing epidemiological evidence implicates lifestyle as being a key component in endothelial dysfunction and, therefore, CVD risk and progression. In contrast to the non-modifiable risk factors for CVD, which include genetics, gender, age and ethnicity, lifestyle modification through changes to behavioural patterns can reduce the risk of CVD and somewhat reverse the pathological processes associated with endothelial dysfunction. Physical inactivity, and obesity arising from poor dietary habits, are continuing to increase across the United Kingdom (Baker, 2017; BHF, 2017) and are considered two of the most important modifiable risk factors for the development of cardiometabolic disorders, in addition to smoking, hypertension and hypercholesterolaemia. Diets rich in sodium and saturated fat are associated with CVD, whereas other dietary components are suggested to be cardioprotective and exert a positive influence upon cardiovascular health, such as fruits and vegetables, spices, omega-3-fatty acids and low-fat dairy products (Feyh *et al.*, 2016; Mangels & Mohler, 2017). As the deleterious effects of sedentary lifestyles have become more apparent and the

beneficial effects of certain dietary components receive wider scientific recognition, dietary interventions have received greater attention as inexpensive tools to combat the ever-increasing global burden of CVD.

The Mediterranean diet is widely reputed to exert a protective effect upon cardiometabolic health, largely achieved via mechanisms related to its richness in plant-derived products, such as olive oil, fruits and vegetables, which contain bioactive compounds known as polyphenols (Ros *et al.*, 2014). These naturally occurring compounds are the most abundant antioxidant in the human diet; to date, in excess of 8,000 polyphenolic compounds have been identified (Pandey & Rizvi, 2009). Polyphenols can be broadly categorised into four subclasses according to their phenolic ring structure and ring-binding elements: flavonoids, phenolic acids, lignans and stilbenes (Pandey & Rizvi, 2009; Amiot *et al.*, 2016). Flavonoids account for the greatest proportion of polyphenols (60%) and were discovered in the 1930s by the Hungarian scientists Rusznyak and Szent-Györgyi, who observed that a substance derived from lemon peel reduced the permeability and fragility of the human capillary (Rusznyak & Szent-Györgyi, 1936; Geleijnse & Hollman, 2008). The substance was subsequently known as "vitamin P" and was later considered a mixture of flavonoids, rather than a pure substance (Bruckner & Szent-Györgyi, 1936; Geleijnse & Hollman, 2008). Interest in flavonoids waned in the 1950s after they lost their vitamin status and were subsequently thought to be detrimental to health in the 1970s (Geleijnse & Hollman, 2008). Scientific focus towards flavonoids was renewed in the early 1990s when red wine was observed to inhibit the oxidation of low density lipoprotein (LDL) *in vitro* and was implicated in explaining why the French diet is high in saturated fat, yet epidemiological observations suggest that the population have a low incidence of coronary heart disease, known as the "French paradox" (Ferrières, 2004). A subsequent Dutch study reported a substantial protective effect of up to a 70% reduction in coronary artery disease-related mortality, following regular consumption of several flavonoids in elderly men (Hertog *et al.*, 1993). The major source of flavonoids in the cohort was tea (61%), followed by onions (13%) and apples (10%).

Following the seminal studies in the early 1990s, a new era of polyphenol research began and the role of dietary factors on CVD risk has been explored in recent years. Epidemiological studies have reported conflicting results, but the consensus of scientific opinion is that a high dietary flavonoid intake is associated with a reduction in CVD risk (Arts *et al.*, 2001; Geleijnse *et al.*, 2002; Mink *et al.*, 2007; McCullough *et al.*, 2012; Cassidy *et al.*, 2013; Ponzo *et al.*, 2015; Kim *et al.*, 2016). Such reductions have been reported in middle-aged (Cassidy *et al.*, 2013; Ponzo *et al.*, 2015) and elderly (Arts *et al.*, 2001; McCullough *et al.*, 2012) populations, with reductions in ischaemic heart disease risk being observed related to flavonoids derived from both tea (Geleijnse *et al.*, 2002) and berries (Cassidy *et al.*, 2013) over 5- and 18-year follow-ups, respectively. Nevertheless, many mechanistic studies have been undertaken *in vitro* and in animal models, with observational human studies often lacking longer-term interventions (Williamson & Manach, 2005) and reporting inconsistent findings due to heterogeneity in the study design, a lack of control group, background lifestyle and dietary factors, in addition to a lack of clarity regarding the polyphenol dosage (Chong *et al.*, 2010). This is particularly true for studies investigating the chronic effects of tea (Deka & Vita, 2011), which is the major source of dietary flavonoids in many countries globally (Yahya *et al.*, 2016).

Several studies have revealed a strong, inverse relation between regular intake of tea and CVD risk (Grassi *et al.*, 2009b; Greyling *et al.*, 2014), with regular consumption of black tea suggested to dose-dependently improve endothelial function in conduit vessels in healthy males (Duffy *et al.*, 2001; Hodgson *et al.*, 2002; Hodgson *et al.*, 2005; Grassi *et al.*, 2009b; Schreuder *et al.*, 2014). Similar benefits of tea ingestion on endothelial function have also been observed in conduit vessels in both healthy individuals with mildly elevated serum cholesterol and triglycerides (Hodgson *et al.*, 2002) and in those with CVD (Duffy *et al.*, 2001). However, to date, no previous study has robustly explored whether beneficial effects of tea are also apparent in smaller vessels, e.g., the skin microcirculation.

The microcirculation represents an important vascular bed in the pathogenesis of many cardiometabolic diseases and acts as a surrogate for generalised vascular function (Holowatz *et al.*, 2008). Furthermore, valuable insight into the mechanisms associated with vascular function can be gained by assessment of microvascular function. The cutaneous microcirculation, therefore, presents an easily accessible site to detect changes in function following dietary interventions, e.g., tea ingestion, that may be representative of other vascular beds. Microvascular integrity can be assessed through non-invasive methods, with local skin heating increasingly used in conjunction with laser imaging techniques, such as laser Doppler flowmetry (LDF), to evaluate skin blood flow (SkBF) responses. Currently, several local heating protocols are used to assess cutaneous microvascular function (Minson *et al.*, 2001; Black *et al.*, 2008b; Choi *et al.*, 2014), with variations in the rate of skin heating and plateau temperature presenting differences in the contribution of the vasodilator pathways in the local heating response. Such protocols are, therefore, useful in achieving an insight into the distinct dilator pathways involved in intervention studies, such as tea. However, the comparable reproducibility of the various local heating protocols is unknown. Therefore, ***aim 1*** of this thesis was to simultaneously determine the inter-day reproducibility of four commonly used local heating protocols for assessing cutaneous microvascular function. Upon establishing the reproducibility of these protocols, appropriate protocols were selected to fulfil ***aim 2*** of this thesis, which was to examine the acute (2-hour) cutaneous vascular responses to local skin heating following ingestion of black tea in a healthy adult population.

The negative consequences of poor lifestyle behaviours are well documented, with modern lifestyles typically characterised by low levels of daily energy expenditure and high fat, high calorie diets that are strongly associated with long-term cardiometabolic disease risk (Hennig *et al.*, 2001). In addition to the vascular effects of tea, regular intake of tea is also associated with a lower risk for developing diabetes (Iso *et al.*, 2006; Stote & Baer, 2008; Jing *et al.*, 2009; Park *et al.*, 2014). The mechanisms for a potential positive relationship between tea ingestion and diabetes are unclear and have not been

thoroughly examined in humans. Acute studies have observed a reduction in postprandial insulin following a single dose of black tea in both insulin-resistant (Fuchs *et al.*, 2016) and healthy individuals (Bryans *et al.*, 2007). Findings from *in vitro* and animal models are suggestive of tea influencing glucose metabolism through mechanisms such as enhanced insulin sensitivity although, to date, limited human trials have been performed, particularly with green tea. Furthermore, no previous work has investigated the simultaneous effects of tea on glucose handling, insulin sensitivity and vascular function using a Western lifestyle model, characterised by inadequate physical activity and excessive caloric intake. Therefore, ***aim 3*** of this thesis was to examine the effect of daily green tea consumption (equivalent to 6 cups/day) on changes in peripheral vascular function and glucose handling after a 7-day 'unhealthy' lifestyle combining physical activity reduction and overfeeding in healthy males.

Flavonoids are also suggested to help maintain and even improve cognitive function, possibly via improved vascular function and glucose metabolism (Mastroiacovo *et al.*, 2015). To date, limited research has been undertaken exploring the acute and chronic effects of flavonoids on cerebrovascular function. Cocoa-derived flavanols have demonstrated increased cerebral blood flow (CBF) both acutely (Francis *et al.*, 2006; Lamport *et al.*, 2015) and following regular (1-12 weeks) ingestion (Fisher *et al.*, 2006; Sorond *et al.*, 2008a; Brickman *et al.*, 2014). Despite tea being the major source of dietary flavonoids for much of the global population, only one study has examined the effect (acute) of tea on CBF which concluded that caffeine was responsible for tea-induced decreases in steady-state CBF and potentially masked any flavonoid related changes in CBF (Vidyasagar *et al.*, 2013). A study of the isolated flavonoid epigallocatechin gallate (EGCG) that is abundant in tea was equivocal (Wightman *et al.*, 2012). Epicatechin is also a natural compound that is present in both tea and cocoa, and has exhibited improved peripheral vascular function (Dower *et al.*, 2016b). Furthermore, (-)-epicatechin has also demonstrated a protective effect on transient ischaemia-induced brain injury in mice (Shah *et al.*, 2010), suggesting that it may have a beneficial impact on the cerebrovasculature. Given the encouraging findings of cocoa-derived flavanols on CBF and the role

that (-)-epicatechin is suspected to play in tea/flavanol-induced acute improvements in vascular function (Dower *et al.*, 2016b), ***aim 4*** of this thesis was, therefore, to examine the effect of acute oral (-)-epicatechin ingestion on cerebrovascular function in healthy adults.

In summary, evidence suggests that acute and chronic tea ingestion may be beneficial for cardiovascular health in humans. However, the effects of tea on microvascular function and cerebrovascular function are not yet understood. Furthermore, given that the limited previous research suggests that tea has a positive influence on vascular function and glucose handling and that poor lifestyle behaviours are shown to have a detrimental impact upon cardiovascular health, further investigation is warranted to determine whether tea ingestion may contribute to the amelioration/prevention of vascular and metabolic impairments induced by an unhealthy lifestyle. Therefore, the overarching aim of this thesis was to explore the impact of tea ingestion on peripheral and cerebral micro- and macrovascular function in humans.

Aims

The aims of this thesis were to:

1. Simultaneously determine the inter-day reproducibility of four commonly used local heating protocols for assessing cutaneous microvascular function.
2. Examine the acute (2-hour) cutaneous vascular responses to local skin heating following ingestion of black tea in a healthy adult population.
3. Explore the changes in peripheral (conduit artery and skin microvessels) and cerebrovascular function and insulin sensitivity after a 7-day metabolic challenge or ‘unhealthy’ lifestyle, combining physical activity reduction (-50% steps per day) and overfeeding (+50% kcal per day, comprising 65% fat) in healthy male participants.

4. Examine the effect of acute oral (-)-epicatechin ingestion on cerebrovascular function in healthy adults.

CHAPTER 2: LITERATURE REVIEW

2.1 Microvascular Function

The cardiovascular system is a complex, interrelated organ system that forms an efficient delivery network for cellular nutrition and removal of metabolic waste products, gaseous exchange and hormonal transport, in addition to being a key regulator of homeostasis for body temperature, pH balance, hydration and blood pressure (Smith & Fernhall, 2011). In order to achieve these vital functions, however, the cardiovascular system must closely interact with other major systems, particularly the respiratory, neural, endocrine, digestive, skeletal, urinary and integumentary systems. In this regard, the vascular tree, particularly the microvasculature, is integral in providing an extensive transport network of vessels to serve each of these systems as a site to facilitate exchange of macromolecules and fluids, in addition to responding to various mechanical forces and chemical signals concerned with homeostasis via thermoregulatory mechanisms (Johnson, 2011).

The microvasculature encompasses the smallest resistance vessels (<15 μm in diameter) embedded within all human tissues and organs, consisting of terminal arterioles, capillaries and venules (Levy *et al.*, 2001; Roustit & Cracowski, 2013). Given the large surface area of these vessels, the microcirculation, therefore, consists of a large proportion (>95%) of the body's vascular endothelium, a squamous epithelial layer that lines the inner surface of all blood vessels and is critical in maintaining vascular homeostasis (Deanfield *et al.*, 2007; Hewett, 2009). Optimal microvascular function is essential for organ systems to function effectively, from the brain, eyes, kidneys, heart, muscle and adipose tissue to the skin. The cerebral microcirculation, for example, serves a pivotal role in homeostatic control to enable adequate central nervous system function. Precise regulation is required due to the brain's high metabolic rate and its limited capacity for energy storage, which is achieved through a combination of complex endothelial, myogenic, metabolic and neural regulatory mechanisms (Willie *et al.*, 2014; Phillips *et al.*, 2016).

The microcirculation continues to receive widespread attention for its contribution towards overt cardiovascular disease and type 2 diabetes (Jaap *et al.*, 1994; Wiernsperger, 2000; Nguyen *et al.*, 2007; Sprague & Ellsworth, 2010). Considered a transitional stage between diet-induced obesity and insulin resistance (de Boer *et al.*, 2012; Houben *et al.*, 2012), microvascular dysfunction is characterised by structural and functional changes to the microvasculature that largely arise from impaired endothelial function. A healthy endothelium is essential for maintaining overall vascular health and as a major regulator of vascular homeostasis, the endothelial monolayer is crucial in maintaining the balance between vasodilation and vasoconstriction (Sena *et al.*, 2013). Endothelium-derived chemical mediators are central to this regulated equilibrium, with any disturbance leading to endothelial dysfunction and the associated pathological processes of cardiovascular and metabolic diseases (Bonetti *et al.*, 2003; Avogaro *et al.*, 2011; Sena *et al.*, 2013). Microvascular dysfunction often precedes microvascular complications and (macro)vascular dysfunction in larger vessels that subsequently leads to pathological interactions and the development of both small and large vessel disease (Krentz *et al.*, 2007), such as atherosclerosis and other plaque-related problems including stenosis and ischaemic vascular disease. Furthermore, microvascular degeneration with advancing age is associated with cerebrovascular disease, such as stroke arising from microbleeds (Nishimura & Schaffer, 2013) and Alzheimer's disease (Farkas & Luiten, 2001). Cerebral hypoperfusion leads to increased systemic blood pressure as a compensatory mechanism to ensure that the brain tissue receives enough oxygen and nutrients in order to function adequately (Qin *et al.*, 2008). This compensation can lead to the development of systemic cardiovascular disease, such as essential hypertension, and demonstrates how local changes can impact systemic cardiovascular health.

Microvascular beds are thought to exhibit detectable changes in endothelial (dys)function earlier than macrovessels (Levy *et al.*, 2001; Bonetti *et al.*, 2003; Minson, 2010; Sena *et al.*, 2013) and can provide an insight into the mechanisms underlying these disease states. The cutaneous microcirculation is

primarily concerned with thermoregulation and to a lesser extent, fulfilling nutritive demands, and represents an easily accessible vascular bed that may be used as a surrogate for generalised vascular function (Holowatz *et al.*, 2008).

2.1.1 The Skin

The skin represents a crucial component of human thermoregulation and is a highly specialised vascular network that is organised into two plexuses within the dermis, in the superficial and deep layers which run parallel to the surface of the skin (Johnson *et al.*, 2014). The majority of vessels, consisting of papillary loops (true capillaries), high-resistance terminal arterioles and post-capillary venules, are located in the superficial papillary dermis, 1-2 mm beneath the epidermal surface (Figure 2.1). The papillary loops are a major determinant for heat exchange, being located in close proximity to the dermal-epidermal junction where there is a high thermal gradient due to a large surface area and high blood flow from the vessels (Charkoudian & Stachenfeld, 2014; Johnson *et al.*, 2014). Highly innervated arterioles control blood flow through the papillary loops, consisting of a lining of endothelial cells encircled by a dual layer of vascular smooth muscle cells. A second vascular plexus is located at the dermal-subdermal junction, where the vessels are typically greater in diameter than those of the upper plexus, with 4-5 layers of vascular smooth muscle (Johnson *et al.*, 2014). At this lower plexus, ascending arterioles connect to the upper plexus, hair follicles and sweat glands.

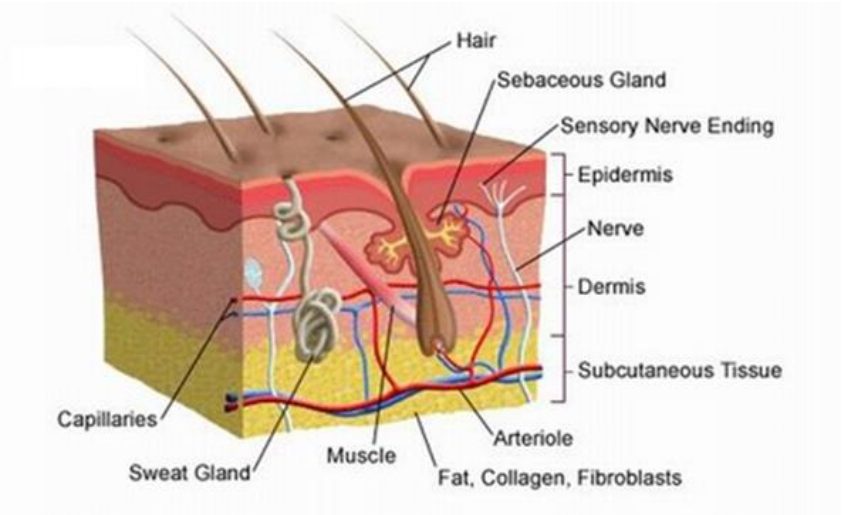

Figure 2.1 Cross-section of the cutaneous membrane and subcutaneous components, comprising of a superficial layer, a relatively thin epidermis and a thicker, deeper dermis. The subcutaneous layer, deep to the skin, attaches the dermis to underlying tissues and organs. The dermis contains the nerves, smooth muscle and sweat glands.

Despite the plexus and papillary loop arrangement being consistent, anatomical differences exist between regions. In the glabrous (non-hairy) skin of the palms, lips and plantar aspect of the feet, arteriovenous anastomoses (AVAs) bypass the resistance vessels, directly connecting the arterioles and venules (Johnson *et al.*, 2014). The AVAs are richly innervated by sympathetic adrenergic fibres and possibly also by cholinergic fibres. As AVAs have a smaller surface area and lie deeper in the dermis than papillary loops, they are less efficient for thermoregulation (Johnson *et al.*, 2014). Non-glabrous (hairy) skin of the limbs, head and trunk is largely governed by dual sympathetic neural control through noradrenergic vasoconstrictor and cholinergic or sympathetic vasodilator mechanisms (Charkoudian, 2003; Cable, 2006; Kellogg, 2006; Johnson *et al.*, 2014), in addition to local effectors, such as endothelial release of nitric oxide (NO). During normothermic conditions, sympathetic vasoconstrictor nerves are tonically active and emit little neural stimulation to the cutaneous arterioles (Kellogg, 2006). Under cold stress or subtle changes in ambient temperature, however, increased noradrenergic vasoconstrictor tone mediates a thermoregulatory reflex to conserve body heat via arteriolar vasoconstriction and a subsequent reduction in skin blood flow (SkBF) (Kellogg, 2006; Johnson *et al.*,

2014). Conversely, during mild heat stress, small variations in both vasoconstrictor and vasodilator activity are responsible for controlling SkBF via a passive vasodilation that provides large changes in heat dissipation from relatively small changes in SkBF (Charkoudian & Stachenfeld, 2014). Any remaining sympathetic vasoconstrictor tone is released, following which the vasodilator system prevails, largely via cholinergic nerves and co-transmitters (Kellogg, 2006; Johnson *et al.*, 2014). However, the vasodilatory pathways underpinning cutaneous hyperaemic responses to local skin heating differ and involve locally controlled mechanisms.

2.1.2 Local Control of Skin Blood Flow – Neural Mechanisms

The cutaneous response to local heating involves complex interactions involving neural and chemically-mediated mechanisms (Figure 2.2) that are dependent upon local skin temperature and are independent of one another (Minson *et al.*, 2001). Vasoconstrictor responses are mediated by an axon reflex mechanism and feature prominent roles for noradrenaline and neuropeptide-Y, which have been extensively reviewed (Johnson *et al.*, 2014). The hyperaemic vasodilatory response to non-painful local heating of non-glabrous skin typically induces a biphasic response that is characterised by an early, transient peak predominantly mediated by neural factors (Minson *et al.*, 2001; Johnson *et al.*, 2014). The peak is succeeded by a brief nadir and a more prolonged secondary increase in SkBF to a plateau that is predominantly mediated by locally produced chemical factors (Minson *et al.*, 2001; Johnson *et al.*, 2014). Sustained heating at the plateau eventually returns SkBF towards baseline, resting levels, despite the continuation of local heating, thereby suggesting limited bioavailability of the local chemical messengers that are involved in cutaneous vasodilation. The rate of local skin heating influences the vascular mechanisms underlying the vasodilatory response, with more prolonged heating (e.g. 0.1°C per min) producing a plateau without the early, transient peak (Johnson *et al.*, 2014). Locally heating the skin to 42-44°C is considered to achieve maximal cutaneous vasodilation and allow SkBF to be normalised for comparison between measurement sites or subject groups (Kellogg *et al.*, 1999; McCord & Minson, 2005; Minson, 2010; Johnson *et al.*, 2014).

The neurogenic changes in SkBF of non-glabrous skin are mediated by local activation of afferent sensory nerves and sympathetic adrenergic nerves, with a greater portion arising from sympathetic influences (Carter & Hodges, 2011). Minson and colleagues (2001) observed a 50% reduction in the initial peak SkBF response to local heating following topical nerve blockade. However, no effect on SkBF was observed following a nerve block proximal to the local heating site that sufficiently blocked all sensory afferent and sympathetic efferent activity, suggesting that no perception of heat was required from the central nervous system, rather activation of local sensory nerves via an axon reflex was required for vasodilation. NO contributes towards the sensory nerve component of local skin heating and studies have demonstrated an absent (Hodges *et al.*, 2008) or delayed (Houghton *et al.*, 2006) axon reflex following blockade of nitric oxide synthase (NOS) with N^G-nitro-L-arginine methyl ester (L-NAME). Further studies suggest a role for transient receptor potential vanilloid type 1 (TRPV-1) channels substantially contributing to the initial peak and nadir, and more modestly the plateau phase, of the hyperaemic response through activation of the NO component of the axon reflex (Wong & Fieger, 2010). Release of the neuropeptide substance P and calcitonin gene-related peptide, which are co-localised in the nerve terminals in the skin (Wallengren, 1997), are also considered to play a role in sensory nerve-mediated cutaneous vasodilation, with substance P believed to be partially dependent on NO (Wong *et al.*, 2005; Wong & Minson, 2006). However, there remains limited research regarding these agents and current theories are reviewed elsewhere (Johnson *et al.*, 2014) so will not be duplicated in this literature review.

Sympathetic adrenergic nerves also play a role in the local control of SkBF in response to local skin heating. Antagonism of vasoconstrictor nerve function demonstrated that the axon reflex is abolished (Houghton *et al.*, 2006; Hodges *et al.*, 2008, 2009) or reduced (Carter & Hodges, 2011; Tew *et al.*, 2011b; Hodges & Sparks, 2014; Del Pozzi & Hodges, 2015), suggesting that cutaneous vasodilation is affected by vasoconstrictor nerve function. Furthermore, in addition to abolition of the axon reflex,

the overall hyperaemic response was attenuated following blockade with bretylium tosylate, an adrenergic neuronal blocking agent (Houghton *et al.*, 2006). Further studies support the role of sympathetic nerves in the hyperaemic response (Carter & Hodges, 2011; Tew *et al.*, 2011b), with the neurotransmitters noradrenaline and neuropeptide Y being implicated in the initiation of the axon reflex and overall hyperaemic response (Hodges *et al.*, 2008). However, in a three-part study, Hodges and Sparks (2014) examined whether the sustained vasodilatation following local skin warming involved noradrenaline, neuropeptide Y and/or NOS, concluding that NOS contributes markedly to the sustained vasodilatation, but that noradrenaline and neuropeptide Y have little, or no, contribution. Nevertheless, the adrenergic system is required for increased SkBF following local thermal hyperaemia, in addition to the sensory nerves and chemically-mediated component.

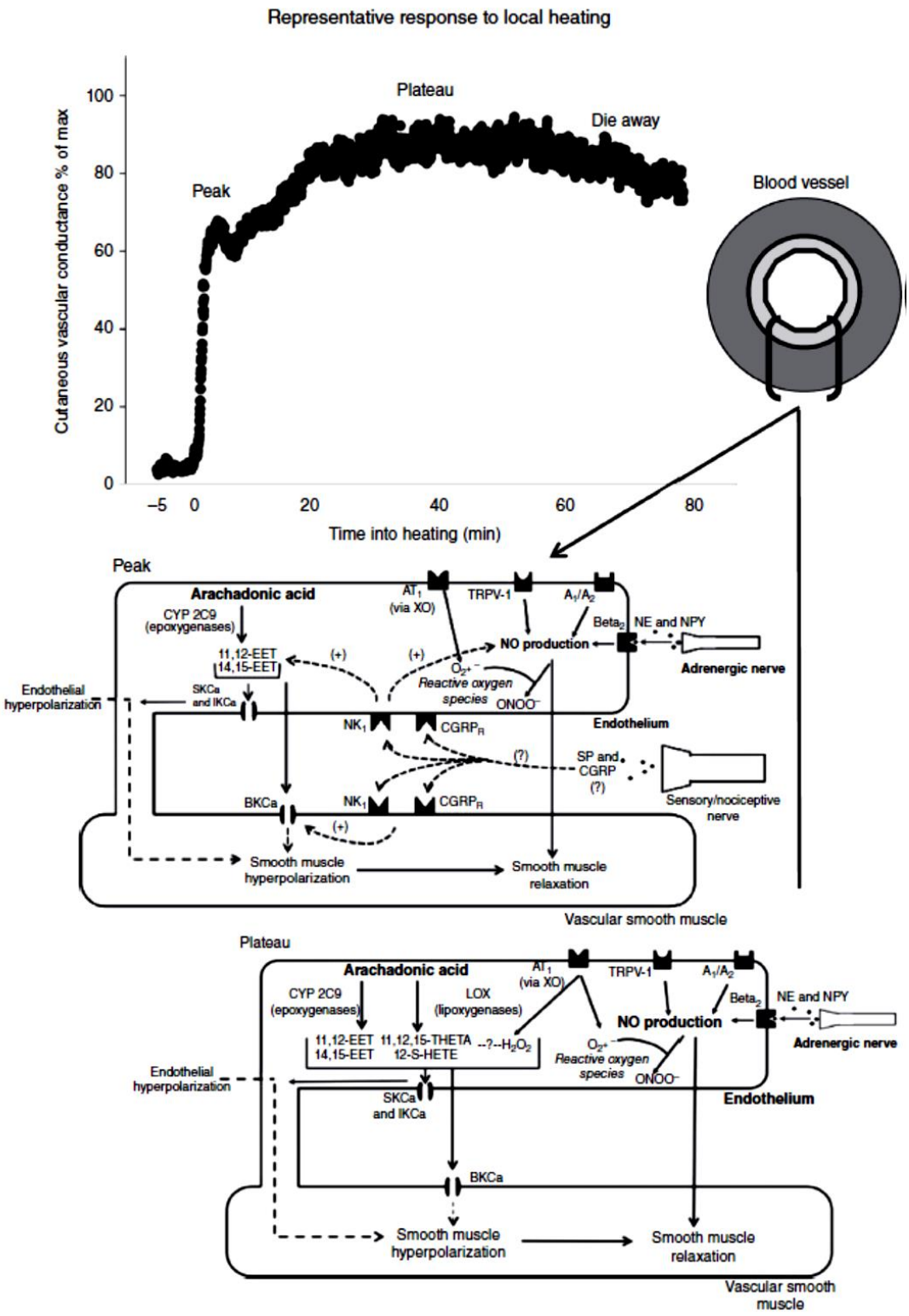

Figure 2.2 Summary of the mechanisms that contribute to local thermal hyperaemia. The top figure represents a typical skin blood flow response to rapid local heating to 42°C, featuring the initial (axon) peak, nadir and sustained vasodilation (plateau). The middle figure depicts the current theory of local heating during the initial peak, with the bottom schematic outlining the theory of vasodilation during the plateau phase, both presenting the pathways in the endothelium and smooth muscle. Taken from (Johnson *et al.*, 2014).

2.1.3 Local Control of Skin Blood Flow – Chemical Mediators

The temperature-dependent sustained plateau phase of the hyperaemic response is largely mediated by NO and endothelial derived hyperpolarisation factors (EDHFs) via hyperpolarisation of the vascular smooth muscle (Johnson *et al.*, 2014). Generated from the amino-acid L-arginine by endothelial nitric oxide synthase (eNOS), NO is the endothelium's most potent vasodilator, and is essential for optimal endothelial function (Maiorana *et al.*, 2001; Di Francescomarino *et al.*, 2009). As an endocrine vasoregulator, NO modulates blood flow within the microcirculation (Datta et al., 2004; Sena et al., 2013) and the role of NO as a major contributor to local thermal cutaneous vasodilation has been reviewed extensively (Johnson *et al.*, 2014). Kellogg and colleagues (1999) observed a ~50% reduction in SkBF following infusion of locally heated skin with the eNOS inhibitor L-NAME, findings which were similarly replicated in later studies (Minson *et al.*, 2001; Gooding *et al.*, 2006). ~60% of the sustained SkBF plateau phase following local heating is thought to be attributable to NO, with the remaining ~40% being linked to EDHFs, although the precise pathways involved remain inconclusive (Brunt & Minson, 2011). Calcium-activated potassium (KCa) channels are important in cutaneous vasodilation for NO and EDHFs such as cyclooxygenase (Garland & Dora, 2017), in addition to prostacyclin which has been shown to modulate SkBF in healthy males (Fujii *et al.*, 2016) and females (Fujii *et al.*, 2017). Scientific knowledge and understanding of the complex chemically-mediated pathways involved in local cutaneous vasodilation remains largely inconclusive, although it is widely accepted that NO is a key mediator of cutaneous vasodilation and in maintaining optimal endothelial function.

2.2 Macrovascular (Conduit Artery) Function

Conduit arteries, or macrovessels, are typically >200 μm in diameter and are characterised by a thick tunica media that contains a large number of collagen and elastin filaments, in addition to a greater number of smooth muscle cells compared to other branches of the arterial tree, such as resistance vessels/arterioles and the cutaneous vessels (Figure 2.3). The contractile structure of the vessel wall

allows active vasodilation via sympathetic neural control or vasoactive chemical mediators, such as NO, released from the endothelium. Once regarded as a passive interface, the vascular endothelium is an important endocrine organ and a healthy endothelium is essential for maintaining overall vascular health, via anti-atherogenic properties that protect against inflammatory responses, smooth muscle cell proliferation and vasoconstriction (Davignon & Ganz, 2004). Numerous paracrine substances are released from the endothelium including NO, which is a lipid soluble gas that is synthesised in the endothelial cells from the amino acid L-arginine through the action of eNOS (Palmer *et al.*, 1988). NO rapidly diffuses into the vascular smooth muscle of the tunica media, binding to and activating the enzyme guanylate cyclase (Ignarro *et al.*, 1986), the resultant increase in cyclic guanosine monophosphate induces smooth muscle relaxation and subsequent vasodilation (Furchgott & Jothianandan, 1991; Green *et al.*, 2004).

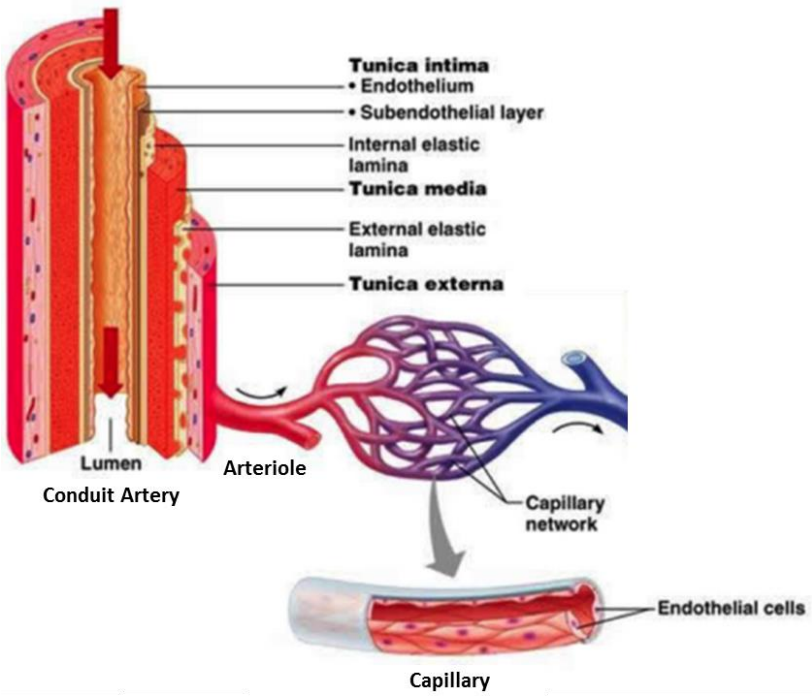

Figure 2.3 The arterial tree, depicting the conduit artery through to the capillaries.

NO is tonically secreted by the endothelium, contributing ~50% to basal vascular tone (Vallance *et al.*, 1989). Upregulation of NO can be physiologically stimulated by increased laminar blood flow and the

resulting shear stress, termed flow-dependent NO formation, or alternatively, NO is produced in response to various endothelial stimulators, such as acetylcholine. Whilst the shear stress induced endothelial mechanotransduction and signalling cascade leading to the secretion of NO is not entirely understood, it is thought that several mechanisms are involved. Increased arterial blood flow and shear stress reportedly induce endothelial potassium channel activation (Oleson & Johnson, 1988), calcium influx in endothelial cells (Dull & Davies, 1991), the release of bradykinin and/or phosphorylation of serine residue (Groves *et al.*, 1995; Kuga *et al.*, 1997), all of which are believed to enhance NO bioavailability. Other vasoactive substances are released by the endothelium and contribute to vasodilation, such as prostacyclin and EDHFs (Grabowski *et al.*, 1985; Spilk *et al.*, 2013), although these are suggested to have a more important role in the smaller arteries (Shimokawa *et al.*, 1996). NO bioavailability is commonly considered the hallmark of endothelial (dys)function (Davignon & Ganz, 2004; Sena *et al.*, 2013) that is regarded as the starting point of progression towards overt CVD and is characterised by a reduced vasodilatory capacity, a proinflammatory and prothrombic state, resulting in an altered endothelial cell phenotype and intracellular signalling pathways (Suganya et al., 2016). NO-mediated vasodilator function is commonly assessed *in vivo* through the non-invasive technique of flow-mediated dilation (FMD), whereby an artery's dilator response is measured in response to a reactive hyperaemia following a brief (5-min) period of artificial limb ischaemia. Impaired FMD responses are associated with future cardiovascular events in healthy (asymptomatic) individuals (Shechter *et al.*, 2009; Inaba *et al.*, 2010; Shechter *et al.*, 2014), and FMD is therefore regarded as a surrogate marker of cardiovascular disease (Vita & Keaney, 2002; Gokce *et al.*, 2003; Green *et al.*, 2011; Mutlu *et al.*, 2011).

2.3 Cerebrovascular Function

The regulation of cerebral blood flow (CBF) is critical for maintaining an adequate supply of oxygen and nutrients to the brain, particularly given the high metabolic rate of brain tissue and its limited capacity for substrate storage (Brown & Ransom, 2007; Willie *et al.*, 2014). The brain occupies only 2-

3% of total human body mass, yet the metabolic demand of the cerebral tissue is ~20% of the body's total oxygen consumption (Bain *et al.*, 2014). Substantial reductions in CBF, therefore, rapidly lead to unconsciousness (van Lieshout *et al.*, 2003) and, if sustained, brain damage and death ensues (Smith *et al.*, 2011). Furthermore, decreases in steady-state CBF (Parkes *et al.*, 2004; Bertsch *et al.*, 2009), blunted task-specific CBF increases (Sorond *et al.*, 2008b) and accentuated reductions in CBF contribute towards impaired cognitive function and cerebrovascular disease, such as in dementia and aging (Farkas & Luiten, 2001; Farkas *et al.*, 2002; Ruitenberg *et al.*, 2005a; Schuff *et al.*, 2009; Spencer, 2009). The control of CBF involves multiple integrated regulatory mechanisms, with the principle regulators involving arterial blood gases, such as the partial pressure of arterial carbon dioxide (P_aCO_2), mean arterial pressure (MAP), cerebral metabolism and the autonomic nervous system (Figure 2.4) (Willie *et al.*, 2014).

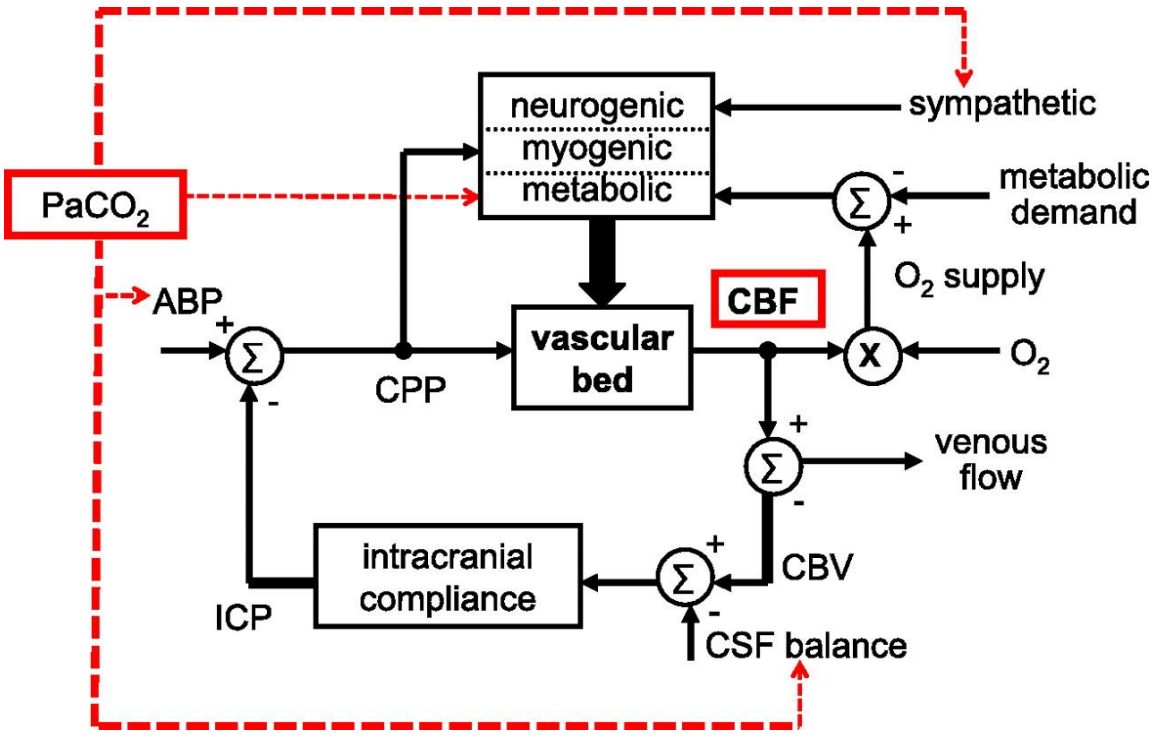

Figure 2.4 A schematic of the primary mechanisms responsible for control of cerebral blood flow (CBF). ABP, arterial blood pressure; CBV, cerebral blood volume; CPP, cerebral perfusion pressure; CSF, cerebrospinal fluid; ICP, intracranial pressure; P_aCO_2, partial pressure of arterial carbon dioxide. Taken from (Ainslie & Duffin, 2009).

2.3.1 Partial Pressure of Arterial Carbon Dioxide (P_aCO_2)

Brain perfusion is highly sensitive to changes in P_aCO_2, with a ~3-6% increase and/or a ~1-3% decrease in flow per mmHg change in CO_2 above and below eupnoeic P_aCO_2 (Willie *et al.*, 2012; Skow *et al.*, 2013; Willie *et al.*, 2014). The entire cerebrovasculature is sensitive to changes in blood gases, with the pial arterioles considered to modulate resistance. In response to increased P_aCO_2, CBF increases following smooth muscle relaxation and dilatation of the vessels, whereas a reduction in P_aCO_2, or hypocapnia, results in a decrease in CBF through increased cerebrovascular resistance (Kety & Schmidt, 1948; Wasserman & Patterson, 1961). The entire cerebrovascular arterial tree is vasoactive, with the arteries of the head and neck also being sensitive to changes in blood gases and perfusion pressure (Willie *et al.*, 2012). The precise mechanism underlying CO_2 regulation is uncertain, but appears to be independent of arterial pH as CBF was unchanged following metabolic acidosis and alkalosis (Lambertsen *et al.*, 1961; Harper & Bell, 1963), whereas changes in arteriolar diameter were induced by manipulation of extracellular pH (Wahl *et al.*, 1970; Kontos *et al.*, 1977). The likely mechanism, therefore, relates to CBF regulation via a change in extracellular pH, such as the cerebrospinal fluid, induced by diffusion of CO_2 molecules across the cerebrovascular blood-brain barrier. The subsequent lowering or elevation of extracellular pH thus alters the vascular smooth muscle tone to induce relaxation or contraction, respectively (Lambertsen *et al.*, 1961; Harper & Bell, 1963; Lassen, 1968). Metabolic regulation of CBF also involves local mechanisms, with local cerebral perfusion being tightly coupled to local neural metabolism due to the anatomical and metabolic relationship between elements of the neurovascular unit, such as the neurons, glial cells and microvasculature (Willie *et al.*, 2014). The mechanisms related to neurovascular coupling and arterial blood gases have been thoroughly reviewed by Willie and colleagues (2014) .

2.3.2 Cerebral Autoregulation

Steady-state CBF reportedly remains relatively stable across a range of blood pressures, requiring reflex adjustments in cerebrovascular resistance concomitantly with fluctuations in blood pressure to maintain adequate blood flow (Lassen, 1959; Paulson *et al.*, 1990). This physiological mechanism is

termed cerebral autoregulation (van Beek *et al.*, 2008; Willie *et al.*, 2014). Current within-subject *in vivo* data suggests that the CBF-MAP relationship is not stable through a broad range of pressures and, furthermore, is dependent upon the severity and direction of change in perfusion pressure (Willie *et al.*, 2014). Experimentally, 'static', or steady-state, cerebral autoregulation has been examined to observe the relationship between CBF and MAP, with 'dynamic' cerebral autoregulation introduced to explore the pressure-flow relationship during transient changes in MAP, such as those induced by changes in posture or when coughing (Willie *et al.*, 2014). Pial arterioles, larger intracranial arteries and the large conduit vessels in the neck are considered to contribute to cerebrovascular resistance and regulation of CBF, with studies demonstrating a change in carotid artery diameter in response to changes in P_aCO_2 and P_aO_2 (Wilson *et al.*, 2011; Willie *et al.*, 2012). Blood pressure is critical for cerebral autoregulation and CBF, with elevated P_aCO_2 and hypoxia impairing the capability of the brain, rendering it unable to defend against changes in blood pressure (Tzeng *et al.*, 2012).

2.3.3 Autonomic Control

Although the precise mechanisms remain poorly understood, neurogenic control is important in autoregulation of the cerebrovasculature, which is richly innervated by adrenergic and cholinergic fibres. A higher proportion of longitudinal nerve bundles are found in the extracranial arteries, whereas the intracranial vessels possess a greater total density of perivascular nerve fibres and neuron terminals (Borodulya & Pletchkova, 1973, 1976; Bleys *et al.*, 1996). In a series of studies, summarised by Willie and colleagues (2014), CBF was observed to increase following ganglionectomy in humans, suggesting sympathetic nerve involvement in CBF regulation, with animal models observing that this is most important in buffering changes in blood pressure and predominantly involves the larger arteries. Impaired cerebral autoregulation in healthy humans has been demonstrated following sympathetic nervous system blockade (Zhang *et al.*, 2004), although the systemic effects of the blockade on the peripheral vasculature are difficult to dissociate from any direct effects on cerebral autoregulation (Willie *et al.*, 2014). This is also true of the relationship between the sympathetic nervous system and the response of the cerebrovasculature to changes in arterial blood gases, with

human studies reporting inconsistent data. Unchanged CO_2 reactivity was demonstrated following augmented sympathetic nerve activity to lower-body negative pressure (LeMarbre *et al.*, 2003) and hand grip exercise (Ainslie *et al.*, 2005), yet other studies have reported attenuation to lower-body negative pressure (Zhang *et al.*, 2011) and ganglionic blockade (Jordan *et al.*, 2000). The contribution of cholinergic nerves to CBF control also remains equivocal, with very few studies performed to date in humans or in animal models, as reviewed elsewhere (Willie *et al.*, 2014).

In summary, current literature supports integrated regulation of CBF control through multiple mechanisms that remain incompletely understood, as previously discussed. However, the lack of understanding of some of the mechanisms involved, such as autonomic control, is partly due to the assessment techniques used to quantify changes in CBF. Use of a vasoactive stimulus to challenge the cerebrovascular system is one such method. Termed cerebrovascular reactivity (CVR), this technique measures the change in CBF per change in vasoactive stimulus (Fierstra *et al.*, 2013). CVR studies currently use various stimuli, such as CO_2, and methods of measuring CBF, including transcranial Doppler ultrasound (TCD) and blood oxygen-dependent magnetic resonance imaging (BOLD-MRI). However, since the variety of stimuli and measurement techniques used do not allow accurate comparison of CVR between individuals, meaningful interpretation of data can be difficult. The cerebrovasculature is, therefore, critical for CBF regulation but provides many challenges with accurate assessment of its function.

2.4 Metabolic Function: Insulin/Glucose

Metabolic homeostasis is closely interrelated with cerebrovascular and peripheral vascular function, with disrupted signalling pathways leading to insulin resistance and endothelial dysfunction. Glucose homeostasis is mediated by several hormones including insulin, glucagon, cortisol, catecholamines, growth hormone and incretins (Xiang *et al.*, 2011). Insulin is secreted from the pancreatic β cells and

regulates glucose homeostasis via disposal of glucose in skeletal muscle and adipose tissue (Muniyappa *et al.*, 2008). Furthermore, insulin is also a key vasoactive hormone in the regulation of cerebral and peripheral blood flow (Hughes & Craft, 2016), and is pivotal in coordinating metabolic and vascular homeostasis (Muniyappa *et al.*, 2007). Under normal conditions, insulin stimulates NO production from the vascular endothelium via activation of eNOS and the phosphatidylinositol-3-kinase (PI-3K) pathway, which further facilitates glucose uptake via increased tissue perfusion (Muniyappa *et al.*, 2008; Olver *et al.*, 2013). However, impaired insulin sensitivity or insulin resistance, typically defined as reduced sensitivity or responsiveness to insulin and its metabolic actions, such as insulin-mediated glucose disposal, causes an accumulation of glucose in the bloodstream (hyperglycaemia) (Wheatcroft *et al.*, 2003; Kim *et al.*, 2006; Muniyappa *et al.*, 2008; Sena *et al.*, 2013). Consequently, a greater amount of insulin is required to allow glucose to enter the cells. However, the pancreatic β cells are unable to fulfil the increased demand for insulin indefinitely and cannot produce adequate quantities to overcome resistance, leading to diabetes. Whereas the etiopathogenesis of type I diabetes involves autoimmune destruction of the pancreatic β cells, resulting in inadequate insulin production and an accumulation of glucose in the bloodstream, type 2 diabetes is characterised by insulin resistance and endothelial dysfunction that is widely linked to environmental factors, such as physical inactivity and obesity (Kim *et al.*, 2006).

Increased CVD risk is associated with disrupted metabolic function, with hyperinsulinaemia, as seen when increased quantities of insulin are secreted in response to glucose accumulation, associated with down-regulation of eNOS and an imbalance in both the mitogen-activated protein kinase (MAPK) and PI-3K pathways (Muniyappa *et al.*, 2008). Excessive levels of circulating insulin subsequently contribute towards morphological maladaptation of the vessel wall due to cellular hypertrophy and an altered extracellular matrix (Maria Assunta *et al.*, 2009). Furthermore, hyperglycaemia-induced vascular damage exhibits reduced NO bioavailability, increased reactive oxygen species and harmful

metabolites, impaired endothelial-dependent vasodilation, increased proliferation and matrix degradation (Sena *et al.*, 2013). Such damaging effects are largely caused by endothelial dysfunction arising as a consequence of an accumulation of glycolytic intermediates through inhibition of glyceraldehyde-3-phosphate dehydrogenase activity, a glycolytic enzyme (Sena *et al.*, 2013; Suganya *et al.*, 2016). Harmful mechanisms are subsequently activated, including protein kinase C (PKC) isoforms, advanced glycation end products (AGEs) and the sorbitol and hexosamine pathways (de Vriese *et al.*, 2000; Sena *et al.*, 2013). Given the reciprocal relationship between metabolic function and cardiovascular health, greater emphasis is increasingly being placed upon establishing corrective strategies for postprandial hyperglycaemia in the treatment and prevention of CVD, with use of overfeeding experiments, or metabolic challenges, providing an insightful tool in unravelling the physiological adaptations to caloric excess.

2.5 Effects of a Metabolic Challenge/Overfeeding on CVD Risk

Human studies are increasingly exploring the effects of Western sedentary lifestyles upon metabolic health, which are typically observed using overfeeding and/or inactivity models that mimic short- to medium-term periods of energy surplus, lasting from a single meal to several days or weeks. Such models are representative of holiday or celebratory periods, when otherwise healthy individuals adopt different dietary and activity patterns compared to their habitual lifestyle (Cornier *et al.*, 2006). Previous models for inducing changes in insulin resistance level have involved complete bed rest and extreme step reduction (>10,000 to <1,500 steps/day), although such models are difficult to implement and are not representative of modern sedentary lifestyles that also typically involve high calorie dietary intake.

Short-term (<7-days) overfeeding interventions have induced transient insulin resistance, without modifying activity levels. Whole-body insulin resistance was observed in lean 'obese-resistant' females

following 3-days of 50% overfeeding (50% carbohydrate, 30% fat, 20% protein), with no difference observed in lean males (Cornier *et al.*, 2006). However, Adochio and colleagues (2009) observed no change in whole-body insulin sensitivity following 5-days of 40% caloric excess in healthy, lean adults. Nevertheless, this study observed distinct differences in insulin sensitivity according to the macronutrient composition of the overfeed. High carbohydrate (20% fat, 60% carbohydrate, 20% protein) overfeeding was associated with changes in *ex-vivo* skeletal muscle signalling compatible with increased insulin sensitivity, suggestive of enhanced glucose disposal that maintained whole-body insulin sensitivity. In contrast, the same study also found *ex-vivo* molecular changes in keeping with reduced insulin sensitivity following high fat (50% fat, 30% carbohydrate, 20% protein) overfeeding (Adochio *et al.*, 2009). Further studies have also observed deleterious effects of high fat overfeeding. In a randomised, crossover trial in young, healthy males, 5-days of high fat (60% fat, 32.5% carbohydrate, 7.5% protein) overfeeding with 50% extra calories, a 26% increase in fasting hepatic glucose production was observed, with a borderline increase in fasting insulin (Brons *et al.*, 2009). Following 7-days of high fat (65%) overfeeding (+50% calories), a significant increase in postprandial glucose and insulin was observed following a mixed meal tolerance test in healthy adults (Parry *et al.*, 2017). Increased fasting insulin and glucose was induced following 8-weeks of overfeeding (+40% calories) in young, healthy adults who followed either a low protein (59% fat, 36% carbohydrate, 5% protein), normal protein (49% fat, 36% carbohydrate, 15% protein) or high protein (39% fat, 36% carbohydrate, 25% protein) diet in a randomised, parallel-arm study (Bray *et al.*, 2016). No significant between-group differences were observed, although the group who consumed the low protein diet, that comprised a greater proportion of fat (59%), exhibited a greater increase in body fat (%) at 8-weeks. It is apparent from these findings that macronutrient composition in overfeeding studies may affect metabolic pathways and energy storage, with higher fat content in particular seeming to exhibit greater changes in insulin resistance.

Medium-term overfeeding has also demonstrated impaired metabolic function. Tam and colleagues (2010) observed an 11% reduction in insulin sensitivity in healthy adults following 28-days of overfeeding (+1250 kcal/day; 45% fat, 15% protein, 40% carbohydrate). Compared to baseline, peripheral insulin sensitivity decreased following 28-days of overfeeding in healthy, non-obese, sedentary adults, with a concomitant increase in oxidative stress that may have contributed towards the insulin resistance (Samocha-Bonet *et al.*, 2012). A further 28-day overfeeding (+1250 kcal/day; 45% fat, 15% protein, 40% carbohydrate) study in healthy adults demonstrated reduced insulin sensitivity, together with serum lipid changes and increased circulating ceramides that may promote systemic insulin resistance (Heilbronn *et al.*, 2013).

Diets high in fat and/or carbohydrate are strongly associated with long-term cardiometabolic risk (Hennig *et al.*, 2001; Liu & Manson, 2001), although few studies have examined the effect of overfeeding on vascular function. Gupta and colleagues (2013) demonstrated a significant increase in CVD risk following 8-weeks of overfeeding (1.4 times habitual calorie consumption, comprising 44% fat, 41% carbohydrate, 15% protein) in healthy adults, that resulted in increased body fat, visceral adipose tissue mass, insulin resistance, systemic inflammation, BP disturbance and endothelial dysfunction (measured by EndoPAT). Consumption of a habitually high fat versus low fat (≥35% and <35% of total calories, respectively) diet was associated with impaired NO-mediated endothelium-dependent vasodilation in sedentary adults (aged 43-67 years), assessed via 4-day dietary records and forearm blood flow responses to acetylcholine via strain-gauge venous occlusion plethysmography, respectively (Dow *et al.*, 2015). Impairment in endothelial function has also been observed in healthy adults 2-4 hours following a single high fat meal (Vogel *et al.*, 1997; Bae *et al.*, 2001; Tsai *et al.*, 2004). Such changes in endothelial function are largely attributed to reductions in NO bioavailability (Bae *et al.*, 2001; Tsai *et al.*, 2004).

Despite a lack of studies examining the effects of overfeeding on changes in vascular function *per se*, the effects of habitual overfeeding following long-term caloric excess and subsequent obesity have been established. Obesity represents a state of energy imbalance, whereby increased energy intake, or nutrient excess, is coupled with disproportionally low levels of physical activity (Cuthbertson *et al.*, 2017). Excess carbohydrate, above the 300-500g typically stored as glycogen, is oxidised or converted to triglyceride that is predominantly stored within adipose tissue, a largely infinite storage facility (Cuthbertson *et al.*, 2017). Endothelial dysfunction arising from obesity is suggested to precede the development of insulin resistance (Suganya *et al.*, 2016), with attenuated NO production and increased vasoconstrictor tone contributing towards blunted endothelium-dependent vasodilation (Okon *et al.*, 2005). Human studies have observed impaired insulin action in dilating resistance vessels (Laakso *et al.*, 1990) and in increasing the elasticity of conduit arteries (Westerbacka *et al.*, 1999) in obese versus lean males. Furthermore, Clerk and colleagues (2006) demonstrated that forearm microvascular responses to insulin were severely blunted in otherwise healthy obese versus lean individuals, suggesting that obesity is associated with generalised endothelial dysfunction throughout the vascular tree. Such obesity-related impairments in endothelial function are likely caused by mechanisms related to inflammatory processes in combination with hyperglycaemia and insulin resistance, whereby NO bioavailability is reduced secondary to increased oxidative stress production (Lantorno *et al.*, 2014; Virdis, 2016). Furthermore, obesity-induced impairments in microvascular function are linked to increased peripheral resistance and elevated BP (de Jongh *et al.*, 2004; Jonk *et al.*, 2007). Disrupted intracellular and endocrine signalling pathways are the likely cause of obesity-related microvascular dysfunction, of which the renin-angiotensin system is thought to play a prominent role (Jonk *et al.*, 2007). Several comprehensive reviews (Jonk *et al.*, 2007; de Boer *et al.*, 2012; Houben *et al.*, 2012) have been published focusing on microvascular dysfunction and its association with obesity, insulin resistance and the potential causal mechanisms.

2.6 Effects of Underactivity on CVD Risk

Physical inactivity represents a major independent risk factor for CVD, with approximately one-third of the global population failing to meet the minimum physical activity required for the maintenance of health (Hallal *et al.*). Largely due to technological advances and a greater increase in more sedentary occupations, physical inactivity is an increasing characteristic of modern Western lifestyles that contributes towards a positive energy balance when combined with excess caloric intake. Methods used to examine the impact of inactivity on cardiovascular health include complete bed rest, space travel or simulated microgravity, lower limb immobilisation, assessment of individuals with spinal cord injury and step-reduction models. However, bed rest is an extreme model of physical inactivity that is limited in its approach as it causes a reduction in plasma volume (Gaffney *et al.*, 1985; Convertino *et al.*, 1986; Fortney *et al.*, 1991), in addition to failing to restrict upper limb movement (Bleeker *et al.*, 2005c). Short-term physical inactivity models (4-8 weeks) demonstrate no differences in BP, BMI or cholesterol (Bleeker *et al.*, 2005a; Bleeker *et al.*, 2005b; Bleeker *et al.*, 2005c; Demiot *et al.*, 2007; Thijssen *et al.*, 2007), with direct effects of inactivity on the vasculature likely having a greater contribution towards increased CVD risk, although the evidence remains mixed. The different models used to explore the effects of physical inactivity upon CVD risk factors may, however, explain some of the conflicting outcomes reported in studies exploring both the acute and chronic vascular adaptations of inactivity.

Bed rest deconditioning demonstrated enhanced brachial artery FMD after 7-days (Bonnin *et al.*, 2001), yet no change was observed following 5-days of bed rest in a study that also found a significant impairment in microvascular function, measured via forearm and calf reactive hyperaemia, in addition to elevated systolic BP and increased cholesterol, triglycerides, fasting insulin and glucose, consistent with the development of insulin resistance (Hamburg *et al.*, 2007). The differences in conduit artery responses may be explained by methodological variation and lack of adherence to consensus

guidelines (Thijssen *et al.*, 2011), or alternatively, may be due to upper limb movement being unrestricted and, therefore, the vessel remaining under the influence of shear stress. In line with this, following 60-days of bed rest, van Duijnhoven and colleagues (2010) observed a 24% decrease in superficial femoral artery diameter and a significant increase in wall thickness and the wall-to-lumen ratio, which were also observed in the carotid artery. These findings suggest that complete physical inactivity induces changes in vessel wall diameter, with conduit artery wall thickening considered a vascular marker for atherosclerotic development (Bots *et al.*, 1994; Lorenz *et al.*, 2007). Exercise countermeasures performed thrice weekly completely abolished the increase in wall thickness in the carotid artery, with partial abolition in the femoral artery (van Duijnhoven *et al.*, 2010), thus demonstrating the impact of physical inactivity versus activity. Further studies examining endothelial function of the femoral artery, however, reported improved endothelial function following 25- and 52-days bed rest (Bleeker *et al.*, 2005c), unilateral lower limb immobilisation (Bleeker *et al.*, 2005a), and in individuals with acute (de Groot *et al.*, 2006) and chronic (de Groot *et al.*, 2004; de Groot *et al.*, 2005) spinal cord injury. However, normalising the femoral artery FMD response for prevailing shear preserves (de Groot *et al.*, 2004) or only slightly increases (de Groot *et al.*, 2005) function compared to able-bodied controls. Upregulation of smooth muscle cell sensitivity to NO may also be responsible for the preservation of endothelial function in deconditioned conduit arteries, possibly arising in response to the chronic reduction in endothelial shear stress and subsequent down-regulation of eNOS, a possible explanation that is discussed in greater detail elsewhere (Thijssen *et al.*, 2010).

Step counting, via pedometers or accelerometers, provides a useful tool for monitoring physical activity levels and habitual patterns amongst the general public. Schmidt and colleagues (2009) reported a substantially higher prevalence of cardiometabolic risk factors, including metabolic syndrome, systolic BP, fasting glucose and triglycerides, in individuals taking <5,000 steps per day. Further studies reported similar findings, with an increased risk of metabolic syndrome associated

with lower step counts (Sisson *et al.*, 2010) and a higher BMI, waist circumference and arterial stiffness in individuals with type 2 diabetes (Jennersjo *et al.*, 2012). Compared to extreme inactivity models such as bed rest, step-reduction models offer a more suitable approach to investigate the effect of inactivity and more accurately reflect Western lifestyles. Significant reductions in peripheral insulin sensitivity, cardiovascular fitness and lean leg mass were observed following a 2-week self-monitored reduction in ambulatory activity to 1,500 steps per day in healthy, non-exercising males (Krogh-Madsen *et al.*, 2010). Mikus and colleagues (2012) observed significant postprandial increases in glucose following reduced physical activity (<5,000 steps per day) for 3-days in habitually active individuals. 5-days of step reduction (<5,000 steps per day) also demonstrated impaired glycaemic control and insulin sensitivity in healthy, young males, although there were no changes in vascular function, assessed as peak blood flow (mL/min) and conductance (calculated as peak blood flow/MAP) of the brachial and femoral arteries, measured via ultrasound (Reynolds *et al.*, 2015).

Combined overfeeding and step-reduction models more closely mimic lifestyle behaviours than models focusing on one component contributing towards an energy surplus, such as physical inactivity models of complete bed rest failing to account for dietary factors. To date, only a handful of studies have explored the combined impact of overfeeding and inactivity, that more closely represents a Western lifestyle, on metabolic and/or vascular function. Insulin resistance was observed following 3-days of overfeeding (+25% calories) combined with abstinence from structured exercise in healthy, habitually active adults (Hagobian & Braun, 2006). A subsequent single bout of exercise, combined with overfeeding, restored insulin sensitivity to baseline levels, demonstrating the deleterious impact of inactivity. Following 14-days of overfeeding (+50% calorie intake) combined with step-reduction (≤1,500 steps per day), Knudsen and colleagues (2012) demonstrated impaired insulin sensitivity after just 3-days in young, healthy males who habitually undertook ~10,000 steps per day and consumed ~2,000 calories per day. A more pronounced reduction in insulin sensitivity was observed after 7- and

14-days of the intervention, with impairment in insulin sensitivity preceding changes in body composition. More recently, hyperinsulinaemia, reduced insulin sensitivity and altered adipose tissue gene expression were observed following 7-days overfeeding (+50% calories) combined with restricted physical activity (≤4,000 steps per day) in heathy, habitually active males, in a randomised parallel group design (Walhin *et al.*, 2013). The addition of a daily exercise bout to a matched model of overfeeding and reduced physical activity largely prevented these changes, again demonstrating the profound effect of exercise compared to inactivity. To date, however, no studies have specifically examined the combined effects of overfeeding and physical activity reduction on both metabolic and vascular function, despite the close interrelationship between the two.

Whilst combined overfeeding and inactivity models offer a more realistic approach to represent habitual lifestyle behaviour, step-reduction targets may not necessarily have been universal for all study participants, potentially influencing individual responses. For example, a targeted step-reduction to ≤1,500 steps per day represents a 70% reduction for an individual who is habitually completing 5,000 steps per day, whereas the same target for an individual completing 10,000 steps per day is a reduction of 85%. Such targets represent dramatic reductions to an individual's activity level and, therefore, their lifestyle. Nevertheless, the combined experimental model offers a more realistic approach to examine the impact of lifestyle behaviours and interventions on cardiometabolic health.

2.7 Dietary Flavonoids

Flavonoids are characterised by two or more aromatic rings bound together by a 3-carbon bridge forming an oxygenated heterocycle, with the degree of oxidation generating further subclasses, in ascending order of oxidation: flavanols (often called catechins), flavanones, flavones, isoflavones, flavonols and anthocyanins (Figure 2.5) (Bravo, 1998; Beecher, 2003). Hydroxylation and conjugation

patterns of the aromatic rings further characterise the individual flavonoids and isoflavones within these subclasses (Beecher, 2003). Phenolic acids are the second most abundant polyphenol subclass, accounting for 30% of dietary polyphenols (Bravo, 1998). These can be categorised as hydroxybenzoic acids, as found in tea, and hydroxycinnamic acids which are found in coffee, cinnamon and fruits such as blueberries, apples and plums (Manach *et al.*, 2004; Pandey & Rizvi, 2009). Lignans are diphenolic compounds formed of 2 phenylpropane units and are found in fruits, legumes, cereals and grains (Manach *et al.*, 2004). Several lignans are regarded as phytoestrogens, such as secoisolariciresinol (Pandey & Rizvi, 2009) which is found in linseed (flax), the richest sources of lignans. Stilbenes are the final of the four polyphenol subclasses and are characterised by two phenyl moieties connected by a two-carbon ethylene bridge. Low quantities of stilbenes occur in the human diet, with resveratrol and pterostilbene considered the main ones. Resveratrol is the most widely known of the two and is found in grapes and its derivative red wine, as well as blueberries, cranberries and peanuts. Pterostilbene is also found in grapes and blueberries, and is suggested to have superior bioavailability compared to resveratrol, in addition to neuroprotective properties in age-related diseases (Chang *et al.*, 2012). Epidemiological research has explored the effects of a variety of polyphenol-rich foods upon cardiovascular health, with a negative correlation identified between the consumption of polyphenol-rich dietary products and CVD incidence (Arts & Hollman, 2005; Vita, 2005; Habauzit & Morand, 2012). Flavonoids are increasingly of scientific interest as they are present in a vast array of dietary sources, such as fruits and vegetables, cocoa and tea, with several studies revealing a strong, inverse relation between regular intake of tea and cardiovascular risk (Grassi *et al.*, 2009b; Greyling *et al.*, 2014).

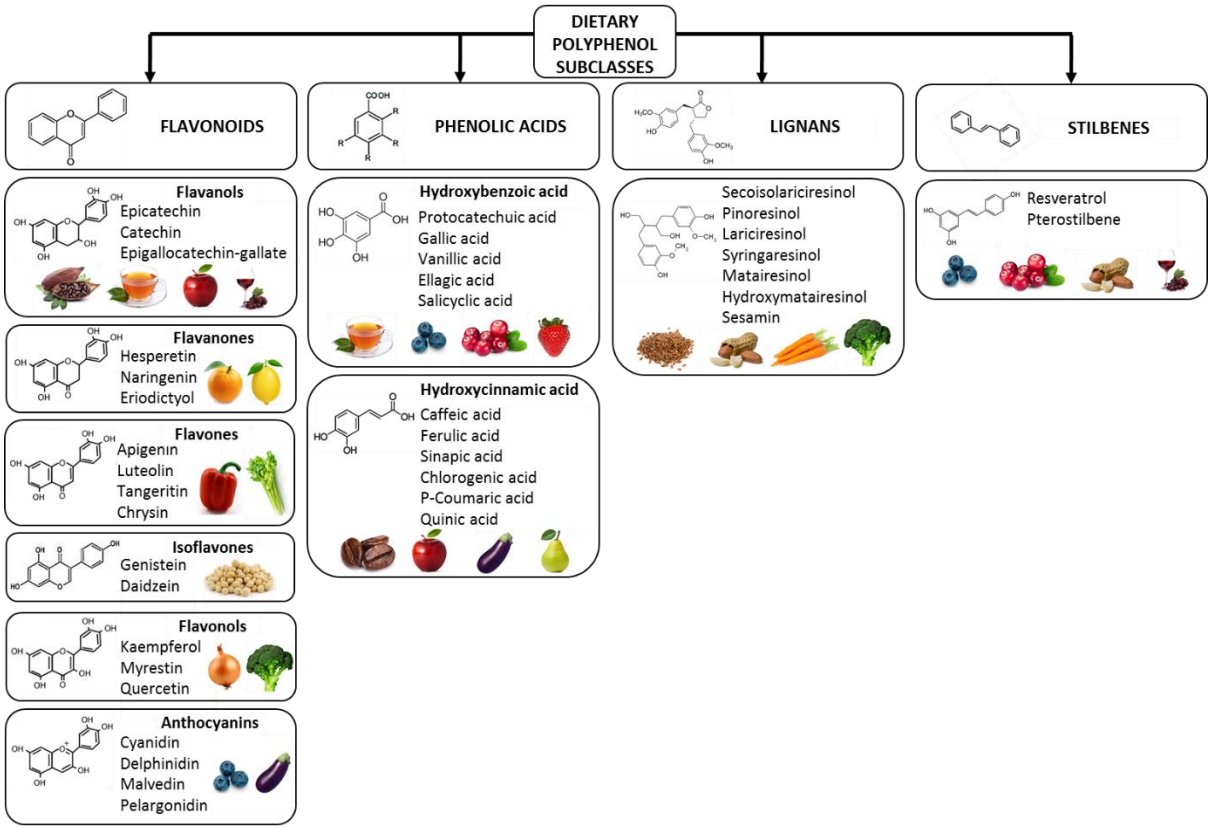

Figure 2.5 Dietary polyphenol subclasses, their basic chemical structure and typical dietary sources. Taken from (Woodward *et al.*, 2017).

2.7.1 Tea

Tea, produced from the plant *Camillia sinesis,* is the major source of dietary flavonoids in many countries globally (Yahya *et al.*, 2016) and has long been associated with medicinal benefits. Tea is classified according to the fermentation process (Figure 2.6), where flavonoids present in the tea leaf are oxidised following the release of intracellular polyphenol oxidase. The four major types of tea are white tea which is produced from very young leaves and buds (not oxidised), green tea (non-fermented), oolong tea (semi-fermented) and black tea (fully fermented) (Cabrera *et al.*, 2006; Dwyer & Peterson, 2013). The associated health benefits of tea are attributed to its richness in polyphenolic compounds called flavonoids, which are found as flavan-3-ols (catechins) in green tea and theaflavins, thearubigins and flavonols in black tea (Hodgson & Croft, 2010; Yarmolinsky *et al.*, 2015). Both green and black teas contain similar quantities of flavonoids, but differ in their chemical structure. The main

catechins present in green tea are epicatechin (EC), epigallocatechin (EGC), epicatechin-3-gallate (ECG) and epigallocatechin-3-gallate (EGCG), the most abundant of which is EGCG (~59%) followed by EGC (~19%), ECG (~14%) and EC (~6%) (Cabrera *et al.*, 2006). During fermentation of green to black tea, these catechins form dimers known as theaflavins, proanthocyandins and large oligomers and polymers called thearubigins (Dwyer & Peterson, 2013). However, the exact constituents within tea that are responsible for the reported improvements in cardiovascular health, along with their mechanisms of action, are unclear. Several biological actions of foods rich in flavonoids support the idea of a cardioprotective effect (Grassi *et al.*, 2008a; Grassi *et al.*, 2009a), with a direct impact of tea on the vasculature suggested as a cardioprotective mechanism, particularly its effects on the vascular endothelium (Grassi *et al.*, 2008a; Grassi *et al.*, 2009a; Ras *et al.*, 2011), such as potentially improving the bioactivity of NO (Grassi *et al.*, 2013a).

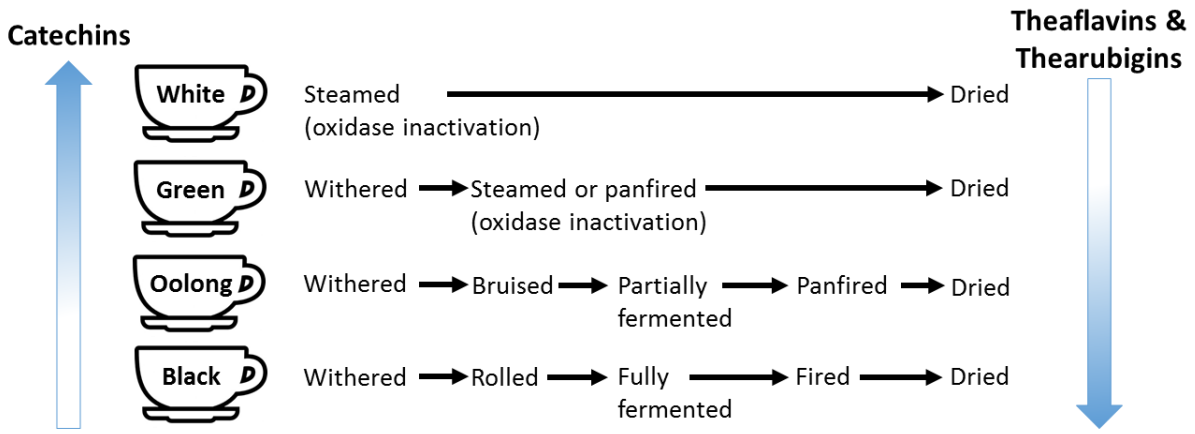

Figure 2.6 Overview of the major tea classifications and summary of the manufacturing processes.

2.7.2 Tea and Microvascular Function

Few studies have examined the impact of tea ingestion on the cutaneous vasculature. Fuchs and colleagues (2016) recently observed the ability of tea to prevent the increase in postprandial vascular resistance of forearm microvessels, assessed using near infra-red spectroscopy (NIRS), within 3-hours following 100 ml black tea in a single-blind, randomised, cross-over study in obese, insulin-resistant

males. Heinrich and colleagues (2011) performed an acute, double blind trial in 15 healthy, overweight females who were randomised into 3 groups, each of whom received a single dose of capsular green tea extract (0.5, 1.0 or 2.0 g). Skin blood flow after all three doses increased at 15-30-min with no difference between doses. A further acute pilot study by Miller and colleagues (2011) investigated the effect of capsular green tea extract (836mg green tea catechins) on laser Doppler responses to ACh and SNP iontophoresis in healthy, non-smoking, overweight individuals, but found no impact of tea. Overall, these studies on the acute effects of tea suggest that flavonoid-rich tea has the capacity to acutely increase microvascular function, although only one study used a tea drink *per se* and both the subject populations and study designs make it difficult to compare the overall findings.

In line with the acute observations, a 12-week double-blind, placebo-controlled study in 60 middle-aged, healthy females observed increased forearm cutaneous blood flow at both the midpoint and end of the 12-week intervention for green tea consumption (Heinrich *et al.*, 2011). Oxygen saturation of haemoglobin was also increased at 6- and 12-weeks following daily consumption of the green tea beverage. However, regular consumption of capsular green tea for 3-weeks was not associated with any difference in endothelium-dependent or endothelium-independent microvascular reactivity in a double-blind, placebo-controlled parallel study in healthy males (Frank *et al.*, 2009). Recently, Wasilewski and colleagues (2016) observed improved microvascular function to rapid local heating in both young (18-35 years) and older (55-75 years) individuals following 14-days consumption of a green tea beverage. However, no placebo control was used and there was poor adherence to fundamental guidelines for vascular assessment (Cracowski *et al.*, 2006; Thijssen *et al.*, 2011).

The microvascular effects of green tea have also been examined in individuals with increased CVD risk. Smoking is associated with impaired endothelial function, even when individuals are otherwise healthy (Heitzer *et al.*, 1996; Papamichael *et al.*, 2004). With this in mind, Oyama and colleagues (2010)

investigated whether daily consumption of green tea catechins for 2-weeks demonstrated any effect on forearm blood flow (FBF) responses to ACh and SNP in healthy, male smokers. Individuals were divided into three groups receiving daily green tea catechins: control (0mg), medium-dose (80mg) or high-dose (580mg). FBF was assessed acutely (2-hours), at the mid-stage (day-7) and at the end of the trial period (day-14). The acute response to ACh was increased for the high-dose group, but no difference was observed for the medium-dose or control groups. No significant acute response to SNP was observed for any group. Similar to the acute responses, chronic consumption of green tea catechins was associated with significantly increased FBF responses to ACh for the high-dose group, but not for the medium-dose or control groups and no difference was observed for any group in response to SNP. These findings suggest an endothelium-dependent vasodilatory response for high-dose green tea catechins, both acutely and following regular consumption. This improvement in endothelial function in at-risk populations is encouraging, however, additional studies are required to explore these effects further.

Despite encouraging data supporting a beneficial effect of tea ingestion on microvascular function, it is difficult to draw any meaningful conclusions from existing data owing to differences in test products, subject populations and a lack of methodological standardisation. The nature of the intervention product may be responsible for some of the observed differences in microvascular function. Studies that used a beverage seem to show improved microvascular function, whilst such effects were not found when using a capsular form of tea. This may be due to the capsular shell material hampering bioavailability, as cellulose based shells and fillers are known to have a negative interaction with polyphenols, and gelatin capsules without a cellulose filler demonstrate more positive outcomes (Draijer & Duchateau, 2015). However, precise details are scarce regarding the shell and filler materials of capsules used in the studies discussed, so it is difficult to establish whether this may be a causal factor for the apparent differences between studies and their intervention products. Studies

that have used a tea beverage are also unclear regarding the tea constituents and, therefore, the flavonoid content of the test product. For example, Wasilewski and colleagues (2016) asked participants to steep a tea bag for 3-4 minutes, which gives no precise indication of the strength or flavonoid content of the tea that has been tested. Furthermore, in the same study, participants were asked to continue with their habitual dietary habits and, therefore, no attempt was made to eliminate confounding influences from other flavonoid dietary sources, such as cocoa, fruits and vegetables.

In summary, the current evidence concerning the effect of tea consumption on microvascular function is promising but remains deficient due to a lack of robust methodology and study design. Further study of tea and its effects on the cutaneous microcirculation are warranted and should account for dietary sources confounding data, ensuring there is an adequate placebo control and that the test product itself can be quantified in terms of its constituents. Furthermore, studies should be adequately blinded and randomised, with adequate washout periods between interventions. Current methodologies do not allow for any interpretation of the potential mechanisms responsible for any effects of tea on the microvasculature. Along with the points outlined above, this should be borne in mind when designing future studies, to provide a more rigorous approach for detecting potential changes in cutaneous blood flow following tea ingestion and how any such changes might have been induced.

2.7.3 Tea and Macrovascular Function

A suggested mechanism for the CVD risk reduction associated with tea consumption relates to a direct effect of tea on the vasculature, particularly the vascular endothelium (Vita, 2003). In healthy males, Grassi and colleagues (2009) observed a dose-dependent increase in endothelium-dependent FMD following twice daily consumption of black tea for 1-week compared to placebo control, in a randomised, double-blind, crossover study. Both systolic and diastolic blood pressure were also reduced with the consumption of tea versus control. Furthermore, endothelial-dependent dilation acutely (90-min) increased following 300 ml black tea in healthy males and females, although no effect

was observed following regular black tea consumption (3-cups daily) for 7-days, versus abstinence from tea (Schreuder *et al.*, 2014). Black tea and green tea demonstrated comparable acute increases in FMD versus water 2-hours after ingestion in healthy females, although no significant difference was observed for endothelial-independent vasodilation (Jochmann *et al.*, 2008).

Tea has also demonstrated positive findings in individuals with increased CVD risk. Green tea was associated with a significant increase in FMD ~90-min following ingestion, compared to water placebo, in heathy males and females, 50% of whom were smokers (Alexopoulos *et al.*, 2008). Similarly, 2-weeks green tea consumption significantly improved FMD in young, otherwise healthy, chronic smokers (Kim *et al.*, 2006). Duffy and colleagues (2001) observed increased endothelium-dependent vasodilation in individuals with coronary artery disease, following both acute (2-hours) and chronic (4-weeks) black tea ingestion versus water, although no difference was detected for endothelium-independent dilation following nitroglycerin. Similarly, a parallel, single-blind study in individuals with elevated cholesterol demonstrated endothelial-dependent dilation following 5-cups of black tea daily for 4-weeks compared to hot water control (Hodgson *et al.*, 2002). However, in contrast to Duffy and colleagues (2001), endothelial-independent dilation was also improved in this cohort. Green tea consumption for 4-weeks was associated with a significant improvement in FMD in a cohort with chronic kidney disease, compared to water control (Park *et al.*, 2010). Overall, these studies are suggestive of a beneficial impact of tea ingestion on macrovascular function in at-risk populations.

Several studies have examined the effect of tea on endothelial-dependent vasodilation in response to an acute oral fat load. In a randomised, double blind, placebo-controlled crossover study of healthy males and females (20-55 years), Corretti and colleagues (2002) examined the FMD response to black tea, green tea and placebo 3-hours following ingestion, together with a 900 kcal high fat meal. Both black tea and green tea blunted the decrease in FMD that was observed following the high fat meal with placebo tea. Further studies in pathological subject groups have demonstrated similar findings.

Hodgson and colleagues (2005) observed increased FMD 4-hours following consumption of 3-cups of black tea with a high fat meal in individuals with coronary artery disease, although no effect of tea was identified when taken without the meal challenge. Black tea has also counteracted fat load FMD impairments in a randomised, double-blind study in hypertensive individuals where tea was consumed regularly for 8-days (Grassi *et al.*, 2012b). Compared to placebo, black tea increased FMD at day 8 compared to baseline, and also demonstrated an acute-on-chronic improvement in FMD 2-hours after ingestion, indicating a vasoprotective effect of tea on macrovascular function.

Current evidence suggests that macrovascular function is generally improved following the consumption of both black and green tea. Tea has induced increased brachial artery FMD in both healthy individuals and in at-risk populations. Furthermore, these encouraging findings have been observed following acute and chronic tea consumption. Two meta-analyses suggest a beneficial effect of tea on FMD (Hooper *et al.*, 2008; Ras *et al.*, 2011), although several studies have been performed since they were published. Despite the encouraging data supporting an endothelial-dependent effect of tea, it is difficult to quantify precise doses of tea, since many studies (Duffy *et al.*, 2001; Hodgson *et al.*, 2002; Hodgson *et al.*, 2005; Jochmann *et al.*, 2008; Schreuder *et al.*, 2014) have used brewed tea leaves and others do not report the tea preparation, which does not allow an accurate indication of flavonoid/catechin content for between study comparisons. As fat rich meals have demonstrated negative effects on vascular function and a reduction in FMD (Rudolph *et al.*, 2007; Gosmanov *et al.*, 2010), the studies observing a counteracting effect of tea to an acute oral fat load suggest that it may exert a vasoprotective effect that may have implications for generalised cardiovascular health. Further investigation is warranted, however, to determine whether tea demonstrates such an effect following a longer term metabolic challenge.

2.7.4 Tea and Cerebrovascular Function

Flavonoids are suggested to help maintain and even improve cognitive function, possibly via improved vascular function (Mastroiacovo *et al.*, 2015). To date, limited research has been undertaken exploring

the acute and chronic effects of flavonoids on cerebrovascular function. Despite tea being a major source of dietary flavonoids for much of the global population, only one study has examined the effect (acute) of tea on CBF. In a randomised, double-blind, four arm crossover study, Vidyasagar and colleagues (2013) investigated the effect of caffeinated black tea (capsules equivalent to 6 cups), decaffeinated black tea, caffeine and placebo on cerebrovascular reactivity to hypercapnia in healthy male adults. Arterial spin labelling BOLD-MRI was used to quantify changes in CBF at baseline and 2-hours after administration of the test product. A significant global reduction in steady-state CBF was observed for both caffeinated tea and caffeine alone, suggesting that caffeine was responsible for the decreased CBF, as no effect was demonstrated for decaffeinated tea. Cerebral CO_2 reactivity was also unaffected by tea, caffeine and decaffeinated tea in the same study (Vidyasagar *et al.*, 2013).

Further human studies have been performed examining the CBF effects of isolated compounds found in polyphenol-rich foods, such as EGCG and EC that are abundant in tea (Dower *et al.*, 2015; Murray *et al.*, 2015). A single low dose (135 mg) of capsular EGCG decreased CBF (measured by NIRS) during computerised cognitive task performance in healthy young adults, whereas a higher dose (270 mg) did not change CBF relative to placebo (Wightman *et al.*, 2012). Several studies investigating cocoa flavanols are potentially relevant to tea, due to EC being present in both cocoa and tea. An acute dose of cocoa flavanols (494 mg; containing 89 mg of EC), consumed as a beverage, increased regional CBF (arterial spin labelling MRI) 2-hours post-ingestion, with increases observed in the anterior cingulate cortex (left parietal lobe) in healthy adults aged 55-65 years (Lamport *et al.*, 2015). Similarly, an acute dose (450 mg) of flavanols increased grey matter CBF (fMRI) 2-hours post-ingestion in a pilot study of healthy young females (n=4) (Francis *et al.*, 2006). In contrast, an acute dose (450 mg) of cocoa flavanols actually decreased CBF (measured by TCD) 2-4 hours post-consumption, likely related to the cocoa's caffeine content (Lunt *et al.*, 2004), with a return to baseline within 4-6 hours (Sorond *et al.*, 2008a). Regular (1-2 weeks) ingestion of cocoa-derived flavonoids (900 mg flavanols per day)

increased CBF through the MCA (via TCD) in elderly (65+ years) individuals (Sorond *et al.*, 2008a). Similarly, daily consumption of a high flavanol (172 mg flavanols/day) cocoa beverage versus a low flavanol (13 mg flavanols/day) beverage for 5-days improved regional CBF during cognitive tasks in healthy young females, when measured via fMRI (Francis *et al.*, 2006). Furthermore, 12-weeks of daily cocoa flavanols (900 mg containing 138 mg (−)-EC) increased CBF (fMRI) in the dentate gyrus region in the healthy elderly, when compared to a low flavanol (45 mg per day) control (Brickman *et al.*, 2014).

Current research is limited regarding the effects of tea, and tea-derived compounds, on cerebrovascular function. The single study that investigated the effect of tea (Vidyasagar *et al.*, 2013) used a capsular test product, so the effect of a tea beverage remains unknown. The few previous studies of cocoa flavanols and isolated compounds have largely investigated the acute effects and, furthermore, across all studies, it is difficult to determine what impact habitual dietary flavonoid consumption may have had on these data. Given the varying doses of flavanols, it is also difficult to make accurate comparisons of study findings to elicit meaningful conclusions. Furthermore, current studies have only assessed steady-state CBF, rather than assess the effect of flavonoids on dynamic changes in CBF (e.g., cerebral autoregulation; a critical modulator of cerebral perfusion), which continuously occur throughout a day, or the effect of flavonoids on cerebral CO_2 reactivity. Therefore, further studies are required to determine the cerebrovascular effects of tea consumption.

2.7.5 Tea and Metabolic Function

Several *in vitro* and *in vivo* studies have observed a reduction in fasting glucose and insulin in diabetic rats following green tea catechins (Quine & Raghu, 2005; Yamabe *et al.*, 2006; Roghani & Baluchnejadmojarad, 2010; Samarghandian *et al.*, 2017), although human trials investigating the effects of green tea and isolated green tea catechins have been inconsistent. Fukino and colleagues (2005) observed no clear effects of a daily green tea beverage on glucose handling or insulin resistance in individuals with borderline diabetes following 2-months consumption. Furthermore, blood glucose

and insulin levels were unchanged in type 2 diabetic adults in a 4-week crossover trial of a daily green tea beverage versus water control (Ryu *et al.*, 2006). Conversely, studies have observed reductions in haemoglobin A_{1c} (HbA_{1c}) in individuals with borderline (Fukino *et al.*, 2008) and type 2 (Nagao *et al.*, 2009) diabetes, following 2- and 3-months consumption of a green tea beverage, respectively. Furthermore, 1500 mg green tea extract daily for 16-weeks in a double-blind, randomised, placebo-controlled trial in type 2 diabetic patients demonstrated a decrease in insulin resistance (Liu *et al.*, 2014) and reductions in HbA_{1c} and insulin (Hsu *et al.*, 2011) compared to placebo.

Several studies have examined the effect of tea in overweight or obese individuals, which are important populations to study given that obesity is one of the most important environmental risk factors for cardiometabolic disease. Chan and colleagues (2006) observed no significant effect on glucose metabolism following 3-months of capsular green tea versus placebo in a randomised, parallel study in obese females (25-40 yrs) with polycystic ovary syndrome. Furthermore, an 800 mg daily dose of EGCG for 8-weeks had no effect on glucose tolerance or insulin sensitivity in middle-aged (40-65 yrs) overweight/obese males (Brown *et al.*, 2009) and no effect was observed in sedentary males following 6-weeks of 800mg green tea catechins in a randomised, crossover trial (Brown *et al.*, 2011). However, 300 mg daily EGCG supplementation combined with regular aerobic exercise in middle-aged (45-70 yrs) overweight/obese post-menopausal women for 12-weeks significantly reduced plasma glucose concentrations compared to placebo (Hill *et al.*, 2007). Additionally, in a 6-month randomised, double-blind, placebo-controlled pilot study, consumption of a decaffeinated green tea beverage (960 ml daily) in overweight, female breast cancer (stages I-III) survivors demonstrated a decrease in insulin (Stendell-Hollis *et al.*, 2010). Further parallel design studies observed a non-significant reduction in glucose levels following green tea extract (Suliburska *et al.*, 2012) and significant reductions in fasting serum glucose and insulin resistance (Bogdanski *et al.*, 2012) following 3-months of green tea extract

(208 mg EGCG) versus placebo in obese and hypertensive obese, heterogeneous middle-aged adults, respectively.

Few studies have examined the metabolic effects of tea in healthy individuals. Sone and colleagues (2011) observed no significant difference in fasting plasma glucose in a 9-week randomised controlled trial following green tea consumption (400 mg catechins/day), compared to a low-catechin (100 mg/day) control, in heathy males and females aged 20-70 years. However, in postmenopausal females, reductions in glucose and insulin were reported following ingestion of capsular green tea catechins for 2-months compared to placebo, with no difference reported between the 400 mg and 800 mg catechin doses (Wu *et al.*, 2012). Furthermore, black tea demonstrated a reduction in postprandial glucose 2-hours following both low- and high-dose black tea polyphenols in normoglyaemic and prediabetic individuals in a randomised, double-blind, placebo-controlled crossover study, although no significant differences were observed for insulin between both tea doses and placebo (Butacnum *et al.*, 2017). An earlier acute study, however, observed no reduction in glucose or insulin following ingestion of 300 ml green tea compared to water, in a crossover trial of young (22-35 yrs), healthy adults (Josic *et al.*, 2010). Furthermore, in a 5-day controlled diet study comparing the effects of oolong tea, catechin-enriched oolong tea, polyphenol-enriched oolong tea, caffeine-enriched water and unsupplemented water, no significant differences were observed for fasting glucose or insulin measures in healthy adult males (Baer *et al.*, 2011).

Overall, findings from studies investigating the effects of tea on glucose metabolism and insulin sensitivity are equivocal. Differences between trials make it difficult to determine any tea-derived effects, largely due to the study design, variations in both the type and dose of tea product used and confounding influences from habitual dietary intake. Current evidence suggests that green tea consumption is associated with a reduction in fasting glucose in individuals who are at a greater risk

of CVD and metabolic syndrome e.g. overweight/obese, and that such effects are largely observed in studies where there is a greater consumption of catechins. However, few studies have standardised habitual dietary intake and limited consumption of polyphenol-rich foods, making it difficult to determine whether these may have influenced study outcomes. There is a clear lack of data in healthy individuals and further studies are required to examine the effects of tea ingestion on glucose handling and insulin sensitivity, ensuring that habitual dietary intake is monitored to reduce confounding influences and ensure that it is the tea-derived effects that are being assessed.

2.8 Summary

In summary, the incidence of CVD continues to increase globally, largely due to the rise in habitual overfeeding and physical inactivity, and therapeutic strategies need to be identified to help combat the economic burden of CVD. Tea consumption demonstrates encouraging benefits for cardiovascular health, although there is insufficient evidence for its effects on microvascular function and little is known about its effects on the cerebrovasculature. Current research suggests that tea may exert a vasoprotective effect and has counteracted the negative macrovascular effects of an acute oral fat load, although the cardiometabolic effects of tea following a longer-term metabolic challenge are unknown.

CHAPTER 3: GENERAL METHODS

The majority of the measurements and protocols undertaken in this thesis were utilised throughout *Chapters 4-6*. Therefore, the general information for the techniques used for the physiological measurements are described in this methods chapter, including the reliability and limitations of these techniques. The specific protocols and participant cohort used for each study are detailed within the respective methods section for each chapter.

3.1 Participants

The population groups differed between the individual studies regarding gender and age (please refer to *Chapters 4-6*). However, across all studies, all participants were healthy and non-smokers. Participants were recruited through local advertisement. Individuals with a medical history of hypercholesterolaemia (total cholesterol >6.5 mmol/l) (Reiner *et al.*, 2011), cardiovascular disease and/or hypertension (systolic blood pressure ≥140 mmHg, diastolic blood pressure ≥90 mmHg) (NICE, 2011; Yarmolinsky *et al.*, 2015) were excluded. Individuals with food allergies, special dietary requirements, currently following a diet and/or those with dietary/vitamin supplements were excluded. Participants were not taking any vasoactive medications or supplements. All participants fasted for at least 6-hours and refrained from alcohol, food products high in polyphenols (dark chocolate, red wine), caffeine and exercise for 24-hours prior to testing (Thijssen *et al.*, 2011). Participants were asked to refrain from drinking all types of tea for a period of one week prior to each laboratory visit, in addition to avoiding exceptional/irregular physical activity (e.g. marathon) in the preceding week. Sips of water (500 ml) were permitted prior to testing to ensure that participants were euhydrated. After being fully informed of the methods verbally and in writing, written informed consent was obtained from all participants. All studies conformed to the *Declaration of Helsinki* and were approved by the local research ethics committee.

3.2 Anthropometric Measurements

Stature was measured in the freestanding position to the nearest 0.1 cm (seca stadiometer, model 217, Birmingham, UK). Body mass was measured to the nearest 0.05 kg using calibrated electronic digital scales (seca, model 767, Germany). Using these variables, body mass index (BMI; mass (kg) / height $(m)^2$) was calculated. Resting blood pressure (mmHg) and resting heart rate (beats/min) were determined from an average of three measures using an automated blood pressure monitor (Dinamap, G & E Medical, Tampa, Florida).

3.3 Peripheral Vascular Function

All vascular function assessments were conducted in a quiet, temperature-controlled laboratory (22-24°C) (Cracowski *et al.*, 2006; Thijssen *et al.*, 2011) and at the same time of day to reduce any circadian influences on vascular function (Jones *et al.*, 2010; Thijssen *et al.*, 2011). Upon arrival in the laboratory, participants rested in a supine position for ~20-minutes to ensure accurate assessment of stable baseline mean arterial blood pressure (MAP) and heart rate (HR). MAP was subsequently determined (MAP = [(2 x diastolic) + systolic] / 3) from an average of three measures taken from the non-dominant arm.

3.3.1 Microvascular Function

An index of forearm local cutaneous blood flow was obtained using the non-invasive techniques of laser Doppler flowmetry (LDF: Periflux System 5001, Perimed AB, Sweden) and full-field laser perfusion imaging (FLPI: Moor Instruments, UK). Participants assumed a comfortable, supine position on a bed throughout testing, with the head slightly elevated and the hand of each testing arm relaxed, supinated and supported by a vacuum cushion to minimise microcirculatory fluctuations resulting from motion artefact (Cracowski *et al.*, 2006; Thijssen *et al.*, 2011). If necessary, forearm measurement sites were shaved 24-hours prior to testing to avoid any inflammatory response that may affect cutaneous blood flow; the forearms were inspected prior to each trial to ensure that no skin damage

was present that may adversely influence cutaneous blood flow responses. Measurement sites were randomly chosen, avoiding visible veins, hair follicles and dermatological lesions (Cracowski *et al.*, 2006). Upon completion of the first experimental trial, the location of the LDF and FLPI assessment sites were marked on the skin, with digital photographs and measurements taken to the nearest millimetre using anatomical and skin-surface landmarks for reference, to ensure accurate re-selection of the probe placement site for the repeat trial(s).

3.3.1.1 Laser Doppler Flowmetry (LDF)

Laser Doppler flowmetry is a non-invasive technique that is routinely used to study microvascular function (Minson *et al.*, 2001; Cracowski *et al.*, 2006; Cracowski & Roustit, 2015), and is sensitive in detecting changes in skin perfusion over a period of time and in response to a physiological challenge or stimulus, such as local thermal hyperaemia (Cracowski *et al.*, 2006). LDF is concerned with the reflection of a laser beam that undergoes a change in wavelength, or Doppler shift, when it detects moving red blood cells (Figure 3.1), the magnitude and frequency of which is related to the concentration and velocity of blood cells, respectively, and is recorded as a signal of red blood cell flux (RBCF) (Cracowski *et al.*, 2006; Cracowski & Roustit, 2015). The measurement depth of LDF is 0.5-1.0mm in individuals with normal skin morphology and measurements are continuous, providing high spatial and temporal resolution beyond that of venous occlusion plethysmography (Charkoudian & Stachenfeld, 2014).

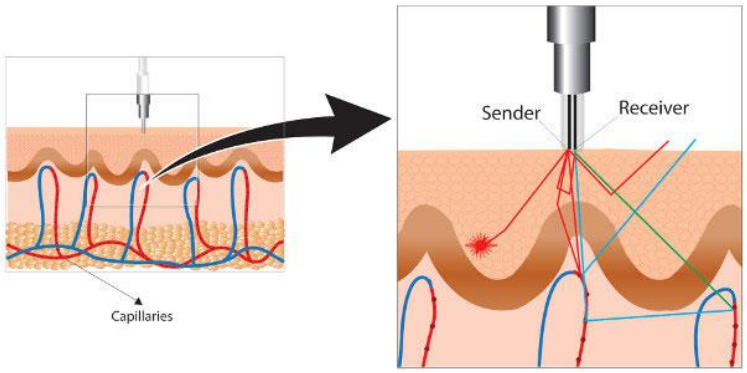

Figure 3.1 Laser Doppler assessment of skin blood flow (SkBF) where a beam of laser light is emitted and undergoes a change in wavelength when it detects moving red blood cells.

Following a 20-minute stabilisation period, the laser Doppler equipment was calibrated using two generic points, 0 and 250 PU with a zeroing disk and motility standard, respectively, according to manufacturer's guidelines (Perimed AB, Järfälla, Stockholm, Sweden). Following sterilisation of the forearm, heating discs (Perimed 355, Perimed AB, Järfälla, Stockholm, Sweden) were placed ~5cm apart on the dominant forearm, into which 7-laser array probes (PF 413, Perimed AB, Järfälla, Stockholm, Sweden) were placed and firmly attached to the skin using adhesive stickers and medical tape. Following a 20-minute acclimation period, cutaneous blood flow was measured as RBCF at the chosen probe sites using a laser Doppler flowmeter (Periflux system 5000, Perimed AB, Järfälla, Stockholm, Sweden). The local heating discs were connected to a heating unit (Peritemp 4005 heater, Perimed AB, Järfälla, Stockholm, Sweden) which was manually controlled to perform the temperature stages of the local heating protocols. Laser Doppler flux was continuously recorded online at 250 Hz (LabChart 8.0, AD Instruments, Dunedin, New Zealand).

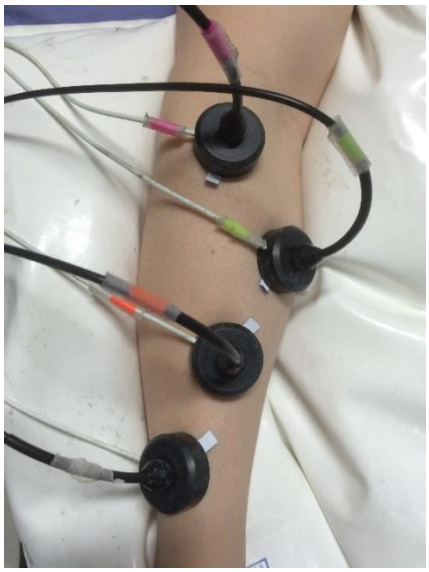

Figure 3.2 Forearm of a subject with LDF probes in situ.

3.3.1.2 Local Heating Protocols

Rather than assessing systemic microvascular function, using local thermal hyperaemia provides a more comprehensive assessment of microvascular reactivity and the complex neural and chemically-mediated pathways underlying local microvascular function. There are a range of distinct rapid and gradual local heating protocols available for the assessment of cutaneous vascular function that all provide different patterns of vasodilation that likely relate to different vasodilator pathways. Baseline skin RBCF was recorded with the local heating disc temperature set at 33°C for 10-minutes for each measurement site. Subsequently, local skin temperature was increased according to four distinct heating protocols (Figure 3.3: please refer to *Chapters 4 to 6* for the specific protocols used). Regardless of the protocol, local skin temperature is ultimately increased to 44°C; a temperature considered to elicit maximal cutaneous vasodilation (Kellogg *et al.*, 1999; Charkoudian, 2003; McCord & Minson, 2005; Minson, 2010).

Rapid 39°C (Choi *et al.*, 2014). This recently introduced protocol (0.5°C per 5 s to 39°C, 30-min at 39°C, 20-min at 44°C) induces a rapid, transient axon-reflex followed by a gradual plateau. By holding the local temperature at 39°C, the plateau phase is largely NO-mediated and causes dilation that is equivalent to approximately 50% of the maximal response (Choi *et al.*, 2014).

Rapid 42°C (Minson *et al.*, 2001). This classic local heating protocol (0.5°C per 5 s to 42°C, 30-min at 42°C, 20-min at 44°C) induces a rapid, transient axon-reflex which is followed by a more gradual, sustained vasodilatory response. The plateau phase represents 80-90% of the maximal response, and is mostly (60-70%) NO-mediated (Kellogg *et al.*, 1999; Minson *et al.*, 2001).

Gradual 42°C (Black *et al.*, 2008b). This adapted, shortened version of the *Slow 42°C* local heating protocol increases local skin temperature to 42°C (0.5°C per 2-min 30 s to 42°C, 30-min at 42°C, 20-min at 44°C) and induces a slow heating response without axon reflexes that is largely NO-mediated (Black *et al.*, 2008b).

Slow 42°C (Black *et al.*, 2008b). This longer version of the former heating protocol induces a gradual, slow heating response without axon reflexes (0.5°C per 5-min to 42°C, 30-min at 42°C, 20-min at 44°C) that is largely NO-mediated (Black *et al.*, 2008b).

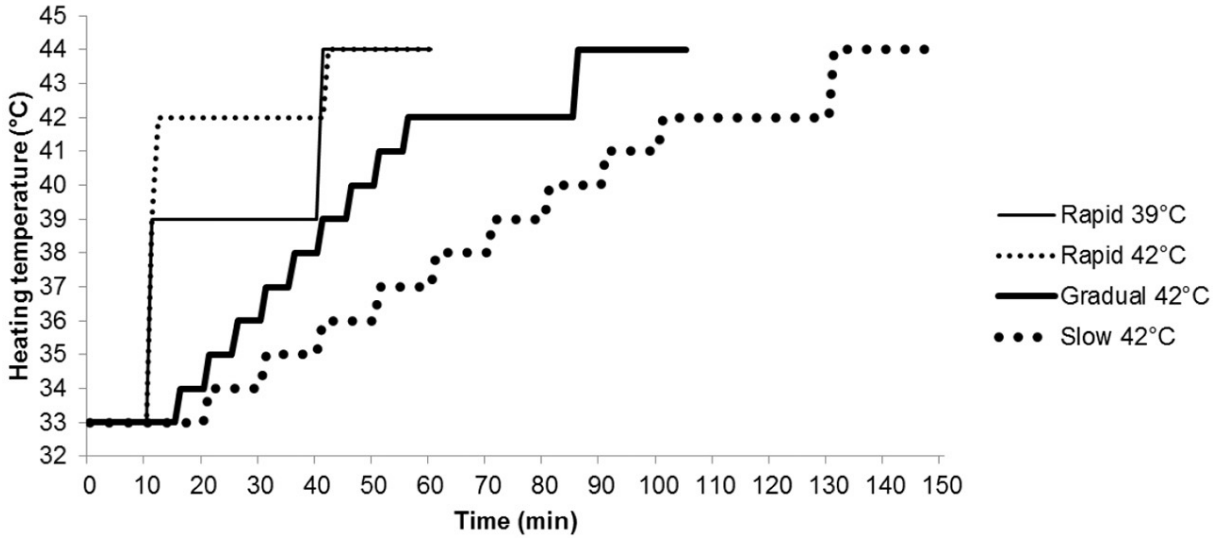

Figure 3.3 Step-wise temperature increments with corresponding times for all four local heating protocols. Taken from (Roberts *et al.*, 2017).

3.3.1.3 Haemodynamics

Heart rate and BP were recorded at the beginning and at the end of the 20-minute acclimation period using an automated sphygmomanometer (Dinamap V100, GE Healthcare, Buckinghamshire, UK; please refer to *Chapters 4-6* for the position). Thereafter, mean arterial pressure (MAP, mmHg) and HR were recorded at 5-minute intervals throughout the local heating protocols. MAP was used to calculate cutaneous vascular conductance.

3.3.1.4 Data Analysis

Data analysis was performed blind. Artefact in the data, due to unwanted subject movement, was identified and removed prior to analysis. Baseline laser Doppler RBCF was averaged over a stable 10-minute baseline period. For the *Rapid 42°C* and *Rapid 39°C* protocols, following initiation of heating, initial peak and nadir values were calculated over a stable 60-second period (Minson *et al.*, 2001), with

the initial peak identified as the highest value and the nadir as the lowest value during the first 5-10 minutes of local heating (van Duijnhoven *et al.*, 2009). A clear nadir was not detected in all measurement traces, which is typical of this type of thermal provocation test. In those traces (~5%), data was included from a 60-second period, 1-minute after the initial peak. This value was always lower than the initial peak. RBCF was calculated over a stable 60-second period for the final minute of each temperature increment (34-41°C) of the *Gradual 42°C* and *Slow 42°C* local heating protocols. For each of the four protocols, *Rapid 42°C*, *Rapid 39°C*, *Gradual 42°C* and *Slow 42°C*, plateau phases during heating (42°C, 39°C and maximal 44°C) were averaged over the last 5-minutes of each phase (Figure 3.4). Data at baseline, at the various temperature increments and at the plateau phases were also expressed as cutaneous vascular conductance (CVC) and also normalised to the maximal CVC achieved at 44°C.

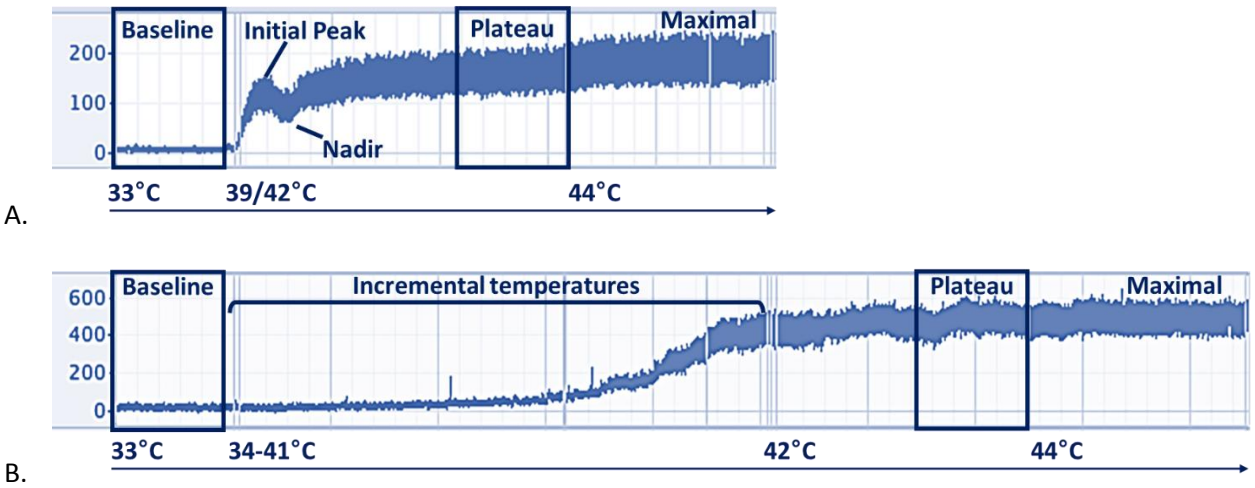

Figure 3.4 Exemplar skin blood flow traces for **A.** rapid and **B.** gradual local heating.

3.3.1.4.1 Cutaneous Vascular Conductance

Cutaneous vascular conductance (CVC) is important in studies of skin blood flow as it accounts for changes in skin blood flow resulting from variations in blood pressure (Minson *et al.*, 2001; Cracowski *et al.*, 2006; Dawson *et al.*, 2015). Calculation of CVC enables comparison of values across

measurement sites for a given individual and/or between participants/trials (Kellogg *et al.*, 1993). CVC was calculated as:

$$CVC = RBCF\ (PU) / MAP\ (mmHg)$$

where CVC (PU/mmHg) is cutaneous vascular conductance, RBCF (measured in perfusion units, PU) represents red blood cell flux and MAP is mean arterial pressure recorded in 5-minute intervals.

3.3.1.4.2 Local Maximal Microvascular Function

CVC values were also normalised to the maximal CVC (%CVC_{max}) achieved during heating of the skin to 44°C which, in addition to CVC, is considered the preferred method of data expression in previous microvascular literature (Minson *et al.*, 2002; Cracowski *et al.*, 2006; Black *et al.*, 2008b; Minson, 2010). Normalised maximal cutaneous vascular conductance (%CVC_{max}) was calculated as:

$$\%CVC_{max} = (CVC / CVC_{max}) * 100$$

where %CVC_{max} is cutaneous vascular conductance relative to maximal cutaneous vascular conductance, CVC is cutaneous vascular conductance and CVC_{max} is maximal cutaneous vascular conductance achieved during skin heating at 44°C.

3.3.2 Macrovascular Function – Flow-Mediated Dilation

Conduit artery endothelium-dependent function was measured using the FMD technique, which provides an assessment of peripheral conduit artery diameter following a brief period of distal limb ischaemia (Thijssen *et al.*, 2011). Shear stress is the key physiological stimulus evoking endothelium-mediated vasodilation during the FMD response (Melkumyants *et al.*, 1989; Thijssen *et al.*, 2011) and is associated with dose-dependent increases in artery diameter (Betik *et al.*, 2004; Padilla *et al.*, 2009). Increased shear stress is detected by cell membrane mechanoreceptors, following which a signalling cascade is activated and subsequently stimulates the production and release of vasoactive substances that diffuse across the endothelial cell membrane into the smooth muscle cell (Thijssen *et al.*, 2011). Signal transduction in the smooth muscle cell causes a reduction in calcium concentration and

subsequently, vasorelaxation. Biological variability in between-subject FMD responses largely arises from differences in the transduction of the vasodilatory responses to the smooth muscle cells, in addition to the structural characteristics of the vessel wall, such as the wall-to-lumen ratio, potentially influencing the resultant diameter change (Thijssen *et al.*, 2011). FMD assessment, therefore, provides a means assessing endothelial function via interrogation of such biological differences and allows the macrovascular impact of lifestyle factors, disease status, exercise training status and various interventions to be examined.

Simultaneous assessment of the brachial artery, at the distal third of the non-dominant upper arm, and the femoral artery of the right leg was performed (Figure 3.5). The non-dominant arm was extended and positioned at an angle of ~80° from the torso, whilst the right leg was extended in a comfortable position. Rapid inflation and deflation pneumatic cuffs (D.E. Hokanson, Bellevue, WA, USA), connected to a rapid inflator (D.E. Hokanson, Bellevue, WA, USA), were positioned on the forearm, immediately distal to the olecranon process, and around the right thigh, proximal to the patella, to provide the stimulus for ischaemia (Thijssen *et al.*, 2011). With a stable image obtained, the ultrasound parameters were set to optimise the longitudinal B-mode image of the lumen-arterial wall interface. Continuous Doppler velocity assessment was collected using the lowest insonation angle (<60°), which was standardised across all measures. Baseline images for the assessment of resting vessel diameter, shear rate and flow were recorded for 1-minute, following which the occlusion cuffs were inflated (>220 mmHg) for 5-minutes to completely block the arterial inflow. Diameter and velocity recordings resumed 30-seconds prior to cuff deflation and continued for 3-minutes thereafter, according to methodological guidelines (Woodman *et al.*, 2001; Thijssen *et al.*, 2011; Greyling *et al.*, 2016). Peak brachial artery diameter, peak blood flow velocity and the time taken to reach these peaks post cuff release were recorded. Ultrasound images were recorded using specialised recording software (Camtasia Studio, Techsmith, USA). The reliability of FMD is largely

dependent upon the adherence to current methodological guidelines (Thijssen *et al.*, 2011), which were followed closely for all assessments performed during the current research programme.

Figure 3.5 Simultaneous assessment of brachial and femoral flow-mediated dilation (FMD).

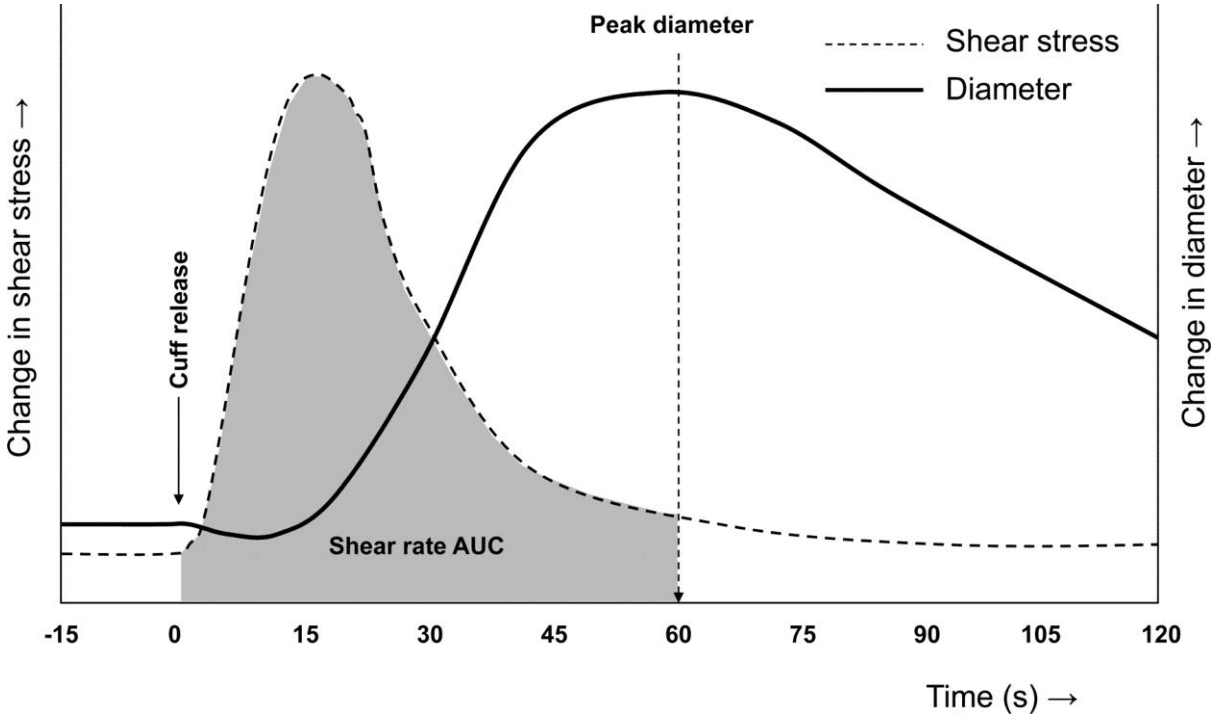

Figure 3.6 Schematic representation of diameter and shear-stress responses following cuff deflation, in response to a 5-minute ischaemic stimulus during FMD assessment. The grey area represents the shear rate area-under-the-curve (SRAUC), which is considered to be the main stimulus for peak diameter. Taken from (Thijssen *et al.*, 2011).

3.3.2.1 Artery diameter and blood flow analysis

Post-test analysis of brachial and femoral artery diameter and velocity was performed using custom-designed edge-detection and wall-tracking software (Dicom Encoder, V.3.0.5 LabVIEW V.7.0, National Instruments Corporation), which is largely independent of investigator bias and provides continuous measurements of arterial diameter and blood flow velocity. The software is written in icon-based graphical programming language (LabVIEW V.7.0, National Instruments) and uses an IMAQ vision toolkit for image handling. Arterial diameter on the B-mode image and velocity on the Doppler strip were calibrated for each individual assessment. An optimal region of interest (ROI) was identified for analysis on the B-mode image, chosen according to the image quality depicting a clear distinction between the vessel walls and lumen (Figure 3.7). Within the selected ROI, a pixel-density algorithm measures the mean diameter changes according to changes in pixel density from the far- and near-wall lumen-intima interface via a Rake routine, which measures 30 points per second throughout the analysis. The edge-detection algorithm assessed the peak velocity envelope from the Doppler gate which was placed in the centre of the artery. Mean diameter derived from the B-mode ROI was subsequently synchronised with the velocity derived from the Doppler ROI at 30 Hz.

From the synchronised diameter and velocity data, blood flow (the product of cross-sectional area and Doppler velocity) and shear rate (four times the velocity divided by the diameter) were calculated at 30 Hz (Black *et al.*, 2008a). Peak blood flow was taken over the initial 10-second post cuff deflation, with peak artery FMD defined as the percentage change of the post cuff deflation peak diameter from baseline diameter. Total shear rate (SRAUC) was calculated, which is considered to be the main stimulus of the FMD response, rather than peak shear rate. All data were written to file and retrieved for analysis in the custom-designed analysis software package. The edge-detection and wall-tracking software is semi-automated and provides significantly better reproducibility of diameter

measurements (coefficient of variation = 6.7%) compared to manual methods, whilst simultaneously reducing observer error (Woodman *et al.*, 2001).

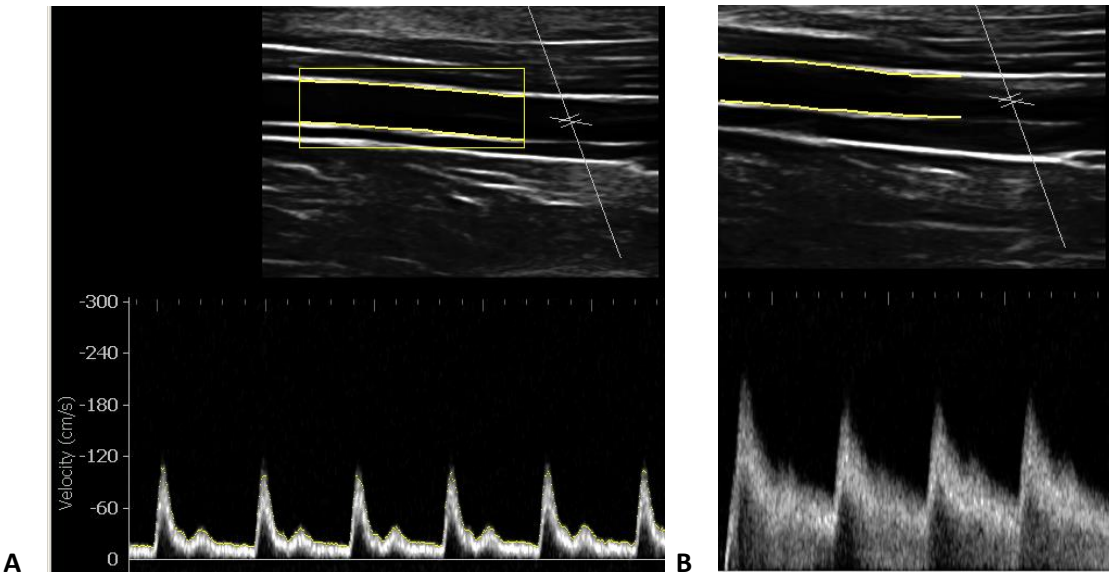

Figure 3.7 Ultrasound image of the brachial artery and blood flow velocity trace during **A.** baseline, and **B.** post cuff deflation during flow-mediated dilation (FMD).

3.4 Haemodynamic Function

3.4.1 Beat-to-Beat Arterial Blood Pressure

Continuous beat-to-beat arterial BP was measured by finger photoplethysmography (Finometer Pro, Finapres Medical Systems, Biomedical Instruments, Amsterdam: The Netherlands). Developed by Wesseling and colleagues (1995), the Finometer uses the voltage-clamp method (Penaz, 1973) to track variations in intra-arterial pressure through clamping the diameter of the digital artery in a constant "unloaded" condition (Imholz *et al.*, 1992; Imholz *et al.*, 1998; Bogert & van Lieshout, 2005). The Finometer apparatus comprises of three main components; a main unit containing an air pump and electronics, a finger cuff with an in-built inflatable air bladder and infrared plethysmograph containing the light detector and light source, and a fronted unit with a fast-acting servo-controller system (Figure 3.8A).

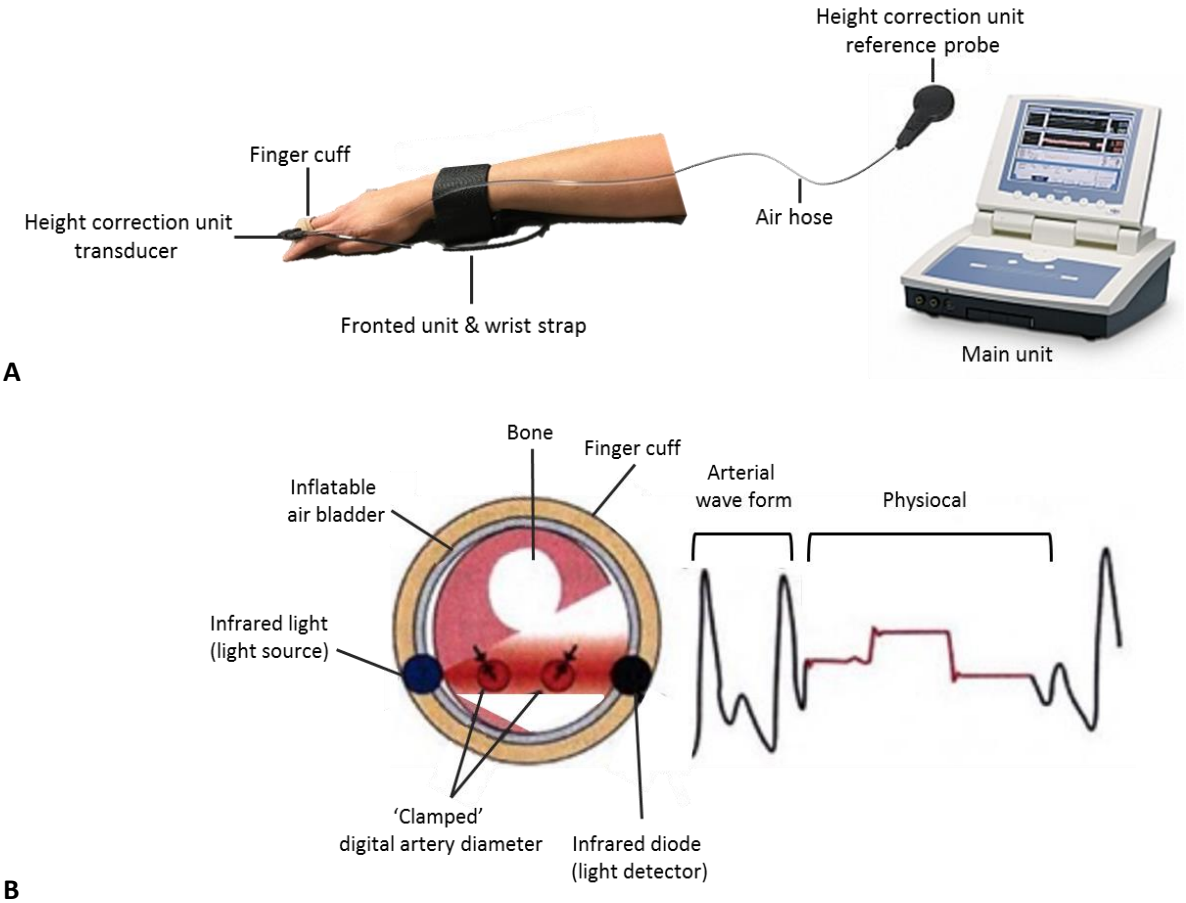

Figure 3.8 A. Finometer apparatus consisting of a finger cuff, fronted unit containing the servo-controller and a main unit housing the air pump and electronics; and **B.** a cross-sectional view of the finger cuff and waveform.

Finger photoplethysmography is based upon dynamic pulsatile unloading of the digital arterial walls, whereby an appropriately sized finger cuff ensures that the arterial diameter is kept constant/clamped at a specific diameter (set point), regardless of any changes to arterial pressure, during each heartbeat. An inbuilt infrared photo-plethysmograph detects changes in diameter and the servo-controller system detects the difference between the light detector signal and set point (Figure 3.8B), subsequently dispatching a signal to the unit microcontroller. Should arterial diameter increase during systole, the diameter change is prevented in the digital artery through immediate inflation of the finger cuff via increased air delivery to the inflatable bladder, thereby ensuring that the transmural pressure of the artery remains "unloaded" (maintained at zero), at which point the finger cuff pressure equates to the intra-arterial pressure (Bogert & van Lieshout, 2005). An in-built calibration system

(Physiocal, Finometer Medical Systems, Amsterdam: The Netherlands), consisting of a dynamic servo set point adjuster, ensures that the optimal unloaded diameter of the finger artery is defined and maintained, the optimal diameter being close to the average diameter at a pressure where the amplitude of pulsations in the plethysmogram is largest (Wesseling *et al.*, 1995; Imholz *et al.*, 1998; Bogert & van Lieshout, 2005).

An appropriately sized finger cuff was placed around the mid-phalanx of the middle or index finger (according to closest fit) of the right hand, ensuring that the emission and detection light sensors were positioned symmetrically and that the cuff was securely fitted. A hydrostatic height correction unit (FMS Height Correction Unit), consisting of a liquid-filled tube attached at one end to a transducer and at the other end to an adjustable reference component, was used to correct hand height to heart level; the transducer and reference component were attached to the finger cuff and at heart level, respectively. Prior to commencement of data collection, a "zeroing" procedure was performed whereby the transducer and reference component were held together to ensure the height difference was 0 cm. The reference component was subsequently attached to the participant's torso at heart level, thereby ensuring that any changes in vertical displacement of the finger cuff relative to heart level were corrected for. In participants experiencing cold hands and/or fingers, a warm compress was used to gently warm the hand and prevent peripheral vasoconstriction. During each experimental trial, the raw arterial pressure waveform was visualised and recorded online at 1 kHz (LabChart 8.0, AD Instruments, Dunedin, New Zealand), from which SBP, DBP and MAP (1/3 SBP + 2/3 DBP, mmHg) were calculated.

Finger photoplethysmography is limited in that systolic blood pressure may be over-estimated, with both mean and diastolic pressure also being lower than intra-brachial pressure (Imholz *et al.*, 1991; Imholz *et al.*, 1998). These discrepancies in arterial pressure arise due to the variable arterial

waveforms throughout the arterial tree, causing augmentation between more proximal arteries, such as the brachial artery, and finger BP. The pressure gradient along the arterial tree and reflection as the pressure wave nears the periphery causes the shape of the pressure wave to change, thereby amplifying the wave and increasing systolic pressure (Imholz *et al.*, 1998). Furthermore, mean and diastolic pressure tend to be lower in the hands than in the arm as a result of this pressure gradient. However, the photoplethysmographic technique is able to continuously track changes in the arterial waveform and several studies have investigated the differences in finger photoplethysmography versus invasive, intra-arterial measurements of blood pressure with little differences reported between the two methods during Valsalva (Imholz *et al.*, 1988), head-up tilt (Jellema *et al.*, 1996), pharmacological manipulation (Imholz *et al.*, 1992), exercise (Silke *et al.*, 1994) or during postoperative patient monitoring (Triedman & Saul, 1994). Finger photoplethysmography is also widely used in scientific research and is a useful tool in monitoring static and dynamic changes in blood pressure (Ainslie *et al.*, 2008; Carter *et al.*, 2014; Low *et al.*, 2016). To verify and accurately calibrate the finger blood pressure (described previously), an automated blood pressure cuff (Dinamap, G & E Medical, Tampa, Florida) was worn on the contralateral upper arm to periodically monitor the finger photoplethysmography values.

3.4.2 Electrocardiogram

Heart rate was obtained from a 5-lead electrocardiogram (ECG; AD Instruments, Oxford, UK) which was recorded continuously (Francis, 2016). An antiseptic medical swab was used to clean each electrode site, prior to the application of the ECG electrodes (3M Red Dot Monitoring Electrode, St Paul, MN, USA; Figure 3.9). The ECG signal was continuously recorded online (LabChart 8.0, AD Instruments, Dunedin, New Zealand) and heart rate was calculated from the beat-to-beat R-R interval values using the formula: HR = 60 / R-R interval, where HR is in beats·min^{-1} and R-R is in seconds.

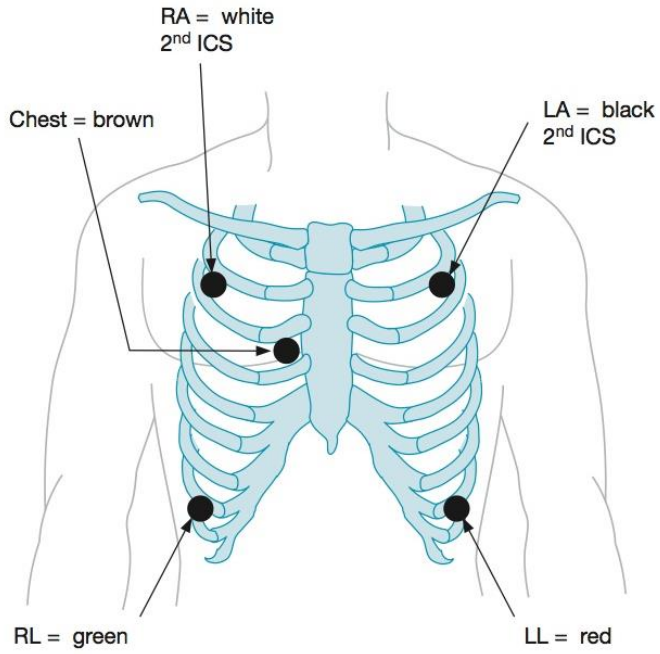

Figure 3.9 5-lead electrode system.

3.4.3 Cold Pressor Test

Haemodynamic function can be evaluated using the cold pressor test (CPT), which measures blood pressure and HR in response to an external cold stimulus, typically a cold water ice slurry, and is a useful predictor of future hypertension (Godden *et al.*, 1955; Victor *et al.*, 1987; Kasagi *et al.*, 1995; Zhao *et al.*, 2012). In healthy individuals, the cold stress typically induces vascular sympathetic activation leading to vasoconstriction and a concomitant sustained increase in blood pressure, although the HR response demonstrates high inter-individual variability (Victor *et al.*, 1987; Mourot *et al.*, 2009). Blood pressure reactivity is calculated as the difference between the baseline and peak blood pressure during the cold stimulus, with greater or "hyper" reactivity corresponding with a greater risk of developing hypertension and CVD (Zhao *et al.*, 2012). However, no best practice guidelines exist concerning what constitutes a "normal" or "hyper" response to the external cold stimulus and various thresholds are used by different research groups (Kasagi *et al.*, 1995).

An index of coronary artery disease risk and cranial conduit artery endothelial function can be calculated through assessment of carotid artery reactivity (CAR%; carotid artery diameter), via an ultrasound scan of the carotid artery performed during the CPT (Rubenfire *et al.*, 2000). During exposure to the cold stimulus, healthy individuals typically demonstrate vasodilation of the carotid artery, which is independent of carotid intima-media thickness (cIMT) (Rubenfire *et al.*, 2000). Recent data demonstrates a good correlation between the response of the coronary arteries and CAR%, and that the presence of traditional cardiovascular risk factors, in addition to older age, is associated with lower CAR% (van Mil *et al.*, 2017). Assessing CAR% via the CPT, therefore, represents a valuable method of assessing cardiovascular risk and the potential changes in CAR% following periods of altered lifestyle behaviours.

Following a 20-minute supine rest, participants were comfortably positioned to enable them to easily move their left hand off the bed without excessive bodily movement. Participants were asked to slightly extend their neck to allow simultaneous ultrasound imaging of the left common carotid artery for the duration of the CPT. A 10 MHz multifrequency linear array probe, attached to a high-resolution 2D duplex ultrasound machine (Terason u-Smart 3300, Teratech, Burlington, MA, USA) was used to assess the left common carotid artery reactivity (diameter and blood flow velocity). Following a 1-minute baseline period, participants were instructed to immerse their left hand (up to the wrist) in iced slush (1-5°C) for 3-minutes. Participants were instructed to breathe normally throughout the CPT and to avoid breath holding/hyperventilation, whilst remaining as still as possible. The reproducibility of the CPT is generally considered to be stable following both short-term (Durel *et al.*, 1993; Saab *et al.*, 1993; Fasano *et al.*, 1996) and long-term (Fahrenberg *et al.*, 1987; Sherwood *et al.*, 1997; Hassellund *et al.*, 2010; Zhao *et al.*, 2012) studies of <2-weeks and between 1 to 18-years, respectively.

Beat-to-beat arterial blood pressure and 5-lead ECG were recorded online throughout the CPT (LabChart 8.0, AD Instruments, Dunedin, New Zealand), from which SBP and DBP were exported in 10-second intervals. BP reactivity was calculated as the difference between baseline BP and the peak BP recorded during the 3-minute hand immersion. Custom-designed edge detection software (described earlier in section 3.3.2.1) was used to analyse CAR%. Baseline diameter and blood flow were taken in 10-second intervals for 1-minute prior to hand immersion. Post-immersion data was analysed in 10-second intervals, from which peak diameter change (maximum dilation/constriction) and area-under-the-curve (AUC) for the diameter change during the CPT (CAR_{AUC}) were calculated. Peak diameter change relates to the dilation or constriction of the carotid artery, with the directional change being determined by a positive or negative CAR_{AUC}, representing dilation or constriction, respectively. Typically, greater CAR% is observed in younger versus older individuals, and in healthy individuals compared to those exhibiting greater cardiovascular risk profiles (van Mil *et al.*, 2017).

3.5 Cerebrovascular Function

Transcranial Doppler ultrasound (TCD) is a useful, non-invasive tool that provides high temporal resolution for measuring blood velocity in cerebral vessels, measured in centimetres per second ($cm \cdot s^{-1}$) (Willie *et al.*, 2011). As vessel diameter is unknown, TCD is not a measure of blood flow *per se*, rather it measures blood velocity of an insonated vessel and gives a reliable index of blood flow, providing the angle of the Doppler probes remain constant (Giller *et al.*, 1998; Schreiber *et al.*, 2000; Ainslie & Duffin, 2009). Compared to other, often limited in availability and more expensive, methods of assessing cerebrovascular function, such as functional magnetic resonance imaging (fMRI), positron emission tomography (PET) and dynamic perfusion computer tomography (PCT), TCD is advantageous in being portable, allowing repeated measures and continuous monitoring of cerebral blood flow (CBF). Furthermore, having a temporal resolution (<0.1 s) enables rapid monitoring of changes in CBF,

particularly in assessing integrative cerebrovascular function through cerebral reactivity, autoregulation and neurovascular coupling (van Beek *et al.*, 2008; Willie *et al.*, 2011).

A low-frequency (≤2 MHz) pulsed Doppler ultrasonic beam is emitted from the Doppler probe(s) and crosses the intact skull to insonate the basal cerebral arteries through relatively thin bone 'windows' (Moppett & Mahajan, 2004; D'Andrea *et al.*, 2016). Reflection of the ultrasonic beam off moving red blood cells within the field of transmission is detected by the transducer within the Doppler probe(s) (Figure 3.9), with the resultant Doppler shift being proportional to red blood cell velocity (DeWitt & Wechsler, 1988; Willie *et al.*, 2011). Assessment of cerebral perfusion is often difficult due to the natural skull barrier and transmission of the ultrasonic beam can be influenced by the structural characteristics of the cranial bones, with approximately 10-20% of individuals having inadequate transtemporal acoustic windows, often related to age, gender and other factors (Marinoni *et al.*, 1997; D'Andrea *et al.*, 2016).

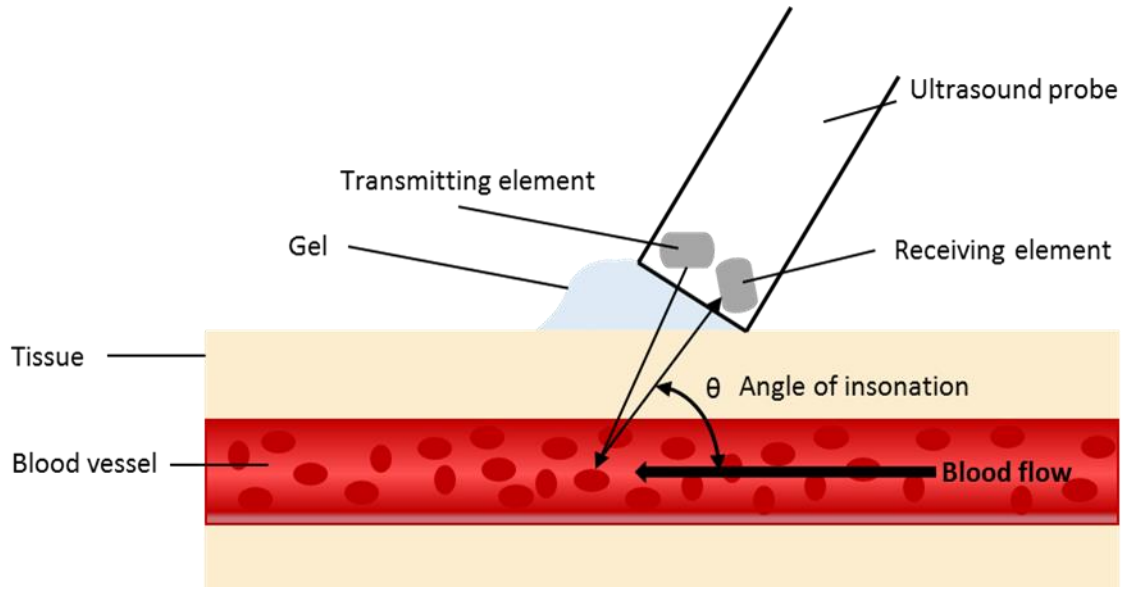

Figure 3.10 Schematic depicting the components of the Doppler ultrasound probe, consisting of two piezoelectric elements; an ultrasound transmitter and a receiver for the returning echoes of detected moving red blood cells.

The intracranial arteries of most notable clinical interest are the internal carotid artery (ICA), middle cerebral artery (MCA), anterior cerebral artery (ACA) and posterior cerebral artery (PCA) (Figures 3.11 and 3.12). The MCA is the most frequently insonated artery due to the ease of access and signal quality obtained through the temporal window. The MCA originates from the ICA and supplies blood to many parts of the lateral cerebral cortex, carrying 50-60% of the ipsilateral ICA blood flow (Moppett & Mahajan, 2004). Furthermore, the MCA receives ~80% of the blood volume delivered to the Circle of Willis (Lindegaard *et al.*, 1987) and is generally considered to represent blood flow to the brain (Moppett & Mahajan, 2004). MCA blood velocity is typically detected at a depth of 45-60 mm, with directional blood flow towards the Doppler probe(s) (Willie *et al.*, 2011; Bouzat *et al.*, 2014). Detailed descriptions of the techniques used for insonating the MCA have previously been documented elsewhere and are outlined in Table 3.1 (Moppett & Mahajan, 2004; Willie *et al.*, 2011; D'Andrea *et al.*, 2016). The angle of insonation is important in ensuring accurate measurement of MCAv and it should remain constant, as the observed velocity is inversely proportional to the cosine angle of incidence between the vessel and ultrasound beam (Moppett & Mahajan, 2004). The MCA provides relatively easy signal acquisition with a small insonation angle and an acceptable error of ~15% has been associated with insonation angles of up to 30° (Aaslid *et al.*, 1982).

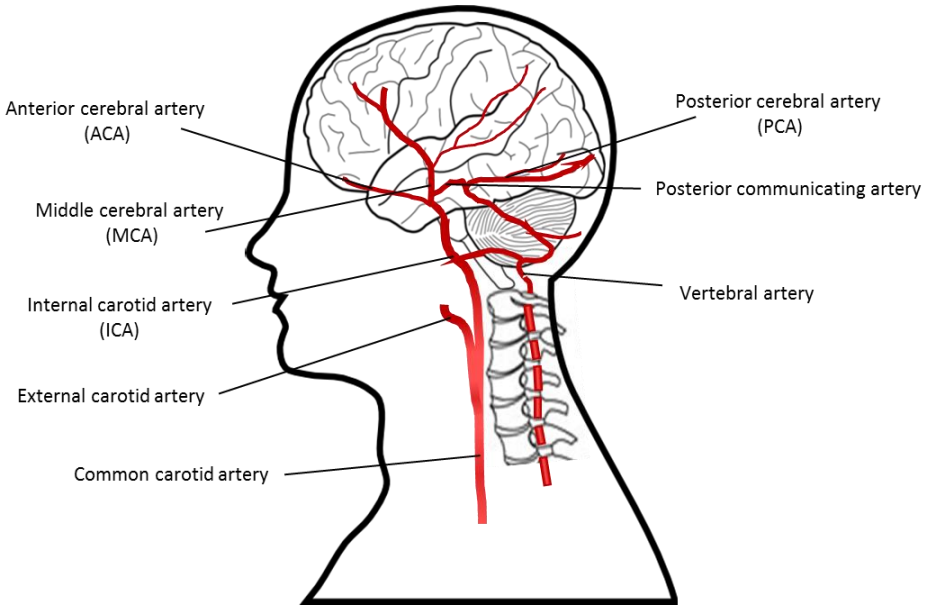

Figure 3.11 Overview of the cerebral circulation.

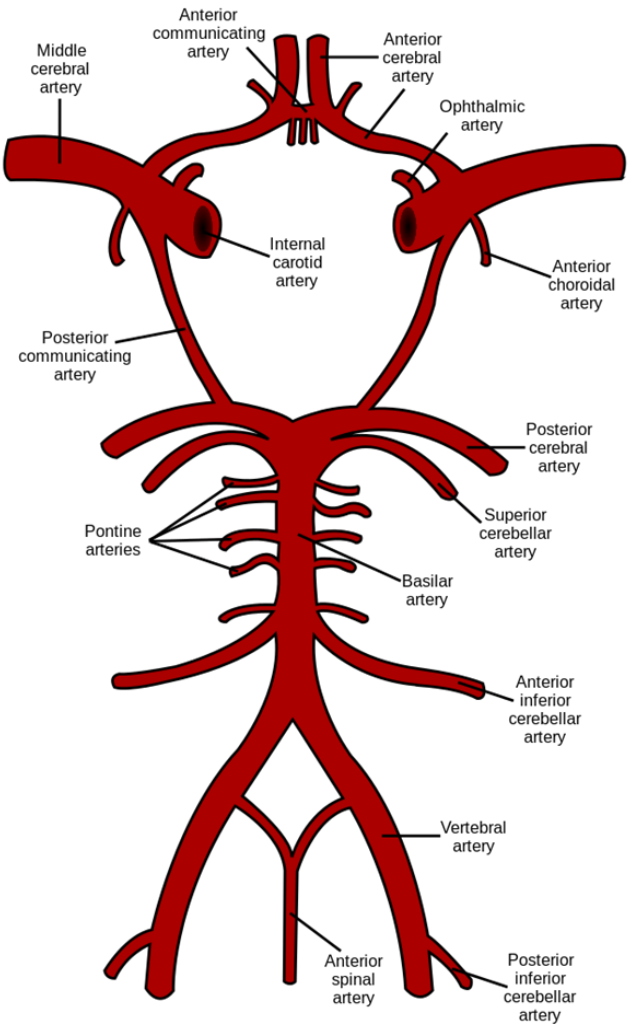

Figure 3.12 Overview of the Circle of Willis that is responsible for delivering cerebral blood flow.

Table 3.1 Typical patterns for identifying cerebral arteries with 'normal' Circle of Willis anatomy and without intracranial or vascular pathology. Adapted from Moppett and Mahajan (2004).

Vessel	Probe direction	Depth (mm)	Flow direction	Ipsilateral carotid compression	Contralateral carotid compression
ACA	Anterior	60-75	Away	Flow reversal	Increased velocity
MCA	Perpendicular	35-60	Toward	Reduced velocity	No change
PCA	Posterior	55-70	Toward	No change or increased velocity	No change

Abbreviations; ACA, anterior cerebral artery; MCA, middle cerebral artery; PCA, posterior cerebral artery.

In this thesis, TCD ultrasonography was used to continuously measure changes in MCAv both at rest (steady-state) and in response to physical manoeuvres (repetitive sit-to-stand movements) that induce fluctuations in BP and alterations in arterial carbon dioxide tension (cerebrovascular reactivity). With the participant resting in a supine position, middle cerebral artery blood velocity (MCAv, 1 cm distal to the MCA-ACA bifurcation) was continuously measured through the temporal window bilaterally via TCD ultrasonography (Doppler-BoxX, DWL Compumedics, Germany). The temporal window was located in the region immediately superior to the zygomatic arch, anterior to the tragus, at which site acoustic gel was applied and a 2 MHz Doppler probe (DiaMon, DWL Compumedics, Germany) was positioned on each side and adjusted until optimal signals were identified and remained consistent, according to previous descriptions (Willie *et al.*, 2011). An adjustable headband (DiaMon, DWL Compumedics, Germany) was used to securely position the Doppler probes to ensure that the same angle of insonation for continuous flow velocity measurement could be maintained and any subtle movements prevented for the duration of the assessment (Figure 3.13).

Figure 3.13 Secure positioning of the Doppler probes using a headband.

Specific criteria were used to insonate the correct vessel (MCA), with an insonation depth initially being set at 50 mm and the Doppler probe being slowly manipulated perpendicular to the assumed vessel direction. Upon identifying a strong signal of blood flow towards the transducer, the signal was optimised through minimal corrective movements of the probe and adjustments to the signal gain. A Doppler spectral waveform demonstrated the blood flow velocity profile on the M-mode screen, which assisted in confirming identification of the MCA through mean and peak blood flow velocity values, reading >50 $cm \cdot s^{-1}$ and ~80 $cm \cdot s^{-1}$, respectively (Willie *et al.*, 2011). However, the large variability in MCA peak blood velocity should be noted when identifying the MCA, as large variability is often inherent in specific patient groups and in ageing populations (Moppett & Mahajan, 2004). Standardisation of within-participant measures during repeated tests was achieved through recording the vessel depth, optimisation settings, mean and peak MCAv values, in addition to using the MCA-ACA bifurcation as an anatomical landmark. The same researcher positioned the Doppler probes for repeat laboratory visits to ensure consistency of placement. Real-time MCAv was displayed and recorded online at 1 kHz (LabChart version 8.0, AD Instruments, Dunedin, New Zealand).

3.5.1 Partial Pressure of End-Tidal Carbon Dioxide ($P_{ET}CO_2$)

Prior to each experimental session, an online gas analyser (ML206; AD Instruments, Dunedin, New Zealand) was calibrated with known oxygen and carbon dioxide (CO_2) beta grade gas concentrations. End-tidal carbon dioxide ($P_{ET}CO_2$) was collected via a sampling tube connected to a Hans Rudolph mouthpiece (Hans Rudolph 9060, Kansas, USA) at a flow rate of 200 $mL \cdot min^{-1}$. Nasal airflow was occluded with a nose clip (Hans Rudolph 9015, Kansas, USA). $P_{ET}CO_2$, mean CO_2 and peak maximum cyclic CO_2 responses for end tidal CO_2 were recorded online (LabChart version 8.0, AD Instruments, Dunedin, New Zealand).

3.5.2 Cerebrovascular Reactivity (CVR)

The partial pressure of CO_2 (P_aCO_2) is one of the principle regulators of CBF, with brain perfusion being highly sensitive to changes in CO_2 (P_aCO_2) (Willie *et al.*, 2014). Following a 1-minute baseline period

recording steady-state CBF, $P_{ET}CO_2$, ECG and BP, cerebrovascular reactivity (CVR) was assessed in response to changes in P_aCO_2 via a hyperventilation exercise (approximately 30-breaths per minute for approximately 1-minute) to induce hypocapnia. This was followed by breathing 5% CO_2 for 3-minutes (Figure 3.14). The strength of the linear relationship between cerebrovascular conductance (CBVC) and $P_{ET}CO_2$ was used as the index of CVR. Cerebrovascular CO_2 reactivity was calculated both in absolute and relative (%) terms as the slopes of the linear regression between CBF (TCD) vs $P_{ET}CO_2$ and CBVC vs $P_{ET}CO_2$.

Throughout baseline and 5% CO_2 breathing, common carotid artery (CCA) diameter and velocities were simultaneously recorded using a 10 MHz multi-frequency linear array probe attached to a high resolution 2D duplex ultrasound machine (Terason u-Smart 3300, Teratech, Burlington, MA, USA) to assess changes in carotid vasomotor activity. Prior to recording, the longitudinal B-mode image of the lumen-arterial walls was optimised and the lowest possible insonation angle (<60°) was used. Ultrasound images were recorded using specialised recording software (Camtasia Studio, Techsmith, USA), with analysis of CCA diameter and flow performed (BloodFlow Analysis, V.3.0.5 LabVIEW V.7.0, National Instruments Corporation; see *General Methods, section 3.3.2.1*). CCA blood flow (the product of lumen cross-sectional area and Doppler velocity) was analysed by averaging 1-minute of baseline data to that of the peak blood flow response and was calculated at 30 Hz. The relative increases (%) in carotid artery diameter, carotid blood flow and carotid vascular conductance (CarVC; ratio of carotid blood flow and mean arterial blood pressure) were calculated using baseline and peak data.

LabChart version 8.0 (AD Instruments, Dunedin, New Zealand) was used to extract the raw MCAv, MAP and $P_{ET}CO_2$ data in 10-second average bins into Microsoft Excel (Microsoft Office 2010, Microsoft Corporation). When bilateral MCAv was obtained, the values were averaged unless a significant difference was present between the two sites, in which case the strongest MCAv was used and

repeated in subsequent tests. To account for the relative time spent in each phase of the cardiac cycle, the weighted mean MCAv (cm/s) was calculated from the peak envelope of the velocity trace for each cardiac cycle using the following equation; Mean MCAv = 1/3 SMCAv + 2/3 DMCAv where SMCAv represents the systolic middle cerebral artery and DMCAv is the diastolic middle cerebral artery (Skow *et al.*, 2013). Linear regression was performed to calculate reactivity slopes. The cerebrovascular conductance (CBVC) index allows for the contribution of arterial BP responsiveness towards changes in CBF (Willie *et al.*, 2014). Therefore, flow conductance in the cerebral vasculature was calculated as: CBVC = mean MCAv / MAP, where CBVC is cerebrovascular conductance, mean MCAv is mean middle cerebral artery velocity and MAP represents mean arterial pressure.

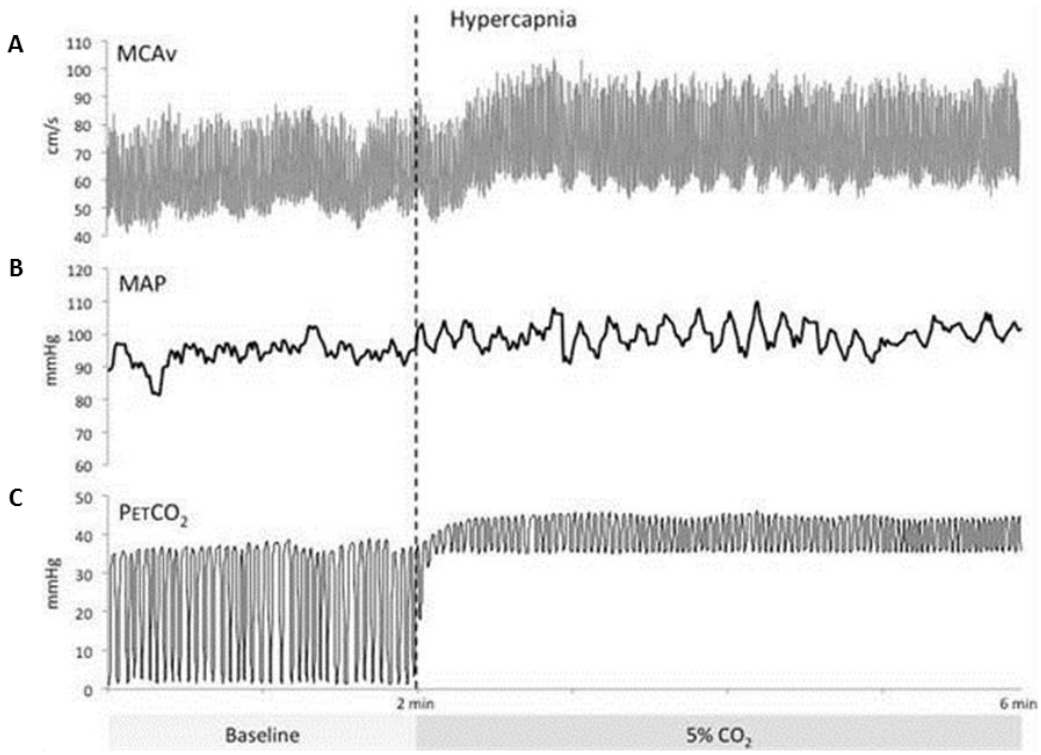

Figure 3.14 Exemplar waveforms for **A.** middle cerebral artery blood velocity (MCAv), **B.** mean arterial pressure (MAP) and **C.** end-tidal carbon dioxide ($P_{ET}CO_2$) at baseline and during hypercapnia (5% CO_2) to assess cerebrovascular reactivity, taken from a representative individual.

3.5.3 Cerebral Autoregulation

Blood pressure is a critical component of cerebral blood flow regulation, with integrated changes in P_aCO_2 and BP occurring throughout the day, particularly during activities such as coughing, exercising and changing posture (Willie *et al.*, 2014). Squat-stand manoeuvres were performed to elicit oscillations in BP and transfer function analysis was conducted on the beat-to-beat BP and MCAv mean signals to determine transfer function estimates of gain, phase and coherence. The transfer gain and phase reflect the relative amplitude and the time relationship between the changes in MAP and MCAv mean, respectively, over a specified frequency range. A high gain reflects reduced autoregulation. Transfer estimates of phase describe the temporal shift required to align the input signal (BP) with the output signal (MCAv mean). For a working autoregulation, output will lead input, whereas for less effective autoregulation, this phase difference will be reduced. The coherence function assesses the linear relationship between two variables; a coherence approaching 1 in a specific frequency range suggests a linear relationship between two signals within that frequency range, whereas a coherence approximating 0 may indicate a nonlinear relationship, severe extraneous noise in the signals, or simply no relationship between signals.

The squat-stand manoeuvres were performed to elicit oscillations in BP within the high-pass filter frequency range (<0.20 Hz) of the cerebrovasculature. These large swings in BP increase the statistical reliability of the phase and gain metrics in a physiologically relevant manner; i.e., the amplitude of these swings represents challenges that the cerebrovasculature endures on a daily basis during coughing, postural changes, exercise, etc. The squat phase engages the muscles of the legs, thereby increasing the skeletal muscle pump resulting in a large transient increase in venous return and BP within 2-3 seconds. Upon standing, the muscles of the legs are relaxed, subsequently decreasing the pressure applied to the veins and enabling venous pooling to increase, resulting in a subsequent decrease in BP. These large swings in MAP are performed at frequencies within the high-pass filter

range (0.05 and 0.10 Hz) and are transmitted to the cerebrovasculature. The large oscillations result in greatly increased coherence values at the frequency of interest (e.g., >0.98). Squat-stand cycles were performed at 0.20 Hz (2.5-seconds squatting, followed by 2.5-seconds standing), 0.10 Hz (5-seconds squatting, followed by 5-seconds standing; Figure 3.15) and/or 0.05 Hz (10-seconds squatting, followed by 10-seconds standing) for 5-minutes each, separated by a 5-minute rest to ensure that $P_{ET}CO_2$ had returned to baseline values prior commencing the second set of squats. These frequencies were selected as they are within the range where cerebral autoregulation is thought to have its greatest influence on the cerebral pressure-flow dynamics. Please refer to *Chapters 6 and 7* for the specific squat-stand manoeuvres and protocols used.

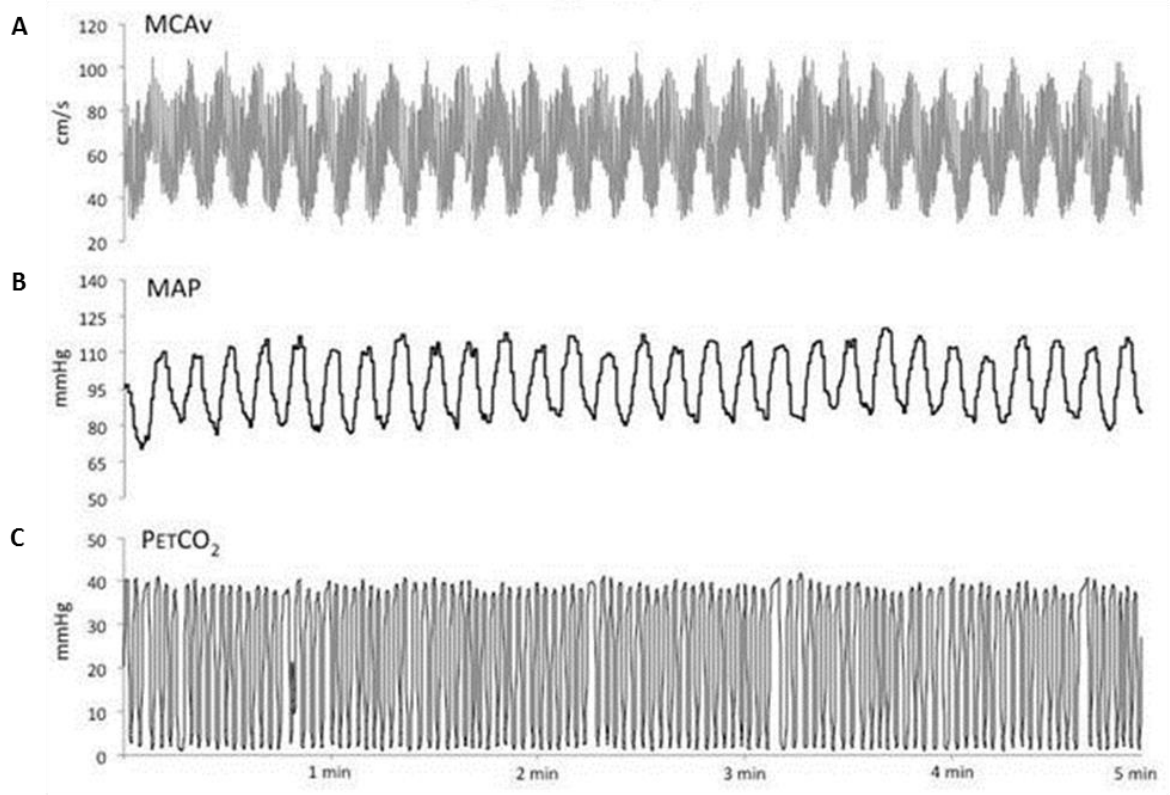

Figure 3.15 Exemplar waveforms for **A.** middle cerebral artery blood velocity (MCAv), **B.** mean arterial pressure (MAP) and **C.** end-tidal carbon dioxide ($P_{ET}CO_2$) during repeated squat-stand manoeuvres at 0.10 Hz (5-s squat, 5-s stand), taken from a representative individual.

Data was extracted from LabChart (version 8.0, AD Instruments, Dunedin, New Zealand) every 0.1-seconds and averaged across the 5-minute squat-stand period. Transfer function analysis was performed to assess the relationship between changes in MCAv and arterial BP using the variables MCAv, $P_{ET}CO_2$ and MAP, according to methodological guidelines (Claassen *et al.*, 2016). Transfer function estimates of gain, phase and coherence were calculated using MATLAB (MathWorks-Inc., Natick, MA) over three different frequencies; very low (0.02 – 0.07 Hz), low (0.07 – 0.20 Hz) and high (0.20 – 0.50 Hz) (Claassen *et al.*, 2016).

3.6 Statistical Analysis

Data were stored and transformed within Microsoft Excel (Microsoft Office 2010, Microsoft Corporation), and statistical analyses were performed using the Statistical Package for Social Sciences Version 22.0 (SPSS, Chicago, IL, USA). Data were expressed as mean ± SD, unless otherwise stated. Statistical significance was delimited at $P \leq 0.05$ and exact *P* values are cited; *P* values of "0.000" provided by SPSS are reported as "<0.001". Please refer to the methodology section in *Chapters 4-7* for the specific statistical analyses performed.

CHAPTER 4: REPRODUCIBILITY OF FOUR FREQUENTLY USED LOCAL HEATING PROTOCOLS TO ASSESS CUTANEOUS MICROVASCULAR FUNCTION

4.1 Introduction

Microvascular dysfunction may predict the manifestation of future cardiovascular disease, preceding abnormalities in larger conduit arteries and arterioles (Levy *et al.*, 2001; Bonetti *et al.*, 2003; Ijzerman *et al.*, 2003; Holowatz *et al.*, 2007; Minson, 2010; Sena *et al.*, 2013). The skin provides an easily accessible site to assess microvascular integrity through non-invasive methods, which can be used as an index of overall systemic vascular function. Control of the cutaneous microcirculation involves both neural and non-neural pathways (Johnson *et al.*, 2014). Neurogenic reflexes and local chemical mediators, such as NO, contribute towards the vasodilatory effect mediated by the vascular endothelium during local skin heating (Houghton *et al.*, 2006; Black *et al.*, 2008b). Protocols that locally heat the skin are increasingly used in conjunction with laser Doppler flowmetry (LDF) to evaluate skin blood flow responses and microvascular function, particularly for comparing between healthy and diseased individuals and/or assessing responses to interventions.

There are currently several local heating protocols that are widely used to assess cutaneous microvascular function (Minson *et al.*, 2001; Black *et al.*, 2008b; Choi *et al.*, 2014). These protocols all aim to increase skin blood flow to maximal/near-maximal levels (39–42°C), but they vary in the rate at which the skin is heated (0.5°C per 5 s, 2-min 30 s or 5-min) and/or the plateau at which the temperature is set (39°C vs 42°C) (Minson *et al.*, 2001; Black *et al.*, 2008b; Pugh *et al.*, 2013; Sprung *et al.*, 2013; Choi *et al.*, 2014; Dawson *et al.*, 2015). Due to the differences in the plateau and the rate of skin heating, a different contribution of the vasodilator pathways to the local heating response is present. Rapid local heating (0.5°C per 5 s) induces a transient axon-reflex (~5–10 min), produced via activation of heat sensitive sensory nerves and adrenergic nerves, followed by a more gradual, sustained vasodilatory response (20–30 min) that is partly (60–70%) NO-mediated (Minson, 2010). A modification of this protocol, by maintaining the plateau phase at 39°C, is believed to lead to a larger contribution of NO to the plateau phase (Choi *et al.*, 2014). Alternatively, gradually heating the skin

(0.5°C per 2-min 30 s or 5-min) evokes a largely NO-mediated vasodilatory response, without producing an axon-reflex (Black *et al.*, 2008b; Dawson *et al.*, 2015). Previous work found moderate to good inter-day reproducibility for all local heating protocols (Agarwal *et al.*, 2010; Roustit *et al.*, 2010; Tew *et al.*, 2011a; Huang *et al.*, 2013; Dawson *et al.*, 2015), especially when data were expressed relative to maximal values (Roustit *et al.*, 2010; Tew *et al.*, 2011a; Dawson *et al.*, 2015). However, no previous study examined the reproducibility of these local heating protocols within the same subjects and/or simultaneously. This latter aspect is of special importance, since simultaneous assessment of distinct heating protocols may achieve better insight due to the distinct dilator pathways involved. Therefore, the aim of this study was to simultaneously determine the inter-day reproducibility of four commonly used local heating protocols for assessing cutaneous microvascular function. Comparable reproducibility of all four protocols was expected, which would facilitate simultaneous use of multiple local heating protocols within the same study.

4.2 Methods

4.2.1 Participants

Fifteen healthy, male participants were recruited through local advertisement. All participants were healthy and non-smokers (28 ± 5 yrs, height 1.79 ± 0.10 m, weight 78.3 ± 8.5 kg, BMI 25 ± 2 kg/m^2, MAP 79 ± 5 mmHg). Individuals with a medical history of hypercholesterolaemia (total cholesterol >6.5 mmol/l) (Reiner *et al.*, 2011), cardiovascular disease and/or hypertension (systolic blood pressure ≥140 mmHg, diastolic blood pressure ≥90 mmHg) (NICE, 2011; Yarmolinsky *et al.*, 2015) were excluded. Participants were not taking any vasoactive medications or supplements. After being fully informed of the methods, written informed consent was obtained from all participants. The study conformed to the *Declaration of Helsinki* and was approved by the Research Ethics Committee of Liverpool John Moores University.

4.2.2 Experimental Design

All participants attended two experimental trials which were 2–7 days apart. During each trial, baseline and maximal thermally stimulated forearm cutaneous blood flow was examined simultaneously at four different sites on the dominant forearm using LDF. At each site, separated by ~5 cm, a different local heating protocol was adopted: 1. *Rapid 39°C* (Choi *et al.*, 2014), 2. *Rapid 42°C* (Minson *et al.*, 2001), 3. *Gradual 42°C* (Black *et al.*, 2008b) and 4. *Slow 42°C* (Black *et al.*, 2008b). The sites at the forearm were kept the same within subjects between the two testing days. Anthropometric measurements were recorded at visit 1. Heart rate and BP were recorded at the beginning and at the end of the 20-minute acclimation period, and thereafter at 5-minute intervals throughout the local heating protocols using an automated sphygmomanometer (Dinamap V100, GE Healthcare, UK) positioned on the contralateral arm. For details of the experimental procedures for these measurements, please refer to *Chapter 3, General Methods*.

4.2.3 Statistical Analysis

Data were expressed as mean ± SD and statistical significance was set at $P \leq 0.05$. Mixed model RMANOVA was used to compare local heating protocols and paired Student's t-tests were used to examine day-to-day systematic bias of each local heating protocol. Bland-Altman plots were constructed to demonstrate individual variability. The coefficient of variation (CV) was calculated to assess the inter-day reproducibility of CVC and $\%CVC_{max}$ at baseline, 39/42°C and 44°C. Prior to calculating the CV, a natural logarithmic transformation was applied to correct for heteroscedasticity of the data. For biological variables, a CV of <10% is considered good and <20% is acceptable (Scott *et al.*, 1989). A two-way ANOVA was used to examine BP within each protocol and between test days (main effects of local temperature and visit). Data were stored and transformed within Microsoft Excel (Microsoft Office 2010, Microsoft Corporation), and statistical analyses were performed using SPSS 22.0 (SPSS, Chicago, IL, USA).

4.3 Results

Figure 4.1 shows representative skin blood flow traces for all local heating protocols, indicating the phases and associated local temperatures (33°C, 39/42°C and 44°C) that were used for analysis. There was a small but significant increase in MAP during the heating protocols (P=0.002), consistent with a circadian variation. This gradual increase in MAP was not different between trials (P=0.39) and no interaction effect was found between local temperature and trial (P=0.49).

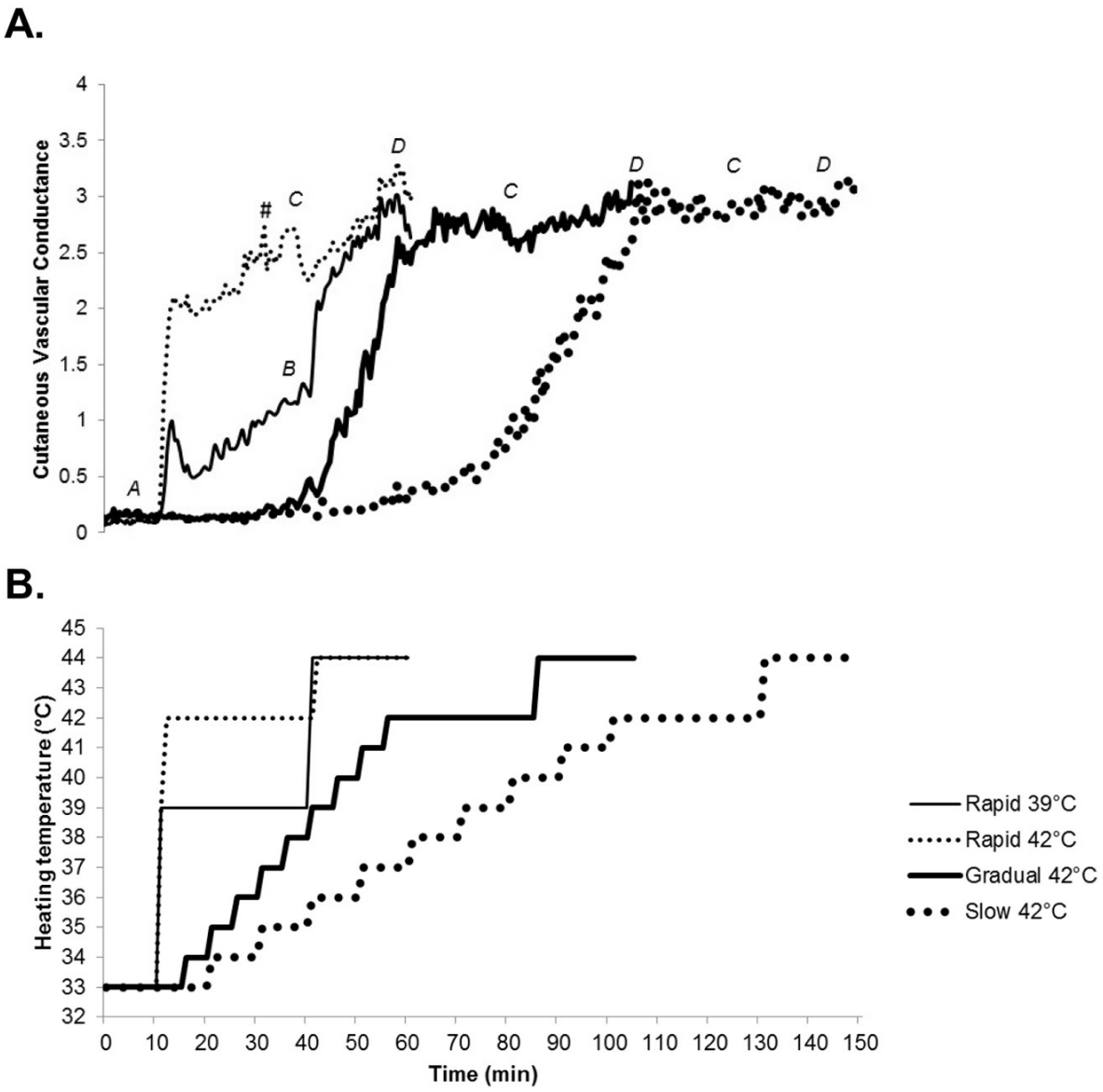

Figure 4.1 A. A representative forearm cutaneous arbitrary flux response for the four local heating protocols. Average flux was calculated over a stable 10-min period of baseline at 33°C (*A*) and the final 5-min of the plateau phases during heating at 39/42°C (*B* and *C*, respectively) and 44°C (*D*). The spike (#) indicates artefact resulting from slight movement of the arm which was removed prior to analysis. **B**. Step-wise temperature increments with corresponding times for all four local heating protocols. Taken from (Roberts *et al.*, 2017).

4.3.1 Baseline

Baseline cutaneous blood flow was not different between days when expressed as arbitrary flux or CVC (all *P*>0.05, Table 4.1). Furthermore, when expressed as %CVC_{max}, cutaneous blood flow was not different between days for the heating sites for *Rapid 39°C*, *Rapid 42°C*, *Gradual 42°C* or *Slow 42°C* (Table 4.1). Inter-day variation of baseline cutaneous blood flow was 17.2–26.1% for flux, 17.4–24.8% for CVC and 18.6–29.2% for %CVC_{max} (Table 4.1), with no differences between local heating sites.

Table 4.1 Baseline cutaneous blood flow results. Data is presented as mean ± SD. Inter-day reproducibility is presented as CV (± 95% CI): light grey shading indicates CV: >21%; mid grey shading indicates CV: 10-20%.

	Trial 1	Trial 2	Between-day CV (%)	Paired t-test
Baseline (33°C)				
Rapid 39°C				
Absolute flux (PU)	21 ± 10	20 ± 9	26.1 (19.1 - 41.3)	0.57
Absolute CVC (PU/mmHg)	0.26 ± 0.12	0.25 ± 0.11	24.8 (18.2 - 39.2)	0.66
Maximal CVC (%CVC_{max})	9 ± 5	8 ± 4	29.2 (21.4 - 46.5)	0.51
Rapid 42°C				
Absolute flux (PU)	20 ± 9	19 ± 10	17.2 (12.6 - 26.8)	0.10
Absolute CVC (PU/mmHg)	0.26 ± 0.11	0.24 ± 0.13	17.4 (12.8 - 27.2)	0.26
Maximal CVC (%CVC_{max})	8 ± 4	8 ± 5	19.3 (14.2 - 30.3)	0.66
Gradual 42°C				
Absolute flux (PU)	19 ± 8	20 ± 12	19.9 (14.6 - 31.2)	0.93
Absolute CVC (PU/mmHg)	0.24 ± 0.10	0.25 ± 0.15	20.8 (15.3 - 32.7)	0.79
Maximal CVC (%CVC_{max})	7 ± 4	7 ± 4	18.6 (13.7 - 29.2)	0.89
Slow 42°C				
Absolute flux (PU)	25 ± 11	22 ± 10	21.1 (15.5 - 33.2)	0.30
Absolute CVC (PU/mmHg)	0.31 ± 0.14	0.28 ± 0.12	22.7 (16.7 - 35.7)	0.43
Maximal CVC (%CVC_{max})	10 ± 4	9 ± 5	24.7 (18.2 - 39.1)	0.73

4.3.2 Local Heating to Plateau (39°C/42°C)

For all four protocols, inter-day cutaneous perfusion at the plateau phase was not different between days when expressed as arbitrary flux, CVC or %CVC_{max} (all *P*>0.05, Table 4.2). Lower inter-day reproducibility was found for the plateau phase of *Rapid 39°C* compared to *Rapid 42°C*, *Gradual 42°C* and *Slow 42°C* when data were expressed as flux, CVC or %CVC_{max} (Table 4.2). When data were presented as %CVC_{max}, CV was lower for *Rapid 42°C*, *Gradual 42°C* and *Slow 42°C* (Table 4.2). Bland-

Altman plots demonstrated no obvious heteroscedasticity for the responses at the plateau phase (Figure 4.2).

Table 4.2 Local heating cutaneous blood flow results. Data is presented as mean ± SD. Inter-day reproducibility is presented as CV (± 95% CI): light grey shading indicates CV: >21%; mid grey shading indicates CV: 10-20%; dark grey shading indicates CV<10%.

	Trial 1	Trial 2	Between-day CV (%)	Paired t-test
Plateau Phase (39°C /42°C)				
Rapid 39°C				
Absolute flux (PU)	138 ± 56	150 ± 57	20.6 (15.1 - 32.3)	0.20
Absolute CVC (PU/mmHg)	1.76 ± 0.67	1.93 ± 0.76	21.2 (15.6 - 33.4)	0.22
Maximal CVC (%CVC_{max})	55 ± 16	57 ± 13	20.7 (15.2 - 32.6)	0.48
Rapid 42°C				
Absolute flux (PU)	233 ± 56	218 ± 45	13.1 (9.7 - 20.5)	0.15
Absolute CVC (PU/mmHg)	3.01 ± 0.73	2.80 ± 0.56	12.8 (9.5 - 20.0)	0.09
Maximal CVC (%CVC_{max})	87 ± 8	86 ± 7	6.8 (5.0 - 10.5)	0.56
Gradual 42°C				
Absolute flux (PU)	240 ± 35	242 ± 54	15.2 (11.2 - 23.7)	0.87
Absolute CVC (PU/mmHg)	3.00 ± 0.51	3.07 ± 0.69	15.9 (11.7 - 24.9)	0.69
Maximal CVC (%CVC_{max})	88 ± 4	90 ± 6	5.2 (3.9 - 8.1)	0.29
Slow 42°C				
Absolute flux (PU)	220 ± 47	231 ± 52	16.6 (12.3 - 26.0)	0.48
Absolute CVC (PU/mmHg)	2.61 ± 0.54	2.81 ± 0.63	18.2 (13.4 - 28.4)	0.26
Maximal CVC (%CVC_{max})	81 ± 11	87 ± 11	10.6 (7.8 - 16.5)	0.09
Maximal Plateau (44°C)				
Rapid 39°C				
Absolute flux (PU)	255 ± 49	261 ± 61	12.9 (9.5 - 20.0)	0.66
Absolute CVC (PU/mmHg)	3.22 ± 0.60	3.37 ± 0.82	13.2 (9.8 - 20.6)	0.38
Rapid 42°C				
Absolute flux (PU)	274 ± 62	254 ± 42	13.7 (10.1 - 21.3)	0.11
Absolute CVC (PU/mmHg)	3.46 ± 0.75	3.26 ± 0.55	12.3 (9.1 - 19.2)	0.14
Gradual 42°C				
Absolute flux (PU)	274 ± 42	275 ± 60	14.6 (10.8 - 22.8)	0.96
Absolute CVC (PU/mmHg)	3.39 ± 0.50	3.41 ± 0.74	14.5 (10.7 - 22.6)	0.93
Slow 42°C				
Absolute flux (PU)	274 ± 47	268 ± 54	13.2 (9.8 - 20.6)	0.71
Absolute CVC (PU/mmHg)	3.21 ± 0.58	3.23 ± 0.61	15.0 (11.1 - 23.5)	0.89

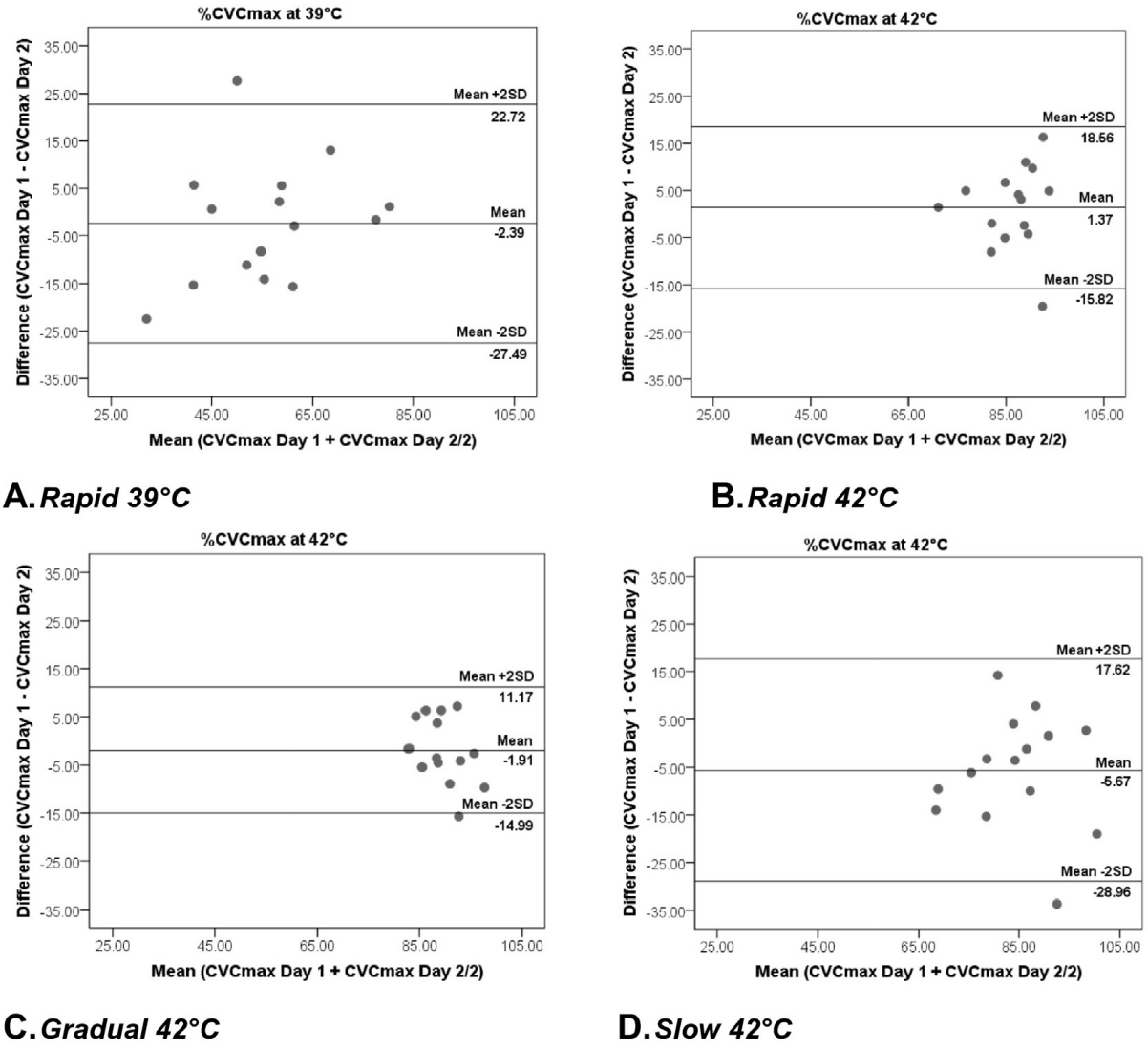

Figure 4.2 Bland–Altman plots of the difference between days in $\%CVC_{max}$ against the mean of the measurements at the 39/42°C plateau for **A**. *Rapid 39°C*, **B**. *Rapid 42°C*, **C**. *Gradual 42°C* and **D**. *Slow 42°C*. Middle horizontal line denotes mean value and upper and lower lines denote 95% limits of agreement. Linear regression demonstrated no evidence of proportional bias. Taken from (Roberts *et al.*, 2017).

4.3.3 Maximal Heating

Perfusion, presented as flux of CVC, during maximal heating (44°C) was not different between days (Table 4.2). The maximum heating response resulted in a reproducibility of 12.3–15.0% when data were expressed as arbitrary flux and CVC (Table 4.2). This observation was valid across all four protocols. Bland-Altman plots demonstrated no obvious heteroscedasticity for the responses during maximal heating (Figure 4.3).

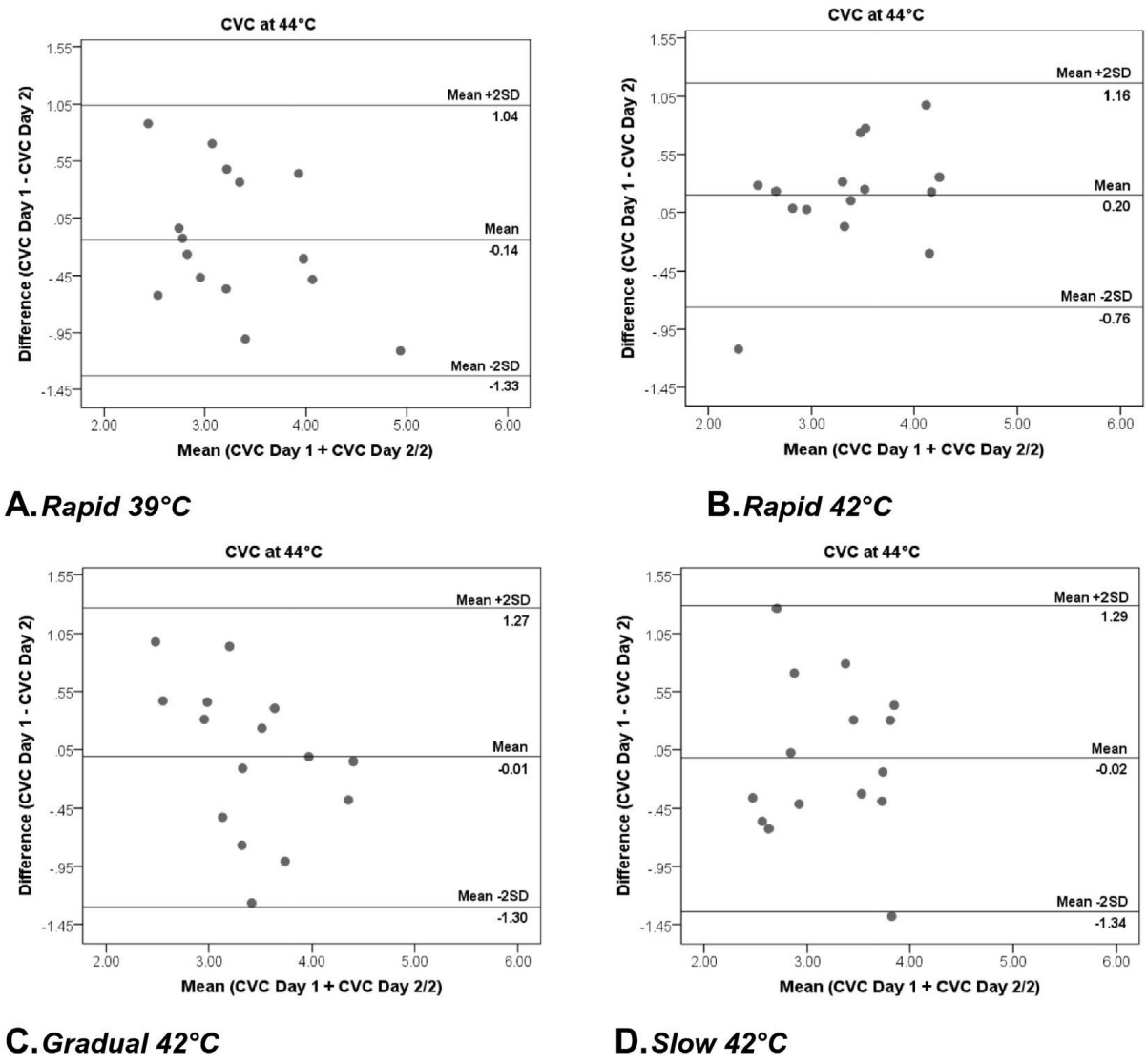

Figure 4.3 Bland–Altman plots of the difference between days in CVC against the mean of the measurements at 44°C for **A**. *Rapid 39°C*, **B**. *Rapid 42°C*, **C**. *Gradual 42°C* and **D**. *Slow 42°C*. Middle horizontal line denotes mean value and upper and lower lines denote 95% limits of agreement. Linear regression demonstrated no evidence of proportional bias. Taken from (Roberts *et al.*, 2017).

4.4 Discussion

The aim of this study was to explore the inter-day reproducibility of four commonly used (and simultaneously performed) local skin heating protocols for assessing cutaneous vascular function. Findings suggest that inter-day variation of baseline cutaneous blood flow demonstrated poor-to-moderate reproducibility. Secondly, inter-day reproducibility of cutaneous blood flow responses to the plateau phase of the *Rapid 42°C*, *Gradual 42°C* and *Slow 42°C* protocols was moderate for flux and CVC, but good when data were presented after correcting for maximal perfusion (%CVC_{max}). In contrast, inter-day reproducibility of perfusion during the plateau phase of the *Rapid 39°C* protocol was moderate-to-poor. Finally, maximal inter-day cutaneous blood flow demonstrated moderate reproducibility for arbitrary flux and CVC across all protocols, which indicates that the maximum response was not affected by the preceding protocol of local heating. These observations have clinical impact for designing future studies, especially when multiple local heating protocols are being used simultaneously.

Protocols that locally heat the skin in combination with LDF are frequently used to assess microvascular integrity and index overall systemic vascular function (Minson *et al.*, 2001). Current protocols vary in methodology with fast (Minson *et al.*, 2001; Choi *et al.*, 2014) or gradual (Black *et al.*, 2008b; Dawson *et al.*, 2015) rates of skin heating. Although studies have explored the reproducibility of individual heating protocols, the current study is the first to simultaneously assess reproducibility of multiple protocols. This is particularly important given that the simultaneous assessment of cutaneous vascular function using these distinct heating protocols may achieve better insight due to the distinct dilator pathways involved. Firstly, moderate to-poor reproducibility was identified for baseline perfusion, in agreement with previous research (Tew *et al.*, 2011a; Dawson *et al.*, 2015). It is important to acknowledge that the reproducibility of baseline perfusion was independent of the site of measurement (i.e. the four different sites that underwent the distinct heating protocols). Similarly,

maximal perfusion at 44°C also demonstrated good agreement between the four measurement sites. This demonstrates that the reproducibility of the LDF technique to assess baseline or maximal perfusion is not dependent on the site of measurement, but also was not affected by the local heating protocol (e.g. rapid or gradual).

Although distinct heating protocols were used, a comparable reproducibility was expected for the plateau phase according to previous studies (Tew *et al.*, 2011a; Dawson *et al.*, 2015). In line with the hypothesis, but also in agreement with previous studies (Tew *et al.*, 2011a; Dawson *et al.*, 2015), moderate reproducibility was found for perfusion during the plateau phases of the *Rapid 42°C*, *Gradual 42°C* and *Slow 42°C* protocols. Again consistent with previous studies (Tew *et al.*, 2011a; Dawson *et al.*, 2015), expressing data as a percentage of maximal perfusion resulted in an improvement of reproducibility. As CVC data is typically normalised to maximum to account for the heterogeneity of cutaneous vessel density, this may explain the increased reproducibility when data were normalised to $\%CVC_{max}$. The use of integrated probes in the current and aforementioned studies, which allow an examination of a greater surface area of skin, may have contributed to the good-to-moderate reproducibility. Indeed, previous work on local heating using single point LDF probes report lower reproducibility (Agarwal *et al.*, 2010; Roustit *et al.*, 2010).

Despite the good agreement between the rapid, gradual and slow heating protocols to 42°C, poorer reproducibility was reported for forearm cutaneous vascular responses to the rapid heating protocol to 39°C. The poorer reproducibility of the *Rapid 39°C* protocol cannot be simply explained by the speed of increasing local temperature, especially since reproducibility of the *Rapid 42°C* and *Gradual 42°C* protocols were similar. The *Rapid 39°C* protocol was developed in order to isolate NO-dependent dilation and/or allow a better assessment of perturbations that may improve microvascular function due to the levels of skin blood flow achieved at this local skin temperature (~50% maximum) compared

to those at 42°C (~90% maximum) (Choi *et al.*, 2014). Differences in reproducibility between the *Rapid 39°C* protocol and the other protocols may therefore relate to the differences in the level of skin blood flow achieved in the protocols. Skin blood flow was lower at the plateau phase of the *Rapid 39°C* protocol (i.e. ~50% of the maximum response) relative to the plateau phases of the *Rapid 42°C*, *Gradual 42°C* and *Slow 42°C* protocols (80–90% of the maximum response). Such levels may provide more space for variation in perfusion, despite correcting the level of perfusion for differences in the maximum perfusion at 44°C. One consequence of this observation is that a larger group size is required for the *Rapid 39°C* protocol compared to the other protocols to detect differences within subjects.

Using data from the present study, the sample sizes required to show significant changes in CVC and $\%CVC_{max}$ at 39/42°C for within subject comparisons (e.g., pre and post interventions; Table 4.3) were calculated. Assuming a power of 80% (α=0.05), the *Rapid 39°C* protocol would require 54 subjects to detect a 5.0% change in $\%CVC_{max}$ at 39°C compared to 27 subjects at 42°C for the *Rapid 42°C* protocol. Again, assuming a power of 80% (α=0.05), the *Gradual 42°C* protocol would require 17 subjects to detect a 5.0% change in $\%CVC_{max}$ at 42°C and the *Slow 42°C* protocol would require 47 subjects. These sample size estimations demonstrate distinctly different requirements for studies that use both rapid and gradual local heating, possibly related to between protocol differences in underlying vasodilatory mechanisms.

Table 4.3. Estimated sample sizes required for a repeated measures study design using the *Rapid 39°C, Rapid 42°C*, *Gradual 42°C* and *Slow 42°C* local heating protocols to detect significant changes in skin blood flow assessed as CVC and %CVC_{max} at 39/42°C using the results from the reproducibility study.

Power, %	α-Error	Change in CVC	Sample size			
			Rapid 39°C	*Rapid 42°C*	*Gradual 42°C*	*Slow 42°C*
80	0.05	0.25	35	29	56	59
80	0.05	0.50	11	9	16	17
80	0.05	0.75	6	6	9	9
80	0.05	1.00	5	4	6	6
90	0.05	0.25	46	38	74	78
90	0.05	0.50	13	11	20	21
90	0.05	0.75	8	7	11	11
90	0.05	1.00	5	5	7	7
Power, %	**α-Error**	**Change in %CVC_{max}**	**Sample size**			
			Rapid 39°C	*Rapid 42°C*	*Gradual 42°C*	*Slow 42°C*
80	0.05	2.5	208	99	58	180
80	0.05	5.0	54	27	17	47
80	0.05	7.5	25	13	9	22
80	0.05	10.0	15	9	6	14
90	0.05	2.5	278	132	77	240
90	0.05	5.0	71	35	21	62
90	0.05	7.5	33	17	11	29
90	0.05	10.0	20	11	7	17

The reproducibility of the *Rapid 42°C*, *Gradual* and *Slow 42°C* protocols were similar. Importantly, these local heating protocols induce skin vasodilation via different mechanisms. Whilst slowly heating the skin prevents significant axon reflexes and leads to a largely NO-mediated response, rapidly heating the skin activates the axon reflex and leads to a NO- and EDHF-mediated response (Johnson *et al.*, 2014). The involvement of multiple vasoactive substances in the rapid heating response might have contributed to a larger variation in response to the *Rapid 42°C* local heating protocol. However, similar coefficients of variation were evident for the CVC and %CVC_{max} data in the *Rapid 42°C* and *Gradual 42°C* protocols. The specific underlying pathways leading to vasodilation in response to the distinct heating protocols may therefore provide useful synergistic insight into complementary vasodilatory mechanisms. Therefore, supported by the comparable reproducibility, it is suggested that measuring multiple local heating protocols is easy, applicable and feasible, but may also provide better

insight into the skin vasodilator mechanisms. An alternative approach would be to use the same heating protocol across multiple sites to minimise any between site differences. Averaging data across these sites would improve reliability and thereby reduce the sample size requirements. However, use of a single protocol is limited in only assessing one vasodilatory pathway, whereas a multi-protocol approach may elicit further mechanistic insight into vasodilatory responses observed.

4.4.1 Limitations

Inter-day reproducibility was assessed in a young, healthy population, thereby limiting extrapolation of our findings to individuals and patient groups with cardiovascular risk, such as older individuals, who commonly exhibit attenuated cutaneous blood flow responses to local heating (Minson *et al.*, 2002; Cracowski *et al.*, 2006; Holowatz *et al.*, 2007; Black *et al.*, 2008b). However, young and older subjects demonstrated comparable reproducibility of local heating in a previous study (Tew *et al.*, 2011a). A further limitation is that females were excluded from this study to control for hormonal influences on vascular function (Charkoudian *et al.*, 1999; Charkoudian & Johnson, 2000; Charkoudian, 2001) and, therefore, the study findings are applicable to males only.

In conclusion, this is the first study to simultaneously examine the inter-day reproducibility of four local heating protocols, which are currently frequently used to assess cutaneous blood flow in humans. The study findings suggest that the reproducibility of baseline forearm skin perfusion assessment is poor to moderate, and is independent of the site of measurement. The *Rapid*, *Gradual* and *Slow 42°C* protocols exhibited superior reproducibility, particularly when data is expressed as $\%CVC_{max}$, compared to the *Rapid 39°C* protocol. The present study data supports the validity of repeated measures of cutaneous blood flow in response to local heating protocols, for use in epidemiological studies as an index of microvascular function. Furthermore, these data provide help in guiding future studies in calculating the sample sizes necessary to detect differences, especially since differences are present between the different protocols. The present data will, therefore, inform the assessment of

cutaneous microvascular function in subsequent chapters in using simultaneous local heating protocols to comprehensively interrogate the microvasculature, whilst also considering the sample size estimations as a guide to detect meaningful differences.

CHAPTER 5: ACUTE BLACK TEA CONSUMPTION IMPROVES CUTANEOUS VASCULAR FUNCTION IN HEALTHY MIDDLE-AGED HUMANS

5.1 Introduction

Cardiovascular disease (CVD) remains the leading cause of global mortality, representing ~30% of all deaths (WHO, 2016). The role of dietary factors on CVD risk has been frequently explored in recent years, with a high dietary flavonoid intake being associated with a reduction in CVD risk (Peterson *et al.*, 2012). Tea, produced from the plant *Camillia sinesis,* is the major source of dietary flavonoids in many countries globally (Yahya *et al.*, 2016) and can be found as catechins and flavanols in green tea and theaflavins, thearubigins and flavonols in black tea (Hodgson & Croft, 2010). Accordingly, several studies have revealed a strong, inverse relation between regular intake of tea and cardiovascular risk (Grassi *et al.*, 2009b; Greyling *et al.*, 2014).

A frequently cited explanation for the cardioprotective effects of black and green tea ingestion relates to the reduction in blood pressure following chronic consumption (Grassi *et al.*, 2009b; Greyling *et al.*, 2014; Grassi *et al.*, 2015). Further research found that acute and regular tea ingestion improves NO-mediated, endothelium-dependent dilation of conduit arteries (Duffy *et al.*, 2001; Hodgson *et al.*, 2002; Hodgson *et al.*, 2005; Grassi *et al.*, 2009b; Schreuder *et al.*, 2014). Both conduit and resistance vessels have demonstrated improved endothelial function following tea ingestion in both healthy individuals (Hodgson *et al.*, 2002; Grassi *et al.*, 2009b) and in those with CVD (Duffy *et al.*, 2001). Thus, the general consensus is that regular tea ingestion improves blood pressure by virtue of a generalised improvement of endothelial function and lowering of peripheral vascular resistance (Duffy *et al.*, 2001; Hodgson *et al.*, 2002; Hodgson *et al.*, 2005; Grassi *et al.*, 2009b; Ras *et al.*, 2011).

Despite encouraging data supporting a beneficial effect of tea ingestion in larger (conduit) vessels, no previous study has robustly explored the effect of black tea on small vessels (skin microcirculation). Therefore, the aim of this study was to examine cutaneous vascular responses to local skin heating. Given the complexity of the cutaneous vascular system and contribution of distinct mechanisms for

skin dilation when gradually or rapidly heating the skin, a comprehensive approach was adopted using both rapid *and* gradual local skin heating protocols simultaneously. It was hypothesised that black tea ingestion would be associated with increased cutaneous microcirculation responses for both rapid and gradual heating protocols.

5.2 Methods

5.2.1 Participants

Twenty middle-aged male (n=9) and post-menopausal female (n=11) participants were recruited through local advertisement. All participants were healthy and non-smokers (58 ± 5 yrs, height 1.70 ± 0.1 m, weight 75.9 ± 16.1 kg, BMI 26 ± 4 kg/m^2, baseline MAP 104 ± 8 mmHg). For details of the inclusion and exclusion criteria, please refer to *Chapter 3, General Methods.* After being fully informed of the methods verbally and in writing, written informed consent was obtained from all participants. The study conformed to the *Declaration of Helsinki* and was approved by the local research ethics committee.

5.2.2 Experimental Design

All participants performed two experimental trials (tea and control), 7-days apart in a randomised, controlled, double-blind, cross-over design (Figure 5.1). The crossover design was chosen to eliminate between-participant variability, taking into account a 6-day washout period between the two interventions to avoid any carry-over effects, which is in accordance with previous similar designed crossover tea vascular function studies (Grassi *et al.*, 2009b; Grassi *et al.*, 2015). Computer-generated randomisation was used to reduce potential selection bias. Participants were asked to adhere to all pre-test instructions regarding fasting, avoiding caffeine etc., as outlined in *Chapter 3, General Methods* (section 3.1). Anthropometric measurements were recorded at visit 1 (see *Chapter 3, General Methods,* section 3.2). Upon arrival to the laboratory, and 2-hours prior to microvascular assessment, participants ingested a tea drink (containing 300 mg flavonoids, 75 mg caffeine and 2.8 g

sucrose) or a taste and appearance matched placebo drink (0 mg flavonoids, 75 mg caffeine, 2.7 g sucrose, tea flavour and caramel colour), prepared by dissolving two sachets in 200 ml hot water. Participants subsequently rested for 2-hours prior to commencement of testing to match peak plasma concentrations of flavonoids and other metabolites such as phenolic acids, with testing of skin microcirculation.

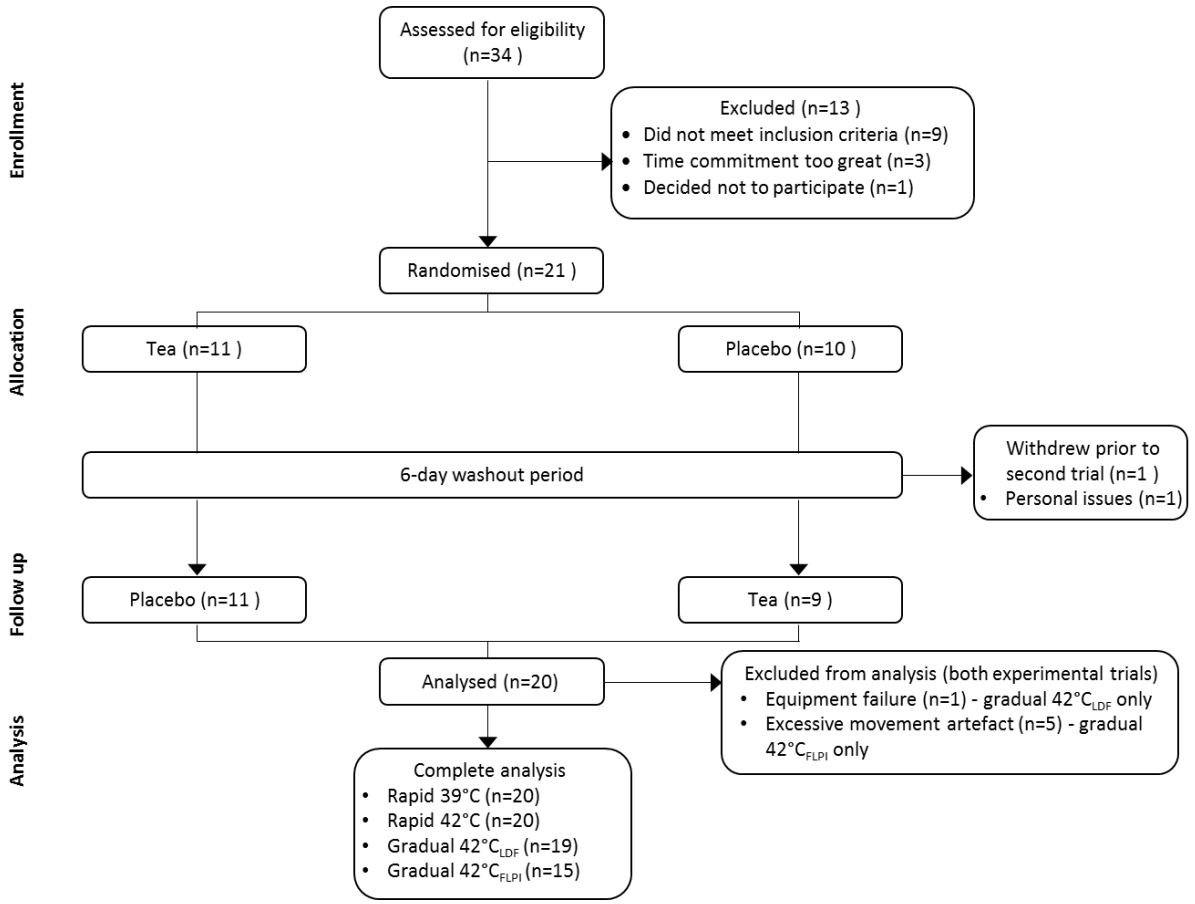

Figure 5.1. CONSORT diagram showing the flow of participants through each stage of the randomised trial. Taken from (Woodward *et al.*, 2016).

At each trial, baseline and thermally stimulated forearm cutaneous blood flow was examined simultaneously using rapid (to 39 and 42°C) and gradual (to 42°C) local heating protocols. Since these protocols reflect different dilator mechanisms and a distinct role of the NO-pathway, they provide complementary insight into the impact of black tea on the cutaneous microvasculature. Rapid local heating was performed at two different sites (i.e. two different local heating protocols) on the

dominant forearm and examined using LDF. Gradual local heating to 42°C was performed on the dominant forearm using LDF and on the contralateral (non-dominant) arm using laser speckle imaging via full-field laser perfusion imaging (FLPI) to provide whole forearm cutaneous microvascular function (Figure 5.2).

The FLPI technique, also known as laser speckle contrast imaging, exploits the fact that the random speckle pattern that is generated when tissue is illuminated by laser light, changes when blood cells move within the region of interest (Briers *et al.*, 2013). High levels of movement (fast flow) produce a more blurred pattern, associated with a reduction in contrast in that region. Low contrast therefore corresponds with high flow and high contrast corresponds with low flow. The strengths of this technique are that video frame rate blood flow images (up to 25 per second) enable the tracking of fast transient blood flow changes and provides high spatial and temporal resolution. This device works with a near infra-red laser diode (785 nm) and is able to scan skin surfaces from 5 mm x 7 mm to 15 cm x 20 cm, to a depth of approximately 150-300 micron and is safe for human use.

Following a 20-minute acclimation period, FLPI recordings were performed using a blood flow imaging system (moorFLPI-1, Moor Instruments, Axminster, UK) with a laser wavelength of 785 nm and sampling frequency of 25 Hz. A ~3 cm^2 area of skin was measured, with the distance between the laser head and skin surface fixed at 15 cm (Mahé *et al.*, 2011). A skin heater module (moorVMSHEAT, Moor Instruments, Axminster, UK) was used to manually set the incremental temperatures. Data were continuously recorded in moorFLPI V3.0 PC Software (Moor Instruments, Axminster, UK).

Both LDF and FLPI provide non-invasive continuous measures of cutaneous blood flow (Roustit & Cracowski, 2012). By using a combination of these techniques, it is possible to simultaneously evaluate

superficial (<300 micron) and deeper (0.5-1.5 mm) skin blood flow via FLPI and LDF, respectively. Anthropometric measurements were recorded at visit 1. Heart rate and BP were recorded using an automated sphygmomanometer (Dinamap V100, GE Healthcare, UK) positioned on the ankle, corresponding to the same laterality as their dominant arm. For details of the experimental procedures for these measurements, please refer to *Chapter 3, General Methods*.

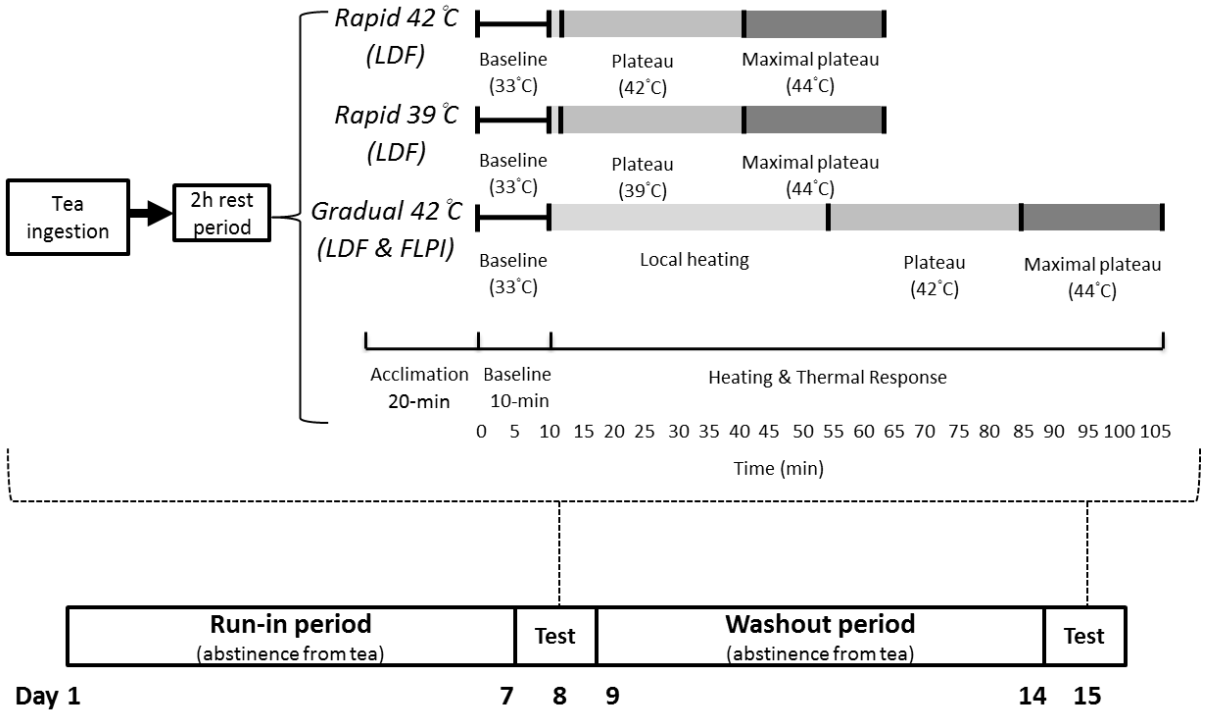

Figure 5.2. Study overview and schematic depicting the stages of the local heating protocols. Light grey shading denotes local heating, mid grey shading represents the plateau and dark grey shading represents the maximal plateau. Taken from (Woodward *et al.*, 2016).

5.2.3 Statistical Analysis

Data were expressed as mean ± SD and statistical significance was set at $P \leq 0.05$. For all protocols, linear mixed models (main effects of condition and time) were used to examine the impact of acute tea ingestion on blood pressure and forearm skin microcirculation. The repeated covariance type was Unstructured and Condition, Time and Condition*Time was specified as Fixed Effects (intercept was included) and as Estimated Marginal Means. Order was added as a covariate to control for the sequence of experimental trials (tea and placebo). The Test of Fixed Effects Condition*Time

interaction was interpreted. Significant main effects of Time or Condition or a Time*Condition interaction were followed up with a simple main effects analysis and the least significant difference (LSD) approach to multiple comparisons (Perneger, 1998). Data were stored and transformed within Microsoft Excel (Microsoft Office 2010, Microsoft Corporation), and statistical analyses were performed using SPSS 22.0 (SPSS, Chicago, IL, USA).

5.3 Results

One participant was removed from the *Gradual 42°C* LDF analysis for both experimental trials (due to probe failure) and five participants were removed from the *Gradual 42°C* FLPI analysis for both trials (linked to excessive movement artefacts), giving a population of n=19 and n=15, respectively. No participants were removed from the *Rapid 39°C* and *Rapid 42°C* analysis (both n=20). There was no order effect for any of the protocols (*P*-value range 0.20-0.87). Baseline MAP was not different between conditions (108 ± 11 mmHg vs 108 ± 11 mmHg, *P*=0.73) and showed no change across time (*P*=0.52). There were no differences in baseline cutaneous perfusion between trials for measurement sites that underwent *Rapid 39°C* or *Rapid 42°C* local heating for absolute flux, CVC or $\%CVC_{max}$ (Table 5.1). Furthermore, the site that underwent *Gradual 42°C* local heating using LDF showed no difference in baseline cutaneous blood flow between trials for absolute flux, CVC or $\%CVC_{max}$ (Table 5.2). However, using FLPI, a significantly higher baseline perfusion was found after tea ingestion for cutaneous flux and CVC, but not for $\%CVC_{max}$ (Table 5.2).

5.3.1 Rapid Local Heating: Impact of Tea

5.3.1.1 Rapid 39°C

Local heating induced a typical pattern of an initial peak, nadir and plateau in cutaneous blood flow. Therefore, a main effect of time was demonstrated for absolute flux, CVC and $\%CVC_{max}$ (Table 5.1). However, there was no effect of the intervention found or a time X intervention-interaction for absolute flux, CVC or $\%CVC_{max}$ (Table 5.1).

5.3.1.2 Rapid 42°C

Local heating induced a typical pattern of an initial peak, nadir and plateau in cutaneous blood flow. Consequently, a main effect of time was demonstrated for absolute flux, CVC and $\%CVC_{max}$ (Table 5.1), whilst no main effect of intervention or time X intervention-interaction was found for absolute flux, CVC or $\%CVC_{max}$ (Table 5.1).

5.3.2 Gradual Local Heating: Impact of Tea

5.3.2.1 $Gradual_{LDF}$ (42°C)

Local heating induced a gradual, slow heating response with no detectable initial axon reflex-induced peak or nadir with a main effect of time (Table 5.2). A higher skin blood flow throughout the heating protocol was observed during the trial preceded by black tea for absolute CVC (P=0.04), with a trend towards significance when data were presented as absolute flux (P=0.06, Table 5.2). No effect of tea was found when CVC was normalised for maximum perfusion ($\%CVC_{max}$, P=0.82, Table 5.2). No time X intervention interaction was found for absolute flux (P=0.93), CVC (P=0.95) or $\%CVC_{max}$ (P=0.98, Table 5.2).

5.3.2.2 $Gradual_{FLPI}$ (42°C)

Local heating induced a gradual, slow heating response with no detectable initial axon reflex-induced peak or nadir (Table 5.2). Tea ingestion was associated with a significantly higher absolute flux (P<0.001) and CVC (P<0.001), but not when CVC was normalised to maximum CVC ($\%CVC_{max}$, P=0.35, Table 5.2). No time X intervention interaction was present for absolute flux (P=0.50), CVC (P=0.66) or $\%CVC_{max}$ (P=1.00, Table 5.2).

Statistical analysis revealed no presence of a carry-over effect.

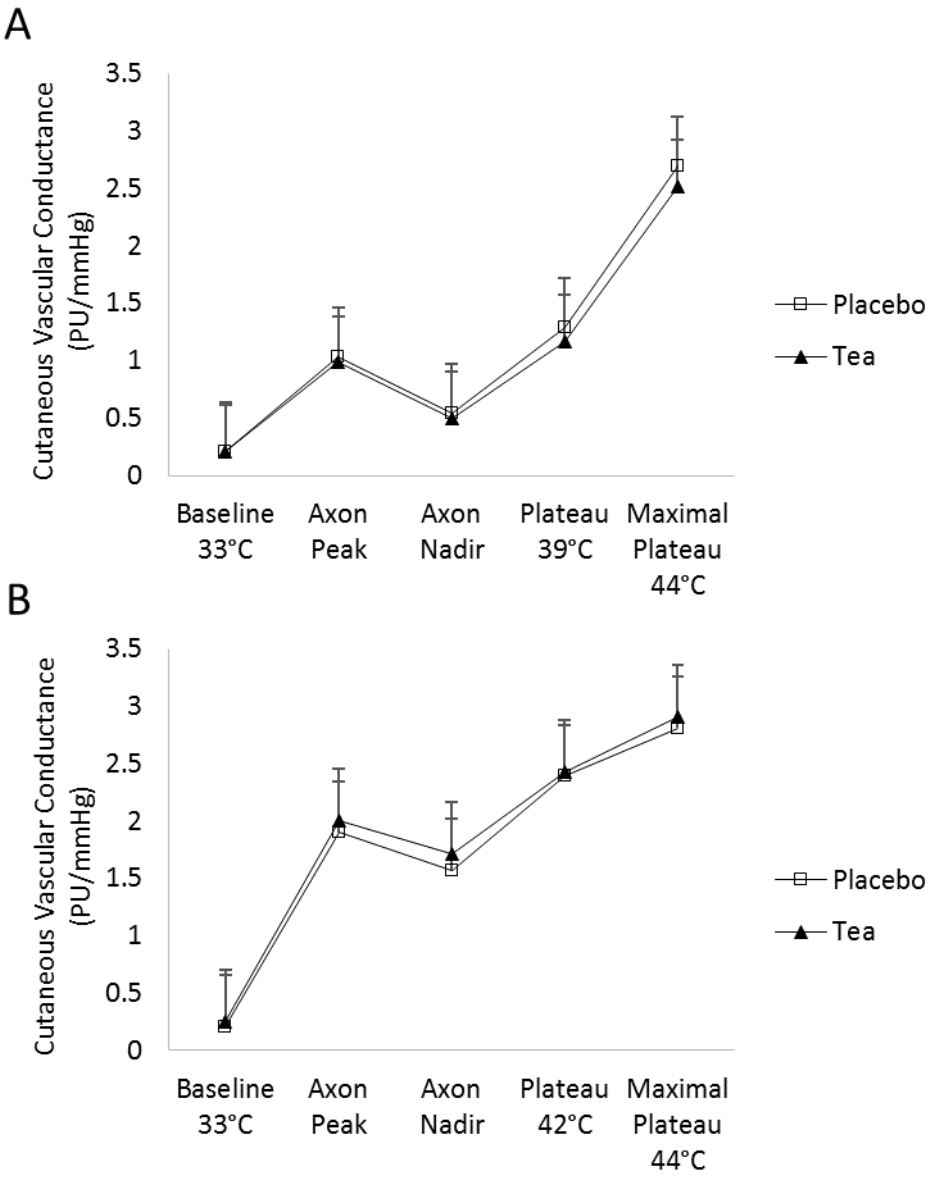

Figure 5.3 Cutaneous vascular conductance (CVC) responses across time points (baseline at 33°C, axon peak, axon nadir, plateau at 39/42°C and maximal plateau at 44°C) following rapid local heating for **A.** *Rapid 39°C* and **B.** *Rapid 42°C* in 20 healthy volunteers when heating was preceded by ingestion of placebo (open squares) or tea (solid triangles). Data are presented as means, with error bars representing SE. Taken from (Woodward *et al.*, 2016).

Table 5.1 Laser Doppler flowmetry cutaneous blood flow responses to local heating for the *Rapid 39°C* and *Rapid 42°C* protocols for placebo and tea interventions.

	Intervention (mean ± SD)		LMM		
Rapid 39°C	**Placebo**	**Tea**	**Time**	**Tea**	**Time*Tea**
Absolute flux(PU)					
Baseline	22 ± 11	21 ± 8			
Axon-reflex	108 ± 38	103 ± 50			
Nadir	57 ± 25	52 ± 26	<0.001*	0.14	0.76
Plateau 39*°C*	136 ± 53	123 ± 70			
Plateau 44*°C*	288 ± 61	263 ± 61			
Absolute CVC (PU/mmHg)					
Baseline	0.21 ± 0.12	0.21 ± 0.10			
Axon-reflex	1.03 ± 0.39	0.99 ± 0.47			
Nadir	0.54 ± 0.25	0.50 ± 0.26	<0.001*	0.27	0.91
Plateau 39*°C*	1.29 ± 0.52	1.17 ± 0.65			
Plateau 44*°C*	2.70 ± 0.67	2.52 ± 0.59			
Maximal CVC (%CVC_{max})					
Baseline	8 ± 4	8 ± 3			
Axon-reflex	39 ± 15	39 ± 15			
Nadir	20 ± 10	20 ± 10	<0.001*	0.76	0.99
Plateau 39*°C*	48 ± 15	46 ± 21			
Rapid 42°C					
Absolute flux(PU)					
Baseline	22 ± 9	25 ± 16			
Axon-reflex	199 ± 60	208 ± 60			
Nadir	165 ± 64	177 ± 74	<0.001*	0.51	0.99
Plateau 42*°C*	252 ± 72	253 ± 67			
Plateau 44*°C*	300 ± 79	302 ± 63			
Absolute CVC (PU/mmHg)					
Baseline	0.21 ± 0.10	0.25 ± 0.16			
Axon-reflex	1.90 ± 0.61	2.00 ± 0.61			
Nadir	1.57 ± 0.64	1.71 ± 0.74	<0.001*	0.29	1.00
Plateau 42*°C*	2.39 ± 0.74	2.43 ± 0.66			
Plateau 44*°C*	2.81 ± 0.81	2.91 ± 0.74			
Maximal CVC (%CVC_{max})					
Baseline	8 ± 3	8 ± 4			
Axon-reflex	67 ± 11	68 ± 11			
Nadir	55 ± 16	57 ± 19	<0.001*	0.65	0.95
Plateau 42*°C*	85 ± 8	83 ± 12			

Data are presented as mean ± SD. LMM, linear mixed model. *Main effect of time $P<0.001$ vs baseline.

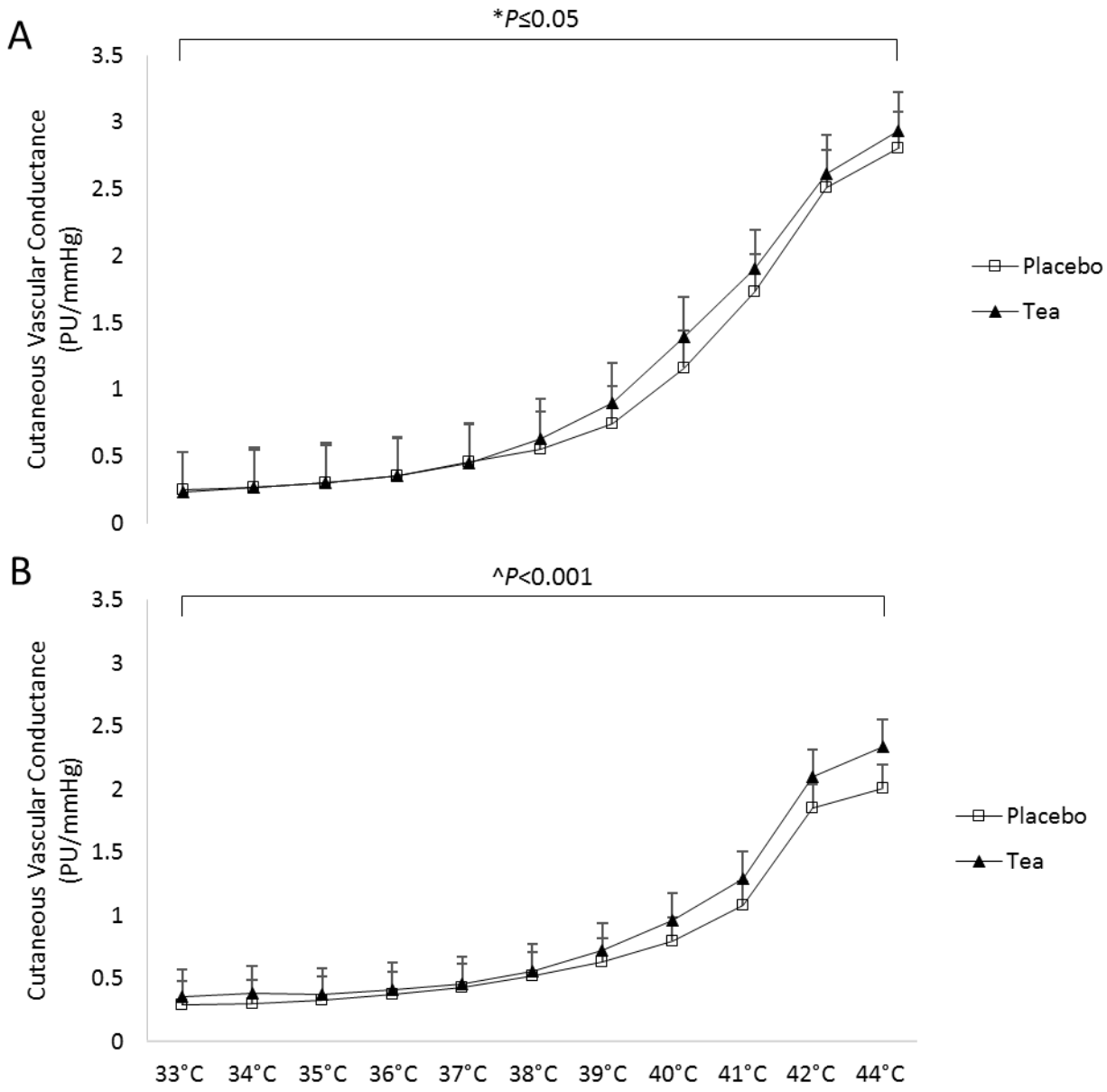

Figure 5.4 Cutaneous vascular conductance (CVC) responses across time points (from baseline at 33°C to maximal plateau at 44°C) following gradual local heating using **A.** laser-Doppler flowmetry (LDF) and **B.** full-field laser perfusion imaging (FLPI) in 20 healthy volunteers when heating was preceded by ingestion of placebo (open squares) or tea (solid triangles). Data are presented as means, with error bars representing SE. *Main effect of condition $P \leq 0.05$ placebo vs tea, ^main effect of condition $P < 0.001$ placebo vs tea. Taken from (Woodward *et al.*, 2016).

Table 5.2 Cutaneous blood flow responses to local heating for the *Gradual$_{LDF}$ (42°C)* and *Gradual$_{FLPI}$ (42°C)* protocols for placebo and tea interventions.

	Intervention (mean ± SD)		LMM		
Gradual$_{LDF}$ (42°C)	**Placebo**	**Tea**	**Time**	**Tea**	**Time*Tea**
Absolute flux(PU)					
Baseline	26 ± 11	24 ± 9			
Plateau 42°C	268 ± 79	278 ± 61	<0.001*	0.06	0.93
Plateau 44°C	302 ± 84	319 ± 45			
Absolute CVC (PU/mmHg)					
Baseline	0.25 ± 0.11	0.23 ± 0.09			
Plateau 42°C	2.51 ± 0.76	2.61 ± 0.64	<0.001*	0.04^	0.95
Plateau 44°C	2.80 ± 0.82	2.93 ± 0.51			
Maximal CVC (%CVC$_{max}$)					
Baseline	9 ± 5	8 ± 3			
Plateau 42°C	90 ± 7	89 ± 14	<0.001*	0.82	0.98
Gradual$_{FLPI}$ (42°C)					
Absolute flux(PU)					
Baseline	30 ± 9	36 ± 8			
Plateau 42°C	197 ± 51	222 ± 50	<0.001*	<0.001^	0.50
Plateau 44°C	216 ± 65	253 ± 68			
Absolute CVC (PU/mmHg)					
Baseline	0.29 ± 0.09	0.36 ± 0.07			
Plateau 42°C	1.85 ± 0.55	2.10 ± 0.57	<0.001*	<0.001^	0.66
Plateau 44°C	2.01 ± 0.64	2.34 ± 0.72			
Maximal CVC (%CVC$_{max}$)					
Baseline	17 ± 11	17 ± 8			
Plateau 42°C	94 ± 10	91 ± 6	<0.001*	0.35	1.00

Data are mean ± SD. LMM, linear mixed model. *Main effect of time P<0.001 vs baseline. ^Main effect of intervention; placebo vs tea P≤0.05.

5.4 Discussion

The primary aim of this study was to test the hypothesis that a single dose of black tea ingestion improves cutaneous microcirculation following both rapid and gradual local skin heating. The study found that gradual local heating of the skin to 42℃ induced a greater vasodilatory response following tea ingestion compared to placebo when expressed as absolute flux and CVC. The ability of tea to improve local gradual heating responses in the skin was reinforced by the observation that both LDF and FLPI, two distinct but accepted techniques to assess skin perfusion, detected this effect. Conversely, rapid local heating did not demonstrate a significant increase in cutaneous microcirculation with tea ingestion, either for *the Rapid 39°C* or *Rapid 42°C* protocols. Taken together, this study provides some further evidence that regular tea ingestion may mediate its potential cardiovascular benefits via improvements in (cutaneous) microvascular function.

This study is the first to explore the acute effects of tea ingestion on the cutaneous microcirculation whilst adopting a rigorous protocol involving blind analysis of rapid and gradual heating protocols as well as two distinct, accepted techniques. This observation fits with the general observation of tea being able to enhance endothelial function in conduit vessels when assessed by FMD (Grassi *et al.*, 2009b; Schreuder *et al.*, 2014). Taken together, these findings suggest that acute tea ingestion improves vascular function across the vascular tree, including skin microvessels, possibly via upregulation of vasodilator mechanisms.

In contrast to gradual local heating, rapid heating of the skin did not alter cutaneous vascular function following tea ingestion when compared to placebo. Findings were similar for both rapid heating protocols (*Rapid 39°C* and *Rapid 42°C*). Interestingly, a recent observational study (Wasilewski *et al.*, 2016) found improved microvascular function following regular consumption of green tea (14-days) using rapid heating (whilst no measure of gradual heating was included). Important differences were present between studies, especially since this previous study did not include a placebo control, did

not fully adhere to guidelines for vascular assessment (e.g. control of menstrual cycle) (Thijssen *et al.*, 2011), and was limited by a lack of control of dietary habits (Grassi *et al.*, 2009b). Furthermore, whilst our study investigated the acute (2-hour) effects of tea, they examined a protocol of 14-days of green tea. Despite the *Rapid 39°C* and *Gradual 42°C* protocols both being linked to the release of NO, distinctly different responses are clearly evident between the gradual and rapid heating protocols in the present study. Different vasodilator pathways directly influence the cutaneous microcirculation, including neurogenic reflexes and local chemical mediators (Minson *et al.*, 2001; Black *et al.*, 2008b; Dawson *et al.*, 2015). The rate at which the skin is heated alters the contribution of these vasodilator pathways, with rapid (0.5°C per 5s) local heating inducing a transient axon-reflex mediated vasodilation that is produced via activation of heat sensitive sensory/nociceptive nerves releasing calcitonin gene-related peptide (CGRP) and substance P and adrenergic nerves releasing norepinephrine and neuropeptide Y (Minson *et al.*, 2001; Johnson *et al.*, 2014). This initial neurogenic response is followed by a more gradual, sustained vasodilation. In both phases, vasodilation occurs through complex pathways that lead to the production of NO and smooth muscle relaxation via hyperpolarization from endothelial derived hyperpolarisation factors (EDHFs) (Johnson *et al.*, 2014), with a greater (but not exclusive) contribution of NO during the plateau phase (Minson *et al.*, 2001; Choi *et al.*, 2014). Furthermore, the relative contribution of NO to the vasodilation during the plateau phase of the rapid heating protocols depends upon the target heating temperature, as the heating response to 39°C seems to depend more on NO than the response to 42°C (Minson *et al.*, 2001; Choi *et al.*, 2014). These studies, therefore, demonstrate that the underlying mechanisms for cutaneous vasodilation differ based on the rate and maximum level of heating. The different vasodilator pathways for these heating protocols may contribute to the distinct findings in this study. From a methodological perspective, the differences between rapid and gradual local heating highlight the importance of using multiple heating protocols simultaneously when exploring the impact of an intervention on skin perfusion.

The higher vasodilatory responses that were observed following gradual heating of the skin were demonstrated for arbitrary flux and CVC values, for both LDF and FLPI techniques. However, the difference in responses between the tea and placebo trials was not significant when data were expressed as $\%CVC_{max}$. The skin is commonly heated to 44°C to reach maximal vasodilation and expressing CVC as a percentage of maximal perfusion is often considered the preferred method of data expression (Cracowski *et al.*, 2006), with improved reproducibility compared to flux or CVC (Dawson *et al.*, 2015). Despite a main effect of tea on flux and CVC, post-hoc analyses revealed no differences between trials at 44°C (LDF: flux = 0.17 and CVC = 0.19; FLPI: flux = 0.09 and CVC = 0.08). However, the magnitude of differences in flux and CVC between tea and placebo are larger than one may expect based on day-to-day variation (Dawson *et al.*, 2015). This provides some indication that the tea intervention may have altered cutaneous perfusion at 44°C local heating.

5.4.1 Clinical Relevance

Tea consumption is known to have cardiovascular benefits, including a reduction in blood pressure after short-to long-term intervention, possibly mediated (in part) by improved endothelial function of conduit vessels (Hodgson *et al.*, 2002; Ras *et al.*, 2011; Schreuder *et al.*, 2014). In the present study, cutaneous microcirculation responses to gradual heating improved following tea ingestion. It may be speculated that these findings may have implications for individuals with microvascular complications and skin endothelial dysfunction, such as type 2 diabetes mellitus. Interestingly, consumption of tea has been associated with a reduced risk for type 2 diabetes mellitus (Yang *et al.*, 2014). The study findings thus support the hypothesis that regular tea consumption may have potential benefit in such patient groups. Future studies are warranted to explore this hypothesis.

5.4.2 Limitations

Due to the modest sample size, the findings of the present study cannot be generalised towards the wider populace. Furthermore, although a middle-aged population was included, who are likely at an increased risk of CVD, the findings cannot be simply extrapolated to clinical groups. Moreover, the study population may have impaired endothelial function as blunted cutaneous NO-mediated

vasodilation has been demonstrated in older individuals (Black *et al.*, 2008b), suggesting that young healthy volunteers may exhibit different results than the older population studied. Therefore, future work is required to explore the potential impact of acute as well as chronic tea ingestion on cutaneous vascular function in both individuals with compromised endothelial function and in young, healthy individuals. A further limitation is that plasma measures of flavonoids or NO compounds were not obtained and, therefore, the present study does not provide any biochemical or biomolecular insight into the mechanisms underlying the improvement in cutaneous microvascular function. However, it is important to emphasise that this was not the purpose of the study, particularly given that this is the first to explore the effects of acute tea ingestion on the cutaneous microcirculation.

In conclusion, the study findings suggest that acute tea ingestion enhances cutaneous vascular function in a healthy, middle-aged population, when measured following gradual local heating to 42°C. Therefore, these data suggest that acute tea ingestion has a beneficial impact on vascular function at the microcirculatory level, which is likely achieved through a mechanism related to activation of endothelium-derived vasodilators. These improvements in cutaneous microvascular function may contribute to the potential cardiovascular health benefits of regular tea ingestion. Future studies are required to explore the acute and chronic effects of tea on individuals with increased CVD risk and in clinical populations with *a priori* endothelial dysfunction. Furthermore, given the detrimental impact of 'unhealthy' lifestyles on increasing CVD risk, further investigation is warranted to determine whether tea may exert a cardioprotective role on peripheral and cerebral vascular function in the context of an 'unhealthy' lifestyle.

CHAPTER 6: GREEN TEA ATTENUATES THE DELETERIOUS IMPACT ON PERIPHERAL VASCULAR FUNCTION AND INSULIN SENSITIVITY ASSOCIATED WITH AN UNHEALTHY LIFESTYLE

6.1 Introduction

Cardiovascular related pathologies have emerged as a major public health burden over recent decades, with scientific evidence increasingly implicating modifiable lifestyle factors in the progression of cardiovascular risk towards overt CVD. Physical inactivity and poor dietary habits are major modifiable risk factors linked with detrimental changes to cardiometabolic health (Lavie *et al.*, 2009; Booth *et al.*, 2012). Physical inactivity has increased significantly in recent decades and is now one of the leading causes for global mortality (WHO, 2015). Low levels of leisure time physical activity are associated with increased risk for CVD (Hamburg *et al.*, 2007; Thijssen *et al.*, 2010; Boyle *et al.*, 2013; Holwerda *et al.*, 2015) and metabolic disease (Hamburg *et al.*, 2007; Thyfault & Krogh-Madsen, 2011). In addition, food intake also impacts cardiovascular and metabolic health, with high fat (most likely trans) (Skeaff & Miller, 2009; Michas *et al.*, 2014; de Souza *et al.*, 2015), high calorie diets being strongly associated with long-term cardiovascular and metabolic disease risk (Hennig *et al.*, 2001; Liu & Manson, 2001; Brons *et al.*, 2009; Dow *et al.*, 2015). Interestingly, even short-term (<7-days) high fat dietary interventions (e.g., >40% extra calories from fat) in healthy adults lead to increased baseline glucose levels (Brons *et al.*, 2009), as well as postprandial increases in glucose and insulin (Parry *et al.*, 2017). Furthermore, habitual high fat dietary consumption is associated with impaired endothelial-dependent vasodilation in sedentary adults (Dow *et al.*, 2015) and a single high fat meal has demonstrated a postprandial decline in endothelial function in healthy adults (Vogel *et al.*, 1997; Bae *et al.*, 2001; Tsai *et al.*, 2004). This work highlights the detrimental impact of both lowering physical activity levels and excessive food intake.

Epidemiological research continues to link increased flavonoid ingestion with a reduction in cardiovascular events, including stroke and dementia (Commenges *et al.*, 2000; Vita, 2005; Fisher *et al.*, 2006; Malar & Devi, 2014). Furthermore, regular tea ingestion, a key source of flavonoids, has been shown to lower BP (Hodgson *et al.*, 2012) and improve NO-mediated, endothelium-dependent

dilation of conduit arteries (Hodgson *et al.*, 2002; Grassi *et al.*, 2009b; Schreuder *et al.*, 2014), particularly in the presence of cardiovascular pathology or following an acute, single high fat meal (Corretti *et al.*, 2002; Hodgson *et al.*, 2005; Grassi *et al.*, 2012b; Grassi *et al.*, 2016). In addition, regular intake of tea is also associated with a lower risk for developing diabetes (Iso *et al.*, 2006; Stote & Baer, 2008; Jing *et al.*, 2009; Park *et al.*, 2014). For example, plasma glucose lowering effects have been observed in both type 2 diabetic individuals (Hosoda *et al.*, 2003) and in healthy adults (Bryans *et al.*, 2007), suggesting that tea may improve metabolism, via changes in glucose handling and insulin resistance.

In this study, it was speculated that tea may represent a potential approach in targeting the cardiovascular and metabolic impairments induced by a lifestyle characterised by too little physical activity and excessive calorie intake (e.g. the Western lifestyle, but also periods of forced unhealthy lifestyle (e.g., illness, injury)). The aim of this study was, therefore, to explore whether daily green tea consumption (equivalent to 6 cups/day) ameliorates the impairments in peripheral (conduit artery and skin microvessels) and cerebrovascular function and insulin sensitivity after a 7-day 'unhealthy' lifestyle, combining physical activity reduction (-50% steps per day) and overfeeding (+50% kcal per day, comprising 65% fat) in healthy male participants. It was hypothesised that daily consumption of green tea would abrogate changes in peripheral and cerebrovascular function following 7-days of reduced physical activity and increased calorie consumption.

6.2 Methods

6.2.1 Participants

Twelve healthy male participants were recruited through local advertisement. All participants were healthy and non-smokers (29 ± 6 yrs, height 1.76 ± 0.1 m, weight 77.0 ± 10.0 kg, BMI 25 ± 2 kg/m^2, baseline MAP 84 ± 8 mmHg). For details of the inclusion and exclusion criteria, please refer to *Chapter*

3, General Methods. Participants' habitual activity levels were established through completion of an IPAQ questionnaire (Lee *et al.*, 2011) and a 4-day self-reported diary. A hip-mounted pedometer (Digi-walker SW-701, Yamax, Japan) and tri-axial accelerometer (GT3X BT+ model, Actigraphy, Pensacola, Florida, USA) were used to count the number of daily steps and to record all non-water based physical activity, respectively. The reliability of tri-axial accelerometry to estimate physical activity has been previously determined (Aadland & Ylvisåker, 2015). Subjects who recorded <8,000 steps per day were excluded from participation. During the 4-day physical activity assessment period, participants also recorded their daily dietary intake in a food diary to determine their habitual consumption. The activity and dietary data were subsequently used to calculate the physical activity reduction of -50% steps per day and overfeeding by +50% kcal per day. After being fully informed of the methods verbally and in writing, written informed consent was obtained from all participants. The study conformed to the *Declaration of Helsinki* and was approved by the local research ethics committee. The study was registered at clinicaltrials.gov (NCT02777853).

6.2.2 Experimental Design

All participants underwent two 7-day periods of lifestyle intervention, both consisting of physical activity reduction (-50% steps per day) and overfeeding (+50% kcal per day, comprising 65% fat), combined with either tea or placebo ingestion (Unhealthy Lifestyle + Placebo, UL-Placebo; or Unhealthy Lifestyle + Tea, UL-Tea), in a randomised, controlled, double-blind, crossover design (Figure 6.1). Each period was separated by a 13-day washout to avoid any carry-over effects. In the week preceding each laboratory visit, participants were asked to refrain from drinking all types of tea (not including the test product) and to avoid all other food sources high in polyphenols, such as berries, dark chocolate, apples and red wine (Perez-Jimenez *et al.*, 2010).

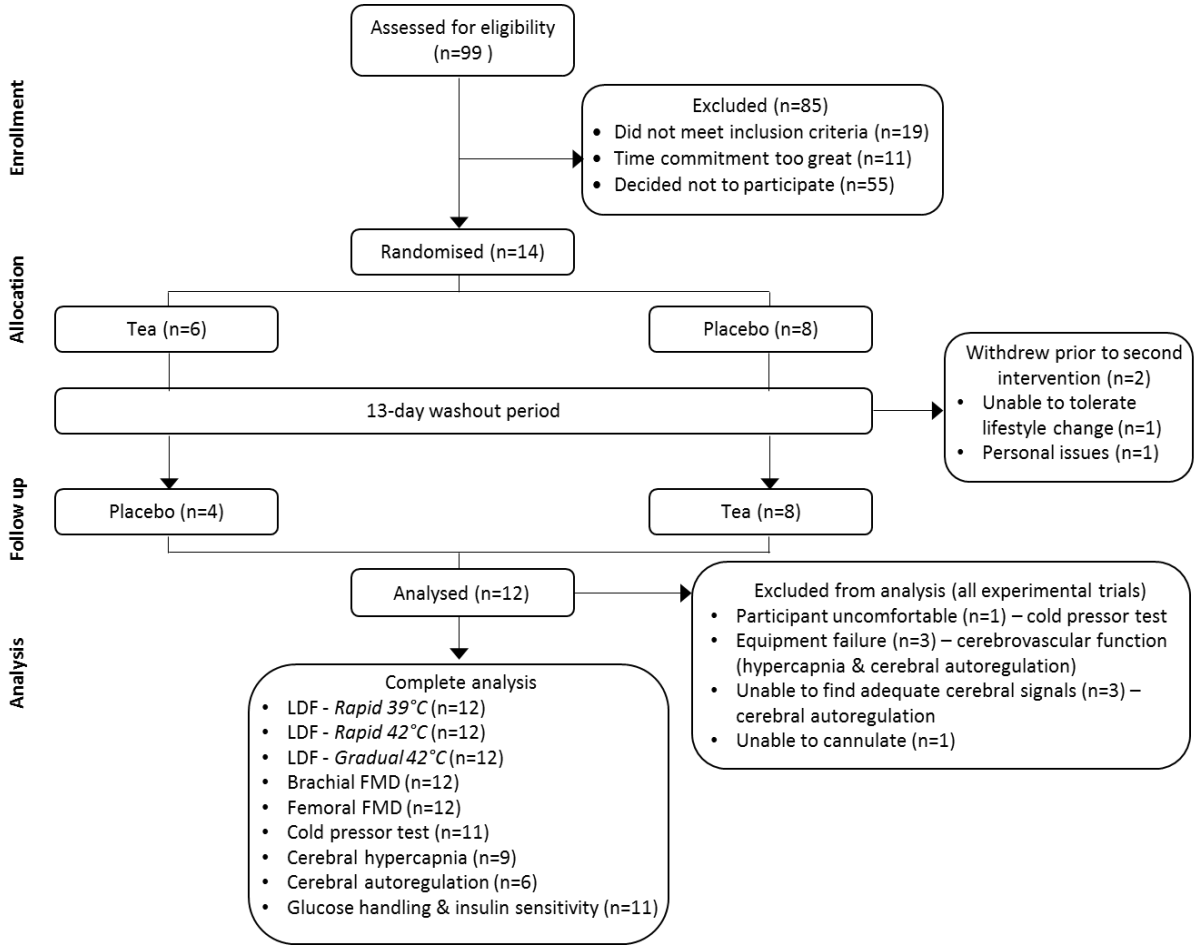

Figure 6.1. CONSORT diagram showing the flow of participants through each stage of the randomised trial (FMD, flow-mediated dilation; LDF, laser Doppler flowmetry).

6.2.2.1 Lifestyle Intervention

During each 7-day intervention period, participants were instructed to reduce their physical activity levels by 50%, comprising of a step reduction target and a reduction in the volume of other physical activities (i.e. gym, running, cycling) which were calculated from the 4-day habitual diary. The physical activity reduction was monitored via a hip-mounted accelerometer (GT3X BT+ model, Actigraphy, Pensacola, Florida, USA), in addition to self-reported step counts using a hip-mounted pedometer (Digi-walker SW-701, Yamax, Japan). Overfeeding was achieved through the provision of "snack boxes" with the equivalent of ~800-2000 kcal for participants to consume on a daily basis; kcal were calculated according to participants' habitual dietary intake during the 4-day dietary diary. Participants

were instructed to maintain their normal diet during each 7-day intervention period, other than restricting foods and beverages high in polyphenols, such as berries, red wine, dark chocolate etc. Habitual dietary patterns were monitored through completion of self-reported food diaries (excluding the additional intake from the “snack boxes”).

6.2.2.2 Tea Intervention

During each 7-day intervention period, participants ingested three doses of an active green tea drink (UL-Tea) or a placebo tea drink (UL-Placebo) per day at regular intervals (morning, afternoon and evening), which participants were advised to take without, or at least 15-minutes before, a meal. Participants and researchers were blinded as to the test product. The crossover design was chosen to eliminate between-participant variability and computer-generated randomisation was used to reduce potential selection bias. The green tea was previously brewed with hot water in a large quantity, prior to being dried into a powder form. A product of similar colour and taste to the green tea, but not containing the presumed actives of tea (polyphenols) was used as the placebo tea drink (Table 6.1). Pre-weighted servings of the test products were supplied to the participants in laminated aluminium foil sachets (1 gram of tea powder per sachet) and participants were given instructions regarding the tea preparation; two sachets of tea powder to be completely dissolved in approximately 300 ml of boiling water, constituting a ‘dose’. It was stressed to the participants that no additives to the tea were permitted (milk, sugar, lemon etc.) and that the tea should be consumed whilst it was still hot. The difference in the maltodextrin content (19 kcal per day) between the active and placebo teas was adjusted for within the overfeeding provision. Participants were instructed to avoid all other types of tea, other than the test product that they were given.

Table 6.1 Composition of the active and placebo tea test products.

	Placebo tea % on dry weight basis	**Active tea** % on dry weight basis
Gallocatechin (GC)	0.00	0.02
Epicatechin gallate (ECG)	0.00	0.05
Gallic Acid	0.00	0.16
Catechin (C)	0.00	0.58
Gallocatechin gallate (GCG)	0.00	0.92
Epicatechin (EC)	0.00	1.53
Catechin gallate (CG)	0.00	2.39
Epigallocatechin (EGC)	0.00	4.07
Epigallocatechin gallate (EGCG)	0.00	5.96
Caffeine	0.00	3.90
Maltodextrin	94.00	21.05
Tea flavour	6.00	0.00

6.2.2.3 Experimental Measures

Participants attended the laboratory before and after each 7-day intervention (4 visits), after adhering to all pre-test instructions regarding fasting, avoiding caffeine etc., as outlined in *Chapter 3, General Methods* (section 3.1). Anthropometric measurements were recorded at visit 1, with body mass repeated at each subsequent visit (see *Chapter 3, General Methods,* section 3.2). During each visit, microvascular function, conduit artery endothelial function, haemodynamic function, cerebrovascular function, glucose handling and insulin sensitivity were examined. Baseline and thermally stimulated forearm cutaneous blood flow was examined simultaneously using rapid (to 39 and 42°C) and gradual (to 42°C) local heating protocols on the dominant forearm using LDF. Heart rate and BP were recorded using an automated sphygmomanometer (Dinamap V100, GE Healthcare, UK) positioned on the contralateral arm. Conduit artery endothelial function (FMD) was examined at the brachial (non-dominant arm) and right femoral arteries. Cerebrovascular function was assessed via CO_2 reactivity and dynamic cerebral autoregulation during squat-stand manoeuvres (SSM) at 0.10 Hz (5-second squat: 5-second stand) and 0.20 Hz (2.5-second squat: 2.5-second stand). For details of the full experimental procedures for all vascular measurements, please refer to *Chapter 3, General Methods*. Following completion of the vascular measures, participants underwent a mixed meal tolerance test (MTT).

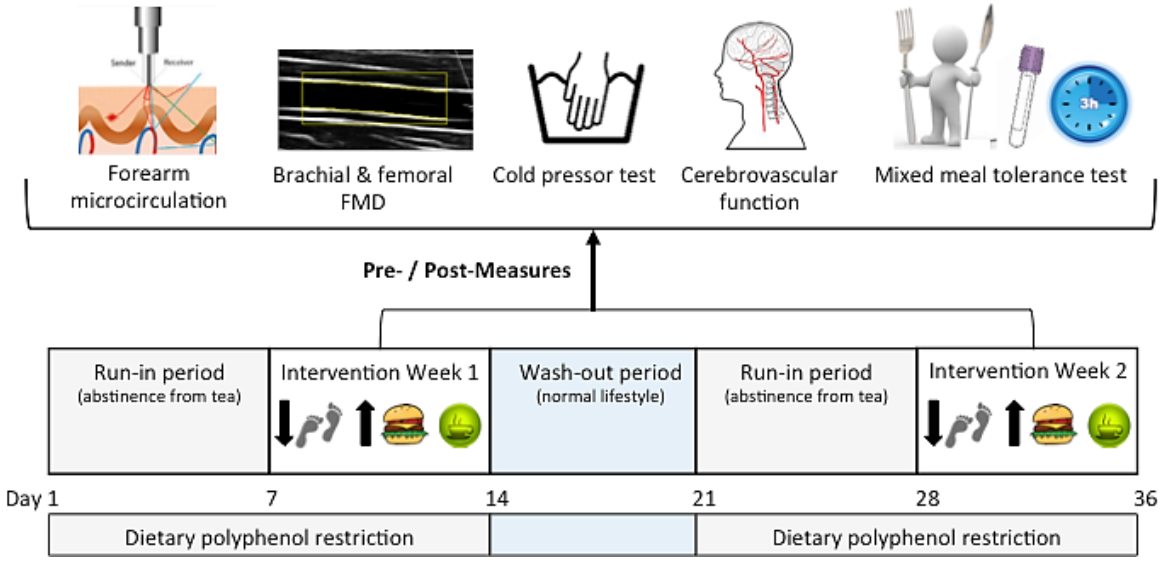

Figure 6.2 Study overview depicting the various stages, restriction of dietary polyphenols and intervention periods, including the measures performed at each of the four laboratory visits.

At the time of venous blood sampling, participants had fasted for ≥10-hours and refrained from vigorous exercise for the preceding 24-hours. Following sterilisation of the sampling site, a 20G cannula (Venflon Pro, BD, NJ, USA) was inserted into the antecubital vein of one arm. A three-way stopcock (BD Connecta, NJ, USA) was subsequently attached to enable multiple venous blood sampling and flushing of the cannula. Baseline samples were collected for glucose (5 ml) and insulin (6 ml), in silica and EDTA vacutainers, respectively, following which participants consumed a mixed meal (1200 kcal, comprising 60% carbohydrates, 33% fat and 7% protein; Table 3.2). Further postprandial blood samples were collected after 30, 60, 90, 120 and 180-min. Following each blood sample, isotonic saline (3 ml; B Braun, UK) was used to keep the cannula patent. All blood samples were centrifuged (1000 g for 10-min at 4°C) to obtain plasma samples, which were subsequently stored in aliquots at −80°C for later analysis.

Table 6.2 Macronutrient content of the mixed meal tolerance test (MTT).

Food Item	Quantity (g)	kcal	Carbohydrate (g)	Fat (g)	Protein (g)
Chocolate spread sandwich					
Thick white bread	150 g	357	66.9	3.3	12.9
Chocolate spread	90 g	513	51.8	32.4	2.7
Medium banana	120 g	105	30.0	0.4	1.3
White chocolate cereal bar	50 g	226	31.0	8.2	4.4
Total		1201	179.7	44.3	21.3
% of Total Calories			60%	33%	7%

6.2.2.4 Data Analysis

For details of the data analysis methods for all vascular measurements, please refer to *Chapter 3, General Methods*. Plasma glucose was determined using an ILab-600 semi-automatic spectrophotometric analyser and glucose hexokinase assay (Randox, London, UK). Plasma insulin concentrations were determined using a direct insulin ELISA kit (Invitrogen, UK) and insulin levels determined using a monochromator microplate reader (Clariostar, BMG LABTECH, Ortenberg, Germany).

6.2.3 Statistical Analysis

The effect size of the interventions could not be estimated or calculated due to the novelty of the study. Therefore the number of volunteers was based upon the sample size of earlier vascular studies (Hodgson *et al.*, 2013; Grassi *et al.*, 2015) and previous chapters contained within this thesis. Data were expressed as mean ± SD and statistical significance was set at $P \leq 0.05$. For all protocols, linear mixed models (main effects of condition and time) were used to examine the differences between Placebo and Tea interventions on vascular function and insulin sensitivity following 7-days of physical activity reduction and overfeeding. The repeated covariance type was Unstructured and Condition, Time and Condition*Time were specified as Fixed Effects (intercept was included) and as Estimated Marginal Means. The Test of Fixed Effects Condition*Time interaction was interpreted. Significant

main effects of Time or Condition or a Time*Condition interaction were followed up with a simple main effects analysis and the least significant difference (LSD) approach to multiple comparisons (Perneger, 1998). Data were stored and transformed within Microsoft Excel (Microsoft Office 2010, Microsoft Corporation), and statistical analyses were performed using SPSS 22.0 (SPSS, Chicago, IL, USA).

6.3 Results

Self-reported compliance to consuming the tea (3 cups per day for 7-days per intervention week) and additional food was 100%. Self-reported compliance to the physical activity reduction was 100%, and was subsequently verified via activity data downloaded from the hip-mounted accelerometer worn during each 7-day intervention period. Compared to baseline screening (11,103 ± 3385 steps), UL-Placebo (5880 ± 1462 steps) and UL-Tea (5710 ± 1390 steps) demonstrated good compliance, with a 47% and 49% step reduction, respectively. Habitual calorie consumption during the intervention weeks remained similar to baseline levels (2373 ± 864 kcal) for both UL-Placebo (2250 ± 771 kcal) and UL-Tea (2330 ± 779 kcal), indicating good compliance to the dietary intervention. Two participants withdrew from the study prior to completion. All remaining participants completed measures of BP, microvascular function and conduit artery endothelial function (both n=12). One participant was unable to complete the cold pressor test due to discomfort. Three participants were unable to complete tests of cerebrovascular function (hypercapnia and cerebral autoregulation) and a further three participants did not complete the cerebral autoregulation measures due to suboptimal TCD signals, leading to a population of n=9 and n=6, respectively. Due to problems associated with venous cannulation, one participant did not complete measures of glucose handling and insulin sensitivity (n=11).

Participants demonstrated a non-significant increase in total body mass following the UL-Placebo (77.4 ± 10.5 kg vs 78.1 ± 11.0 kg) and UL-Tea interventions (76.9 ± 9.0 kg vs 77.6 ± 10.6 kg, main effect of time; *P*=0.07). However, no significant difference was observed between UL-Placebo and UL-Tea conditions (*P*=0.31) and there was no interaction of condition and time (*P*=0.92).

6.3.1 Metabolic Function

In the basal state, prior to the mixed meal tolerance test, there were no significant differences in fasting glucose or insulin levels between pre-UL-Placebo and pre-UL-Tea conditions (Figure 6.3). Glucose displayed a typical initial increase and subsequent decrease during the 3-hour MTT (*P*<0.001). There was no main effect of time (pre vs post) (*P*=0.13) or condition (*P*=0.53) for glucose. There was a significant interaction of condition*time (*P*=0.03); following UL-Placebo, blood glucose was elevated at 60- and 90-minutes compared to pre-UL-Placebo, whereas a reduced blood glucose was demonstrated across all time points following UL-Tea (*P*=0.03, Figure 6.3).

Insulin displayed a typical initial increase and subsequent decrease during the 3-hour MTT (*P*<0.001). There was no main effect of time (pre vs post) (*P*=0.41), but there was a main effect of condition (*P*=0.04) and a significant interaction of condition*time (*P*<0.001). Post-hoc analysis revealed that insulin levels were higher across all time points after 7-days of UL-Placebo, consistent with increased insulin resistance (Figure 6.3). Following UL-Tea, however, the insulin response was reduced and demonstrated an interaction of time*condition (*P*<0.001, Figure 6.3).

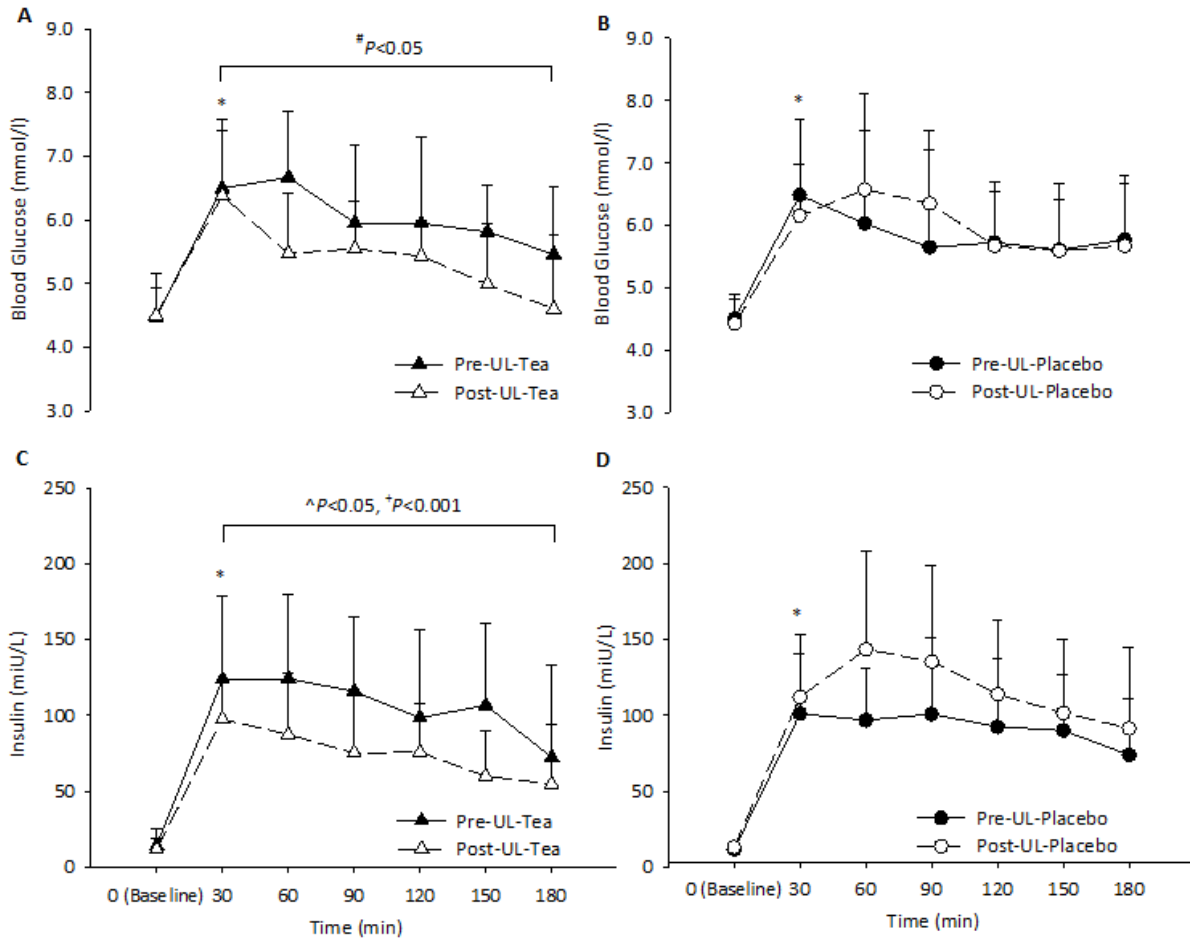

Figure 6.3 Mixed meal tolerance test (MTT) 180-minutes for postprandial glucose for **A.** Tea and **B.** Placebo, and insulin for **C.** Tea and **D.** Placebo interventions. Data are presented as means, with error bars representing SD. *Main effect of time (across 3-hour MTT) *P*<0.001; ^main effect of condition *P*≤0.05; #time*condition interaction *P*≤0.05; +time*condition interaction *P*<0.001.

6.3.2 Microvascular Function

Throughout the duration of the microvascular function assessment periods, MAP was lower post-UL-Tea (84 ± 7 vs 82 ± 6) and was higher post-UL-Placebo (83 ± 5 vs 85 ± 5, time*condition interaction *P*=0.06). There was no main effect of condition (*P*=0.67). There were no differences in baseline cutaneous perfusion between pre-UL-Tea or pre-UL-Placebo trials for absolute flux, CVC or $\%CVC_{max}$ (Figures 6.4 to 6.6). Due to the impact of UL-tea on blood pressure, data below were only presented for CVC and $\%CVC_{max}$. Furthermore, all local heating protocols induced the typical pattern of an initial peak, nadir and subsequent plateau during rapid heating, and absence of a peak and nadir with gradual heating.

6.3.2.1 Rapid Local Heating: Rapid 39°C

When expressed as CVC, there was no main effect of time (*P*=0.84) or condition (*P*=0.24), but there was a close to significant interaction of condition*time (*P*=0.09), whereby a decrease in CVC responses to local heating was observed following UL-Placebo, whereas UL-Tea demonstrated a slight increase (Figure 6.4). When data were expressed relative to maximal data (%CVC_{max}), there was no main effect of time (*P*=0.36) or condition (*P*=0.93), nor were there any interactions of condition*time (*P*=0.86) and condition*time*temp (*P*=0.56).

6.3.2.2 Rapid Local Heating: Rapid 42°C

When expressed as CVC, there was no main effect of time (*P*=0.57) or condition (*P*=0.26) or an interaction of condition*time (*P*=0.18), but there was a close to significant interaction of condition*time*temp (*P*=0.09) whereby a decrease in CVC was observed following UL-Placebo, whereas UL-Tea demonstrated a slight increase (Figure 6.5). When data were expressed relative to maximal data (%CVC_{max}), there was no main effect of time (*P*=0.65) or condition (*P*=0.80), nor was there an interaction of condition*time*temp (*P*=0.22), but there was a significant interaction of condition*time (*P*=0.03) whereby %CVC_{max} was higher post-UL-Placebo but lower post-UL-Tea.

6.3.2.3 Gradual Local Heating: Gradual 42°C

When expressed as CVC, there was no main effect of time (*P*=0.75) or condition (*P*=0.78) or an interaction of condition*time (*P*=0.63), but there was a significant interaction of condition*time*temp (*P*=0.02) whereby no change in CVC was observed following UL-Placebo, whereas UL-Tea demonstrated a larger CVC to gradual local heating (Figure 6.6). When data were expressed relative to maximal data (%CVC_{max}), there was no main effect of time (*P*=0.18) or condition (*P*=0.21), nor were there any interactions of condition*time (*P*=0.20) or condition*time*temp (*P*=0.17).

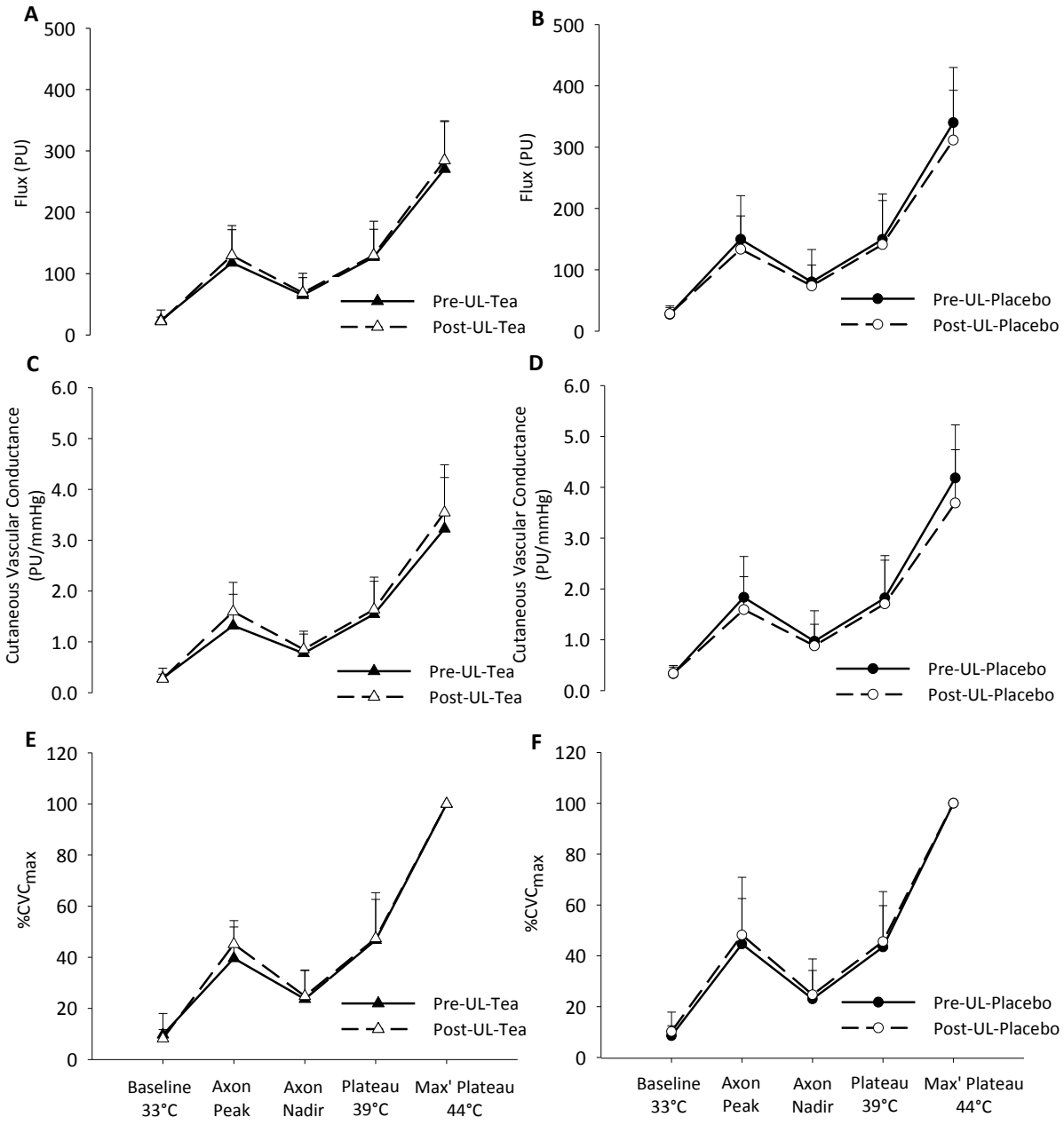

Figure 6.4 Microvascular responses across time points (baseline at 33°C, axon peak, axon nadir, plateau at 39°C and maximal plateau at 44°C) following *Rapid 39°C* local heating for flux (**A** and **B**), CVC (**C** and **D**) and %CVC_{max} (**E** and **F**) for UL-Tea and UL-Placebo interventions, respectively, in 12 healthy male volunteers. Data are presented as means, with error bars representing SD.

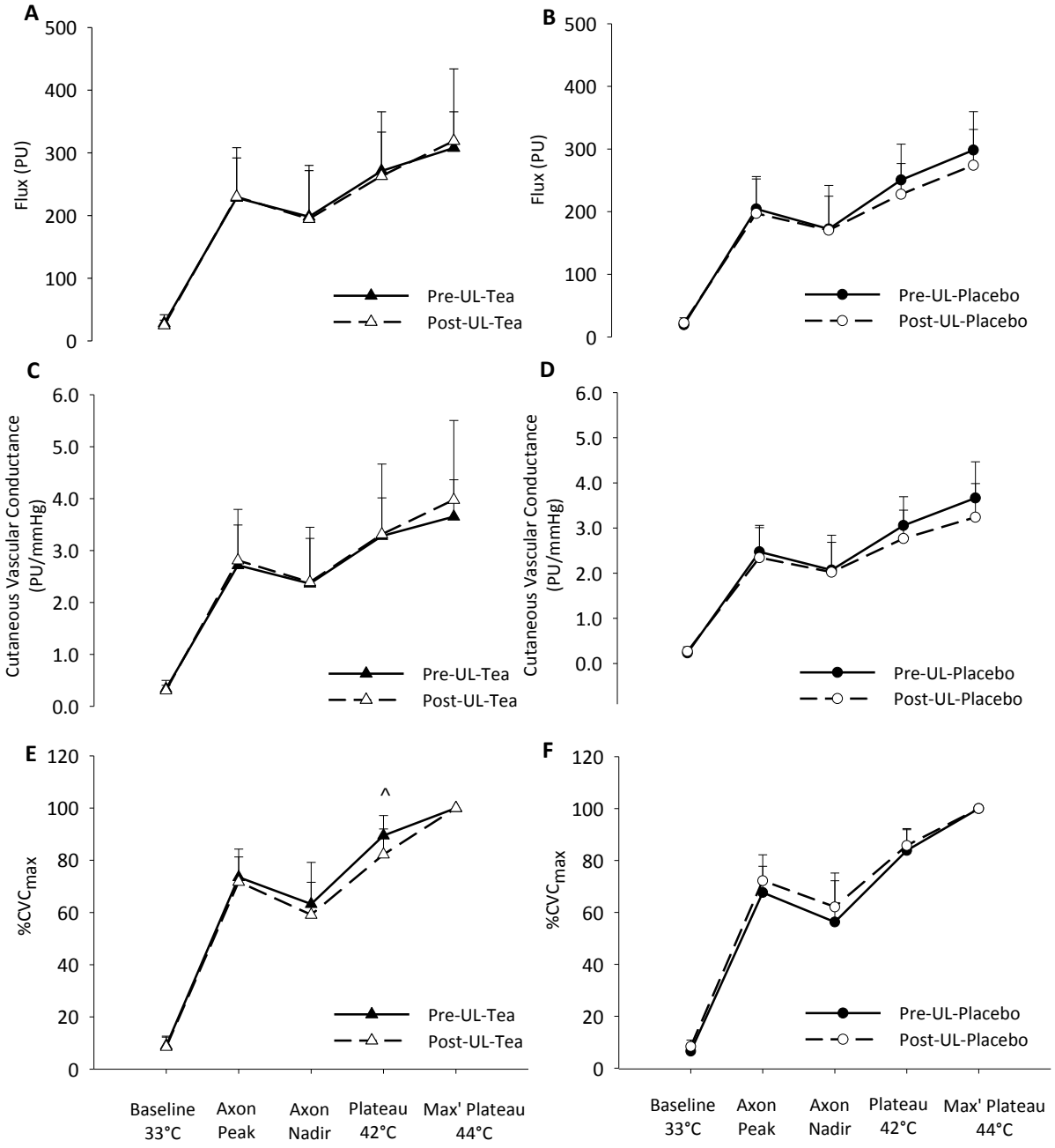

Figure 6.5 Microvascular responses across time points (baseline at 33°C, axon peak, axon nadir, plateau at 42°C and maximal plateau at 44°C) following *Rapid 42°C* local heating for flux (**A** and **B**), CVC (**C** and **D**) and %CVC_{max} (**E** and **F**) for UL-Tea and UL-Placebo interventions, respectively, in 12 healthy male volunteers. Data are presented as means, with error bars representing SD. ^Condition*time interaction $P \leq 0.05$.

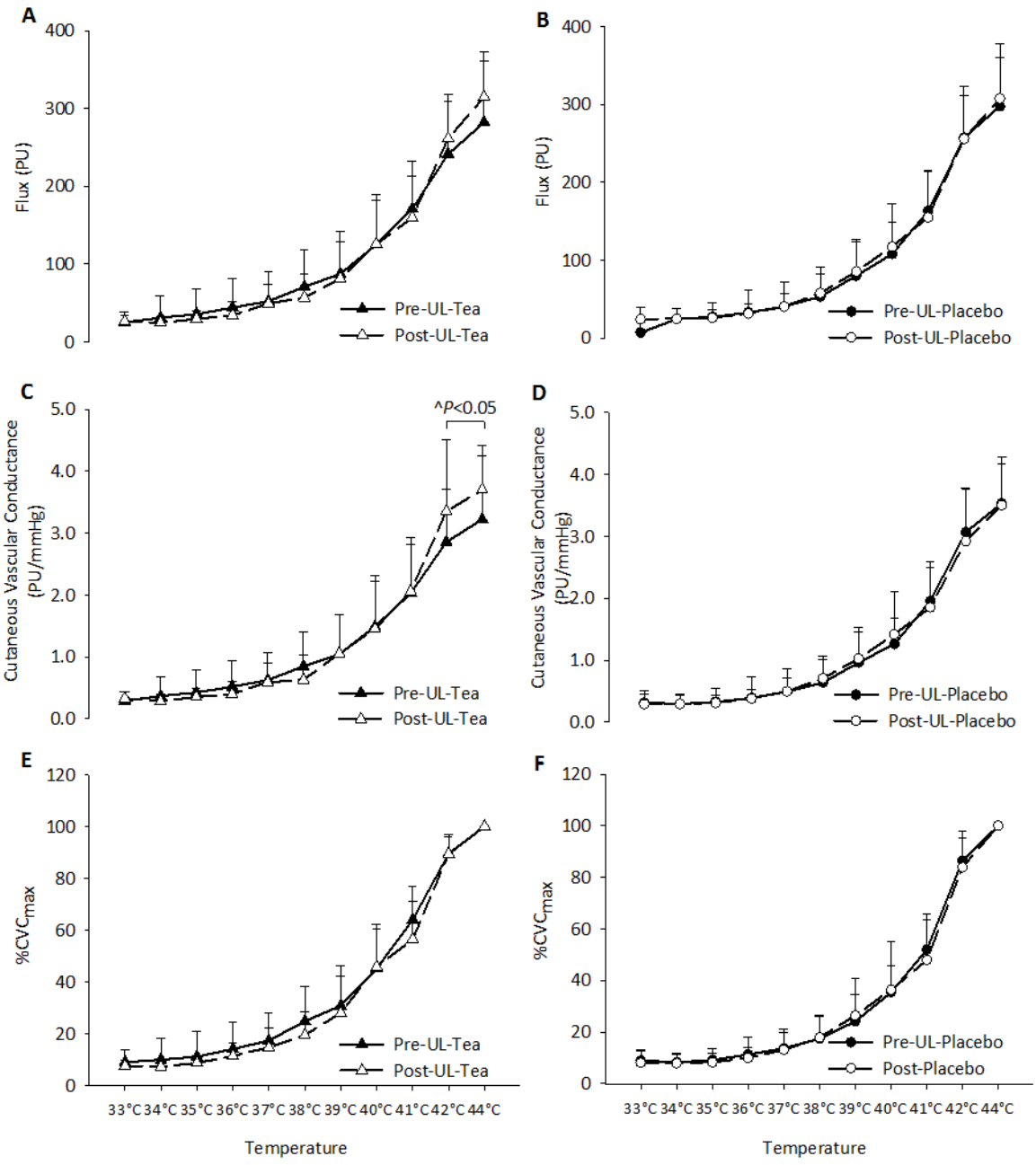

Figure 6.6 Microvascular responses across incremental temperature stages (baseline at 33°C, plateau at 42°C and maximal plateau at 44°C) following *Gradual 42°C* local heating for flux (**A** and **B**), CVC (**C** and **D**) and %CVC_{max} (**E** and **F**) for UL-Tea and UL-Placebo interventions, respectively, in 12 healthy male volunteers. Data are presented as means, with error bars representing SD. ^Condition*time*temperature interaction $P \leq 0.05$.

6.3.3 Macrovascular Function

6.3.3.1 Brachial Artery FMD

Baseline brachial artery FMD was not different between pre-UL-Placebo and pre-UL-Tea (P=0.40). There was no main effect of time (*P*=0.20) or condition (*P*=0.97) and no interaction of time*condition (*P*=0.11, Figure 6.7). No main effect of time, condition or time*condition interaction was observed for time-to-peak, baseline diameter or SRAUC (Figure 6.7).

6.3.3.2 Femoral Artery FMD

Baseline femoral artery FMD was not different between pre-UL-Placebo and pre-UL-Tea (*P*=0.89). There was no main effect of time (*P*=0.10) or condition (*P*=0.21), but there was a significant interaction of time*condition (*P*<0.001). Post-hoc analysis found that femoral artery FMD decreased after UL-Placebo (6.87 ± 3.41 % vs 4.96 ± 2.82 %), which was prevented during UL-Tea (6.72 ± 3.63 % vs 7.27 ± 3.45 %, Figure 6.7). For time-to-peak, there was no main effect of time (*P*=0.77) but there was a main effect of condition (*P*=0.02), whereby time-to-peak was higher for pre- and post-UL-Placebo compared to UL-Tea (Figure 6.7). However, there was no interaction of time*condition (*P*=0.30). No main effects of time, condition, nor interactions of time*condition were observed for baseline diameter or SRAUC (all *P*>0.05, Figure 6.7).

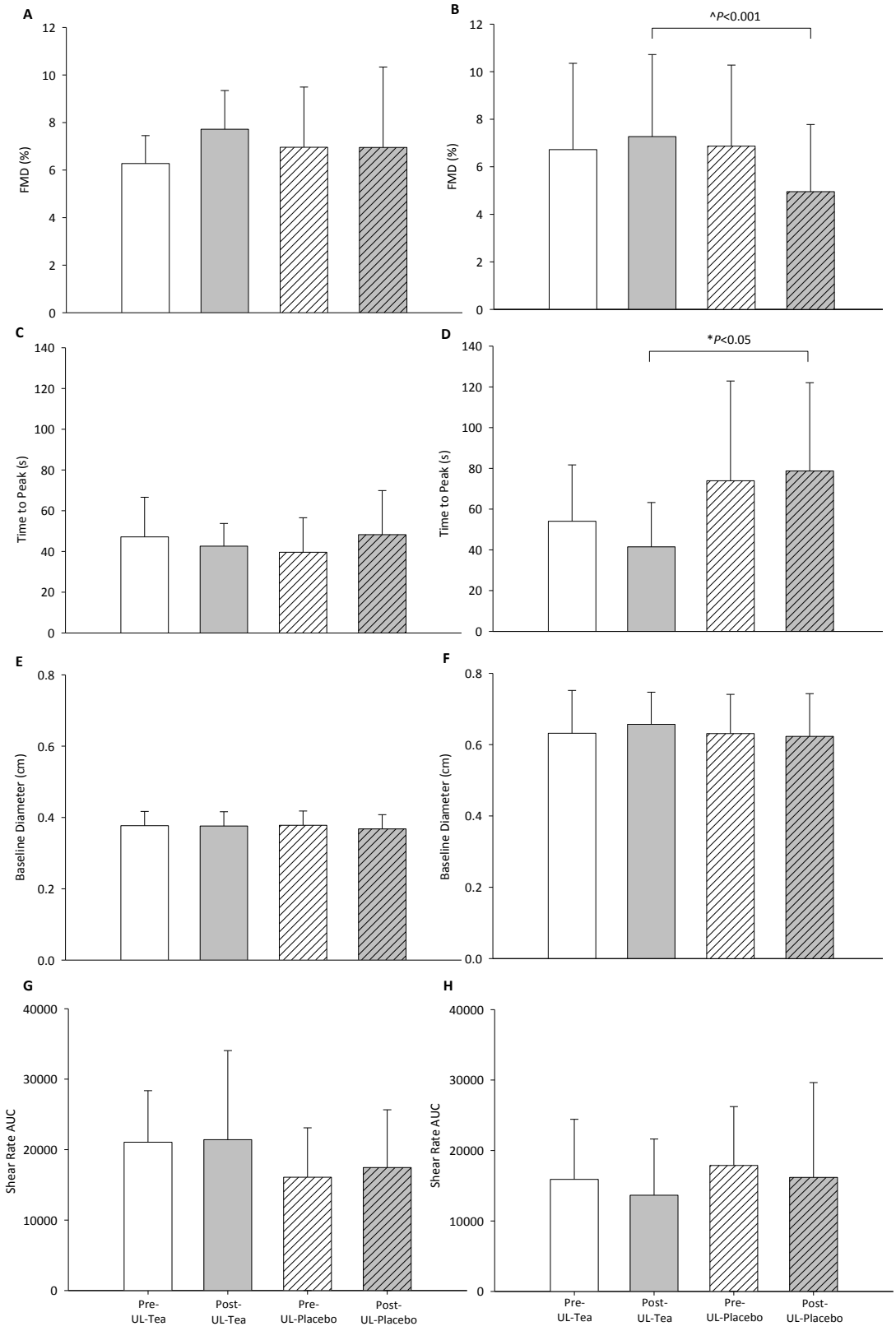

Figure 6.7 Conduit artery endothelial function flow-mediated dilation (FMD%), time-to-peak, baseline diameter and shear rate area-under-the-curve (SRAUC) for the brachial artery (**A**, **C, E** and **G**, respectively) and femoral artery (**B**, **D, F** and **H**, respectively) for UL-Tea and UL-Placebo interventions. Data are presented as means, with error bars representing SD. ^Main interaction of time*condition $P<0.001$, *main effect of condition $P\leq0.05$.

6.3.4 Cold Pressor Test/Haemodynamic Function

Baseline carotid artery reactivity (CAR%) was not different between pre-UL-Placebo and pre-UL-Tea (*P*=0.78). There was no main effect of time (*P*=0.85), but there was a borderline main effect of condition (P=0.05) and a significant interaction of time*condition (*P*=0.04). Post-hoc analysis showed that CAR% decreased following UL-Placebo (5.08 ± 1.51 % vs 3.33 ± 4.25 %), which was prevented during UL-Tea (5.74 ± 5.31 % vs 7.52 ± 4.03 %, Figure 6.8). Similar findings were evident for AUC, which demonstrated no main effect of time (*P*=0.88), but there was a main effect of condition (*P*=0.04) and a close to significant interaction (*P*=0.09, Figure 6.8). Baseline SBP change was not different between pre-UL-Placebo and pre-UL-Tea (*P*=0.27). There was no main effect of time (*P*=0.27) or condition (*P*=0.10). The CPT-induced elevation in systolic and diastolic BP was not different across time, condition or time*condition between UL-Placebo and UL-Tea (all *P*>0.05, Figure 6.8).

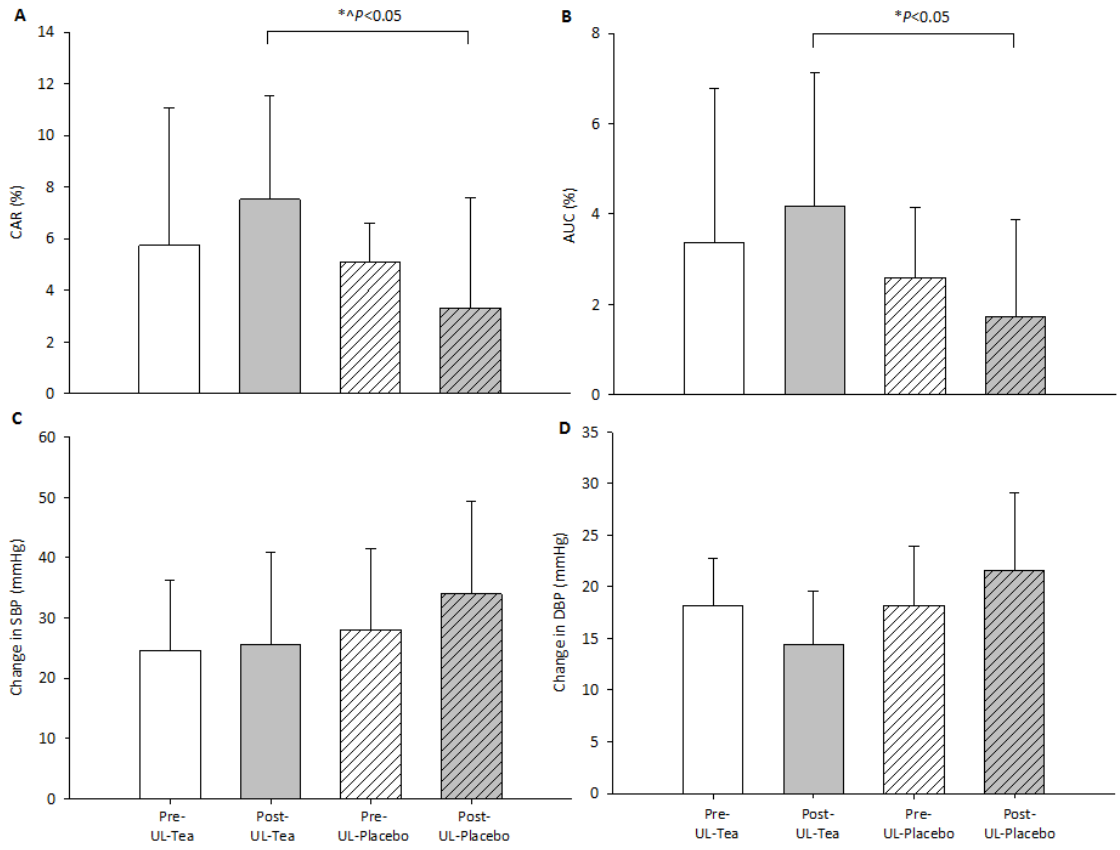

Figure 6.8 Cold pressor test derived carotid artery reactivity (CAR%, **A**), area-under-the-curve (AUC, **B**), systolic blood pressure (SBP, **C**) and diastolic blood pressure (DBP, **D**) changes for UL-Tea and UL-Placebo interventions. Data are presented as means, with error bars representing SD. *Main effect of condition *P*≤0.05, ^time*condition interaction *P*≤0.05.

6.3.5 Cerebrovascular Function

6.3.5.1 *Hypercapnia*

MCAv increased during hypercapnia for all conditions, as evidenced by a main effect of time (P<0.001). Baseline to peak changes in MCAv demonstrated no main effect for time (P=0.67) or condition (P=0.95) and there was no time*condition interaction (P=0.91, Table 6.3). MCAv vs $P_{ET}CO_2$ reactivity slopes were not different between UL-Placebo and UL-Tea, with no main effect of time (P=0.16), condition (P=0.63) or time*condition interaction (P=0.83). When expressed as relative changes, MCAv vs $P_{ET}CO_2$ reactivity slopes demonstrated no main effect of time (P=0.30), condition (P=0.10) or a time*condition interaction (P=0.48, Table 6.5).

No differences were observed in baseline MAP between UL-Placebo or UL-Tea and there was no main effect of time (P=0.22) or condition (P=0.74), nor any interaction of time*condition (P=0.36, Table 6.3). MAP increased during hypercapnia across all conditions (P=0.01), but no differences were observed between UL-Placebo or UL-Tea (P=0.10). MAP at peak MCAv demonstrated no main effect of time (P=0.27) or condition (P=0.80) and there was no time*condition interaction (P=0.30, Table 6.3). Absolute CBVC vs $P_{ET}CO_2$ reactivity slopes demonstrated no main effect of time (P=0.38) or condition (P=0.91), nor any interaction of time*condition (P=0.37, Table 6.5). When expressed as relative changes, there were no differences observed between Placebo and Tea, with no main effect of time (P=0.38) or condition (P=0.77), and there was no time*condition interaction (P=0.84, Table 6.3).

Baseline common carotid artery (CCA) diameter demonstrated no main effect of time (P=0.56), although there was a main effect of condition (P=0.01), but no time*condition interaction (P=0.76). CCA diameter increased during hypercapnia (P≤0.05). Peak CCA diameter during hypercapnia did not demonstrate a main effect of time (P=0.19), but a main effect of condition (P=0.02) was observed, however, there was no time*condition interaction (P=0.84). The change in CCA diameter

demonstrated no main effect of time (*P*=0.14), condition (*P*=0.12) and no time*condition interaction (*P*=0.99, Table 6.3). Furthermore, change in CCA diameter when controlling for baseline diameter did not demonstrate a main effect of time (*P*=0.46) or condition (*P*=0.90) and there was no time*condition interaction (*P*=0.46). There were no differences in the change in carotid blood flow during hypercapnia between UL-Placebo (23 ± 22 % vs 19 ± 17 %) and UL-Tea (20 ± 17 % vs 25 ± 24 %, *P*=0.98), with no main effect of time (*P*=0.80), and no time*condition interaction (*P*=0.60). The change in MAP during hypercapnia demonstrated no main effect of time (*P*=0.97); there was a borderline significant effect of condition (*P*=0.05), but there was no time*condition interaction (*P*=0.97, Table 6.5). No differences were evident for changes in CarVC between UL-Placebo (15 ± 17 % vs 11 ± 13 %) and UL-Tea (17 ± 15 % vs 22 ± 24 %) with no main effect of time (*P*=0.94), condition (*P*=0.36) or time*condition interaction (*P*=0.50, Table 6.3).

Table 6.3 Cerebrovascular and common carotid artery (CCA) variables measured throughout hypercapnia for UL-Placebo and UL-Tea interventions.

	Intervention (mean ± SD)				Two-Way ANOVA *P* Values		
	Pre-UL-Placebo	Post-UL-Placebo	Pre-UL-Tea	Post-UL-Tea	Time	Condition	T*C
Cerebrovascular Variables							
Baseline MCAv (cm·s^{-1})	70 ± 14	66 ± 12	68 ± 14	70 ± 8	0.91	0.57	0.14
Change in MCAv (cm·s^{-1})	14 ± 11	14 ± 12	16 ± 16	14 ± 12	0.67	0.95	0.91
Baseline $P_{ET}CO_2$ (mmHg)	39 ± 4	40 ± 6	37 ± 5	38 ± 5	0.18	0.36	0.82
Change in $P_{ET}CO_2$ (mmHg)	4 ± 5	6 ± 3	8 ± 3	9 ± 2	0.60	0.86	0.73
MCAv vs $P_{ET}CO_2$ absolute (cm·s/$mmHg^{-1}$)	3.30 ± 1.35	4.21 ± 1.87	3.81 ± 1.23	4.35 ± 1.15	0.16	0.63	0.83
MCAv vs $P_{ET}CO_2$ relative (% cm·s/% $mmHg^{-1}$)	4.71 ± 2.51	5.71 ± 2.69	5.63 ± 1.68	6.23 ± 1.81	0.30	0.10	0.48
Baseline MAP (mmHg)	85 ± 14	91 ± 16	90 ± 11	90 ± 19	0.22	0.74	0.36
Change in MAP (mmHg)	2 ± 4	2 ± 3	6 ± 3	6 ± 3	0.98	0.06	0.90
Baseline CBVC (cm·s/$mmHg^{-1}$)	0.92 ± 0.33	0.84 ± 0.30	0.77 ± 0.19	0.83 ± 0.27	0.93	0.87	0.21
CBVC: $P_{ET}CO_2$ absolute (cm·s/$mmHg^{-1}$/$mmHg^{-1}$)	0.03 ± 0.04	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.38	0.91	0.37
CBVC: $P_{ET}CO_2$ relative (% cm·s/$mmHg^{-1}$/%$mmHg^{-1}$)	1.37 ± 1.67	0.94 ± 0.75	1.29 ± 0.72	1.19 ± 0.42	0.38	0.77	0.84
Common Carotid Artery Variables							
Baseline diameter (mm)	0.70 ± 0.06	0.72 ± 0.06	0.67 ± 0.06	0.68 ± 0.05	0.56	0.01*	0.76
Change in diameter (%)	2 ± 2	3 ± 3	3 ± 3	4 ± 3s	0.14	0.12	0.99
Baseline blood flow (mL/min^{-1})	13.07 ± 3.96	16.04 ± 5.30	13.20 ± 3.76	13.28 ± 2.62	0.28	0.49	0.08
Change in blood flow (%)	23 ± 22	19 ± 17	20 ± 17	25 ± 24	0.80	0.98	0.60
Baseline CarVC (mL/min^{-1}/$mmHg^{-1}$)	0.15 ± 0.06	0.18 ± 0.06	0.15 ± 0.04	0.15 ± 0.05	0.42	0.33	0.28
Change in CarVC (% mL.min/$mmHg^{-1}$)	15 ± 17	11 ± 13	17 ± 15	22 ± 24	0.94	0.36	0.50

Data are mean ± SD. T*C, time*condition interaction; MCAv, middle cerebral artery velocity; $P_{ET}CO_2$, end-tidal carbon dioxide; MAP, mean arterial pressure; CBVC, cerebrovascular conductance; CarVC, carotid artery vascular conductance. *Main effect of condition $P \leq 0.05$.

6.3.5.2 Cerebral Autoregulation: Squat-Stand Manoeuvres

6.3.5.2.1 Squat-Stands at 0.10 Hz

Gain: At the very low, low and high frequencies there were no main effects of time (all $P>0.05$), condition or interactions of time*condition (Table 6.4).

Phase: At the very low, low and high frequencies there were no main effects of time (all $P>0.05$), condition or interactions of time*condition (Table 6.4).

Coherence: At the very low, low and high frequencies there were no main effects of time (all *P*>0.05), condition or interactions of time*condition (Table 6.4).

6.3.5.2.2 Squat-Stands at 0.20 Hz

Gain: At the very low, low and high frequencies there were no main effects of time (all *P*>0.05), condition or interactions of time*condition (Table 6.4).

Phase: For the VLF, there was no main effect of time (*P*=0.33) and no main effect of condition (*P*=0.07), nor a time*condition interaction (*P*=0.34). At the LF, there was no main effect of time (*P*=0.94) or condition (*P*=0.94) and no interaction of time*condition was demonstrated (*P*=0.75). For the HF, no main effect of time was demonstrated (*P*=0.74), but there was a main effect of condition (*P*=0.01). However, there was no time*condition interaction (*P*=0.51, Table 6.4)

Coherence: At the VLF, no main effect of time was observed (*P*=0.49) and there was no main effect of condition (*P*=0.74), nor a time*condition interaction (*P*=0.26). For the LF, there was no main effect of time (*P*=0.69) or condition (*P*=0.67). However, an interaction of time*condition was observed (*P*=0.04), whereby UL-Placebo was slightly increased (0.59 ± 0.25 vs 0.67 ± 0.17 $cm \cdot s^{-1} \cdot mmHg^{-1}$) and a slight reduction was demonstrated following UL-Tea (0.68 ± 0.14 vs 0.59 ± 0.14 $cm \cdot s^{-1} \cdot mmHg^{-1}$). At the HF, no main effect of time was demonstrated (*P*=0.94) and there was no main effect of condition (*P*=0.41), nor a time*condition interaction (*P*=0.62, Table 6.4).

Table 6.4 Gain, phase and coherence determined by transfer function at very low (VLF), low (LF) and high frequency (HF) during repeated squat-stand manoeuvres at 0.10Hz and 0.20 Hz whilst breathing normal ambient air for UL-Placebo and UL-Tea interventions.

	Intervention (mean ± SD)				Two-Way ANOVA *P* Values		
0.10 Hz Squat-stands	**Pre-UL-Placebo**	**Post-UL-Placebo**	**Pre-UL-Tea**	**Post-UL-Tea**	**Time**	**Condition**	**T*C**
Gain (cm·s^{-1}·mmHg^{-1})							
VLF	0.6 ± 0.2	0.4 ± 0.1	0.6 ± 0.1	0.7 ± 0.5	0.06	0.86	0.35
LF	0.8 ± 0.2	0.8 ± 0.1	0.9 ± 0.3	0.9 ± 0.2	0.70	0.20	0.23
HF	0.9 ± 0.4	0.8 ± 0.5	1.3 ± 0.7	1.1 ± 0.3	0.64	0.15	0.29
Phase (radians)							
VLF	41.9 ± 20.6	41.3 ± 14.3	29.9 ± 10.9	42.1 ± 25.6	0.50	0.37	0.24
LF	23.7 ± 9.9	21.9 ± 13.8	29.2 ± 20.5	23.0 ± 14.0	0.26	0.25	0.53
HF	34.1 ± 41.6	25.8 ± 43.8	39.7 ± 28.5	45.4 ± 44.3	0.78	0.61	0.89
Coherence							
VLF	0.5 ± 0.0	0.4 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.58	0.44	0.13
LF	0.6 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.80	0.36	0.27
HF	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	0.62	0.43	0.25
0.20 Hz Squat-stands							
Gain (cm·s^{-1}·mmHg^{-1})							
VLF	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.49	0.24	0.50
LF	0.6 ± 0.2	0.8 ± 0.2	0.9 ± 0.3	0.8 ± 0.2	0.74	0.39	0.08
HF	0.7 ± 0.2	0.9 ± 0.3	0.9 ± 0.4	0.8 ± 0.2	0.70	0.18	0.24
Phase (radians)							
VLF	65.6 ± 24.2	62.4 ± 39.0	45.7 ± 14.4	53.4 ± 15.5	0.33	0.07	0.34
LF	22.6 ± 9.6	22.6 ± 20.2	15.2 ± 16.9	15.9 ± 15.7	0.94	0.94	0.75
HF	8.0 ± 15.4	10.1 ± 12.2	10.4 ± 14.7	12.7 ± 11.0	0.74	0.01*	0.51
Coherence							
VLF	0.4 ± 0.2	0.4 ± 0.2	0.4 ± 0.1	0.4 ± 0.2	0.49	0.74	0.26
LF	0.6 ± 0.3	0.7 ± 0.2	0.7 ± 0.1	0.6 ± 0.1	0.69	0.67	0.04^
HF	0.4 ± 0.2	0.4 ± 0.2	0.4 ± 0.2	0.4 ± 0.1	0.94	0.41	0.62

Data are mean ± SD. T*C, time*condition interaction; VLF, very low frequency; LF, low frequency; HF, high frequency. *Main effect of condition $P \leq 0.05$, ^time*condition interaction $P \leq 0.05$.

6.4 Discussion

The primary aim of the present study was to explore whether daily green tea consumption ameliorated changes in peripheral vascular function, cerebrovascular function and insulin sensitivity in healthy male adults, following a 7-day metabolic and physical inactivity challenge designed to represent a Western lifestyle. The novel findings of the study are that following 7-days of high fat overfeeding combined with physical activity reduction, a marked increase in the insulin and glucose responses to a mixed meal tolerance test were observed which are consistent with the development of insulin resistance, but also presence of impaired peripheral vascular function in peripheral and cranial arteries. Remarkably, daily consumption of green tea, equivalent to 6 cups per day, attenuated the observed impairments in insulin resistance and (peripheral and extracranial) vascular function. Following the lifestyle intervention, no effects were observed on cerebrovascular function. Collectively, findings from the present study support the hypothesis that 7-days of reduced physical activity and increased calorie consumption are associated with a decline in peripheral vascular function and reduced insulin sensitivity, and that these detrimental changes are partly mitigated through daily consumption of green tea.

6.4.1 Metabolic Function

The present study observed metabolic changes consistent with impaired glucose handling and a compensatory elevated postprandial insulin response following the unhealthy Western lifestyle intervention, with directionally opposite responses following daily consumption of green tea. Such findings are consistent with previous studies in demonstrating the deleterious impact of reduced physical activity and/or overfeeding on glycaemic control and insulin resistance, for example, bed rest interventions (Hamburg *et al.*, 2007; Sonne *et al.*, 2010) and bed rest interventions combined with a high saturated fat diet (Stettler *et al.*, 2005), short-term (5-days) step-reduction models (Holwerda *et al.*, 2015), and overfeeding (+50 kcal) studies (Brons *et al.*, 2009). Consistent with current findings, a

model more closely mimicking a Western lifestyle that combines overfeeding with a reduction in physical activity induced impaired insulin sensitivity in young, healthy males after only 3-days, which was worse still after 7-days (Knudsen *et al.*, 2012). Increased insulin secretion may precede the development of peripheral insulin resistance (Le Stunff & Bougneres, 1994) and, therefore, hyperinsulinaemia may be causally related to the development of insulin resistance following more prolonged periods of caloric excess, particularly with a high proportion of energy from fats (Adochio *et al.*, 2009; Brons *et al.*, 2009). The current findings demonstrate the development of an insulin resistant state in previously healthy humans after adoption of an unhealthy Western lifestyle for only 7-days. Such profound findings reinforce previous observations that the initial stages of impaired insulin sensitivity are causally linked to the effects of high energy diets and physical inactivity.

As impaired glycaemic control is an independent risk factor in the development of type 2 diabetes (Nathan *et al.*, 2007), it is important to identify therapeutic strategies to improve glycaemic control and mitigate the negative impact of an unhealthy Western lifestyle. The present study suggests a protective role for green tea consumption, with daily ingestion not only attenuating elevated blood glucose levels, but even improving glucose handling and concomitantly enhancing insulin sensitivity. To date, limited intervention studies have been undertaken exploring the effect of tea ingestion on biomarkers of insulin sensitivity and risk factors for diabetes (Hosoda *et al.*, 2003; Fukino *et al.*, 2005; Ryu *et al.*, 2006; Bryans *et al.*, 2007), with inconsistent outcomes. Acute oolong tea consumption reduced plasma glucose in individuals with type 2 diabetes on oral antihyperglycaemic medication (Hosoda *et al.*, 2003). Furthermore, in healthy individuals, Bryans and colleagues (2007) observed a reduction in postprandial plasma glucose, with a corresponding increase in insulin, following acute consumption of black tea, compared to water and water with caffeine. Conversely, a randomised controlled trial in individuals with borderline diabetes or overt diabetes who consumed green tea daily for 2-months demonstrated no difference in blood glucose, HbA_{1c}, insulin resistance or inflammatory

markers, compared to a control group (Fukino *et al.*, 2005). Similarly, daily green tea consumption for 4-weeks was not associated with any change in insulin resistance or arterial stiffness in middle-aged individuals with type 2 diabetes (Ryu *et al.*, 2006). Such observations suggest that green tea may exert a protective role in healthy individuals, but that the outcomes in diabetic populations may not be as encouraging. However, a lack of methodological rigor cannot be excluded, including lack of dietary restriction, self-selected tea strengths (Fukino *et al.*, 2005), the type/dose of tea and unknown physical activity status. Further research is therefore warranted to determine whether tea may be an effective adjunct in the management of diabetes.

6.4.2 Microvascular Function

The present study is the first to examine microvascular function in relation to a Western lifestyle and observed impaired function after only 7-days. Previous studies have demonstrated the deleterious impact of physical inactivity on microvascular function (Shoemaker *et al.*, 1998; Hesse *et al.*, 2005), although studies have tended to involve prolonged periods of complete bed rest that are not wholly representative of a typical Western lifestyle. For example, impaired endothelium-dependent microvascular function and increased circulating endothelial cells, indicative of endothelial damage, were observed following prolonged 2-months bed rest (Demiot *et al.*, 2007) and 5-days of bed rest in healthy adults demonstrated impaired forearm and calf reactive hyperaemia (Hamburg *et al.*, 2007). Such inactivity models do not account for the dietary contributions towards an unhealthy lifestyle, which have seldom been investigated and, furthermore, no study has explored the combined effect of excess energy consumption and physical activity reduction as is typical of a Western lifestyle. In the present study, the lifestyle intervention (UL-Placebo) demonstrated a trend of decreased cutaneous blood flow, expressed as CVC, across all three local heating protocols, indicating impaired microvascular function. These findings suggest that adopting a Western lifestyle for even a short period has a deleterious impact upon microvascular function. Given that microvascular function is considered a surrogate for overall vascular health and that changes present in the microvasculature

typically precede and often predict the development of conduit artery atherosclerosis, current observations indicate that the impact of Western lifestyles manifest after only a few days. Furthermore, as forearm microvascular function was assessed rather than that of a lower limb, which may have been more applicable given the step-reduction, identifying microvascular impairments in the forearm is suggestive of lifestyle-induced systemic dysfunction that may have greater implications for cardiovascular health.

Importantly, the present study also demonstrated a protective role for green tea on an unhealthy lifestyle, as evidenced by daily green tea consumption largely attenuating the impairment in microvascular function. Furthermore, in keeping with findings for acute black tea consumption in *Chapter 5*, green tea caused a greater effect following gradual local heating, with a significant increase in cutaneous blood flow following UL-Tea compared to UL-Placebo. As previously discussed (*see Section 5.4, Chapter 5*), the rate of local heating and maximum heating temperature produce different vasodilatory responses based upon neurogenic reflexes and local chemical mediators, such as NO. The observation that green tea attenuates, and even improves, cutaneous vascular function following gradual local heating, suggests that tea enhances endothelial function via upregulation of local chemical mediators, which is consistent with previous studies in conduit vessels (Grassi *et al.*, 2009b; Schreuder *et al.*, 2014). Given that the *Rapid 39°C* and *Rapid 42°C* protocols are also linked to the release of NO but to a lesser extent, the marginal improvements in cutaneous blood flow observed in the present study are consistent with green tea protecting microvascular function via a mechanism related to upregulation of NO. These observations support the suggestion highlighted in *Chapter 5*, in using multiple local heating protocols simultaneously to interrogate microvascular responses to interventions for mechanistic insight.

Despite a maintained/higher vasodilatory response for green tea in response to *Gradual 42°C* local heating for CVC in contrast to a reduction with UL-Placebo, no significant differences were observed when data were expressed as $\%CVC_{max}$. These latter findings are likely explained by the changes in absolute CVC at maximal vasodilation (e.g., a maintenance/increase with UL-Tea and a reduction with UL-Placebo) that would result in similar $\%CVC_{max}$ responses. As discussed in *Chapter 5*, $\%CVC_{max}$ is often the preferred method of data expression following local skin heating to 44°C to reach maximal vasodilation (Dawson *et al.*, 2015) but consideration of absolute CVC is important and would indicate a higher absolute flow *per se*. Current findings are in keeping with the observations following acute black tea consumption in *Chapter 5* and collectively, are suggestive of tea altering cutaneous perfusion at higher local skin temperatures e.g. at 39-44°C.

6.4.3 Macrovascular Function

Brachial artery FMD demonstrated little change following the lifestyle intervention, with no changes observed following UL-Placebo and a non-significant 1.45 % increase demonstrated following UL-Tea. In the femoral artery, however, a significant difference in FMD was observed between UL-Placebo and UL-Tea, with a ~2 % decline for UL-Placebo and UL-Tea both attenuating and slightly improving (+~0.5 %) FMD. Given that a 1 % decline in FMD reportedly represents an 8 % risk of future cardiovascular events (Inaba *et al.*, 2010), the current findings suggest that adoption of an unhealthy lifestyle for only seven days is associated with an elevated CVD risk, yet daily tea consumption mitigates the negative effects and is cardioprotective. As FMD is a reliable measure of NO-mediated endothelial function (Thijssen *et al.*, 2011), current findings indicate that a direct effect of NO on the endothelium is likely responsible for both the lifestyle-induced impairment in vasoreactivity and for the mitigating effect of green tea, related to reduced shear-stress and NO upregulation, respectively. Reduced activity of the lower limbs is likely to result in a decrease in local shear stress which may subsequently reduce vasodilation through inhibition of eNOS (Hamburg *et al.*, 2007). This mechanism explains the lifestyle-

induced decline in femoral FMD, but not brachial FMD, in the present study, whereby the inactivity component targeted the lower limbs through step-reduction, with the upper limbs remaining largely active. Previous studies in lower limb inactivity models report similar findings, with no difference in brachial artery FMD (%) following 5-days of bed rest (Hamburg *et al.*, 2007) or following 4-weeks of unilateral lower limb suspension (Bleeker *et al.*, 2005a). Furthermore, Boyle and colleagues (2013) observed a ~3 % reduction in popliteal artery FMD following step-reduction in recreationally active men, although brachial artery FMD was unchanged. However, significant decreases in both brachial and femoral artery FMD were observed in a 5-day bed rest study of healthy adults, although the subject population was small (n=5) (Nosova *et al.*, 2014).

Few studies have examined the impact of overfeeding on macrovascular function, with the acute effects of a high fat load demonstrating impaired brachial FMD postprandially in adults (Plotnick *et al.*, 1997; Vogel *et al.*, 1997; Bae *et al.*, 2001; Plotnick *et al.*, 2003). Diets high in fat (particularly trans) are associated with increased CVD risk (Skeaff & Miller, 2009; Michas *et al.*, 2014; de Souza *et al.*, 2015), largely attributed to endothelial dysfunction arising from increased oxidative stress and reduced NO bioavailability (Bae *et al.*, 2001). Increasingly, tea consumption has demonstrated improved NO-mediated endothelial function in both healthy and diseased humans (Ras *et al.*, 2011), possibly arising from activation of eNOS (Loke *et al.*, 2010), reduced oxidative stress (Łuczaj & Skrzydlewska, 2005) and/or an improved antioxidant and anti-inflammatory capacity (Neyestani *et al.*, 2010). Several studies have observed attenuated/improved brachial artery FMD following acute tea ingestion compared to control, together with an oral fat load (Corretti *et al.*, 2002; Hodgson *et al.*, 2005; Grassi *et al.*, 2012a), with the present study being the first to explore the effects of tea following a 7-day unhealthy lifestyle. Taken together, these findings are highly suggestive of a vasoprotective mechanism for tea consumption, although the precise pathways remain unclear.

6.4.4 Cold Pressor Test/Haemodynamic Function

The CPT induces sympathetic activation that elevates HR, arterial BP and myocardial oxygen demand via the neurotransmitters epinephrine and norepinephrine (Victor *et al.*, 1987). Assessment of carotid artery reactivity (CAR%) provides an index of coronary artery disease risk, with a vasodilatory response that counteracts the effects of the sympathetic activation considered to be a "normal" response in healthy individuals, and vasoconstriction indicating the presence of CVD risk (Rubenfire *et al.*, 2000). However, the CPT can also be considered a test of cranial artery endothelial function, similar to FMD assessment of the peripheral arteries. Whilst the carotid artery vasodilatory mechanisms underpinning CAR% in response to the CPT are uncertain, they are suggested to involve endothelial-dependent vasodilation via endothelium-derived relaxing factors, such as NO, whereas vasoconstriction arises from a direct effect of norepinephrine on smooth muscle cells (van Mil *et al.*, 2017). Findings from the present study demonstrated a decrease in CAR% associated with the unhealthy lifestyle, that was attenuated and improved by daily green tea consumption (P=0.04). Furthermore, the impairment in CAR% was accompanied by concomitant increases in basal BP, which were reversed following green tea, and, similarly, the magnitude of the elevation in BP during the CPT was greater after UL-Placebo compared with no change after UL-Tea, suggesting that BP reactivity was elevated after UL-Placebo. Such findings suggest that adoption of a Western lifestyle induces impaired vasodilation and elevated BP, even following a short period of 7-days. Furthermore, these observed changes on cranial artery function are consistent with those in the peripheral vasculature, in that UL-Placebo demonstrated impaired peripheral vascular function which was partly prevented following UL-Tea. Collectively, these findings suggest that the Western lifestyle affects general vascular health and that these detrimental changes can be prevented by daily green tea consumption. However, whilst CAR% is a useful measure to establish CVD risk, it is seldom used and, therefore, limited comparisons of present findings can be made against previous research.

6.4.5 Cerebrovascular Function

Excessive changes in CBF, e.g., hypoperfusion, have been linked to the development of cerebrovascular disease such as stroke and dementia (Roher *et al.*, 2012), particularly a diminished vasodilatory response to CO_2 (Gur *et al.*, 1996; Claassen *et al.*, 2009). Epidemiologic reports link a higher intake of dietary flavonoids with a reduction in the development of dementia (Commenges *et al.*, 2000; Engelhart *et al.*, 2002), likely related to direct effects of flavonoids on the vascular endothelium, given that endothelial dysfunction impairs cerebral vasodilatory capacity (Zimmermann & Haberl, 2003; Lavi *et al.*, 2006). The present study, however, observed no significant changes in cerebrovascular function (CO_2 reactivity and cerebral autoregulation) after the intervention *per se,* nor with any effect of green tea on the cerebrovasculature. Furthermore, no meaningful changes were observed in extracranial reactivity, assessed via CCA function, following the lifestyle intervention or daily tea consumption, thereby suggesting that the short-term inactivity/overfeeding intervention or green tea did not impair cerebrovascular function (CO_2 reactivity and cerebral autoregulation). The lack of an effect may be due to several reasons. Firstly, the intervention was likely not severe enough or of sufficient duration to challenge the CCA or cerebrovasculature, particularly since the cerebral regulatory mechanisms differ from the peripheral vessels and the brain's plasticity was likely adequate to cope with a short 7-day intervention. Previous bed rest studies have demonstrated significant reductions in transfer function gain after 18-days in young, healthy adults (Jeong *et al.*, 2014) and altered autoregulation after 60-days (Greaves *et al.*, 2007). It is also reasonable to assume that the small sample size influenced the cerebrovascular findings of the present study, as it is likely that any subtle changes in function were not detectable with such small numbers.

Despite regular tea consumption being associated with a reduced risk of stroke (Arab *et al.*, 2009), to date, only a single study has examined the effect of tea on CBF. In a randomised, double-blind, placebo-controlled study, Vidyasagar and colleagues (2013) assessed cerebrovascular reactivity to

hypercapnia in healthy males and observed reduced steady-state CBF following an acute dose of black tea equivalent to 6 cups. However, the authors suggest that caffeine was responsible for this change, rather than the tea *per se*, given that decaffeinated tea demonstrated no differences. Tea-derived flavonoids, such as EC and EGCG, have demonstrated variable outcomes, with a single low dose (135mg) of capsular EGCG reducing CBF (measured by NIRS) during computerised cognitive task performance in healthy humans, whereas a higher dose (270mg) did not change CBF relative to placebo (Wightman *et al.*, 2012). As outlined above, the duration of the tea consumption and/or the dosage may not have been high enough to influence cerebrovascular function and the small sample sizes limit the interpretation of the findings. Present findings are inconclusive regarding the effects of tea consumption, or lifestyle changes, on cerebrovascular function and further research is, therefore, required to determine whether the beneficial effects of tea seen in the peripheral vasculature are evident in cerebral vessels.

6.4.6 Clinical Relevance and Perspectives

The present study has produced several important observations. Principally, it is the first study to comprehensively explore the effects of regular tea consumption on vascular and metabolic function in the context of an unhealthy Western lifestyle. Importantly, current findings suggest that tea exerts a beneficial effect on function across the vascular tree that is protective against the deleterious impact of an unhealthy Western lifestyle. The present study has also demonstrated the adverse impact of an unhealthy lifestyle in inducing peripheral vascular dysfunction and insulin resistance in a cohort of previously healthy males after a period of only 7-days. As this lifestyle is increasingly adopted by portions of the general population on a daily basis, and many normally 'healthy' individuals often experience it during holidays and festivities, the current findings are widely applicable in establishing the detrimental impact of such a lifestyle upon cardiovascular health. Insulin resistance and vascular dysfunction share common underlying mechanisms, which are largely centred around endothelial dysfunction, in addition to contributions from oxidative stress, inflammatory factors and subcellular signalling pathways (Wheatcroft *et al.*, 2003). The present study observed improved cutaneous

vascular function and conduit artery FMD following daily green tea consumption which suggest that tea exerts a protective effect on the vasculature at the level of the endothelium, and it may be speculated that this likely results from activation/upregulation of endothelium-derived vasodilators, such as NO. Tea, therefore, may represent an inexpensive therapeutic approach for improving cardiovascular health, particularly in relation to a Western lifestyle.

6.4.7 Limitations

There are several strengths of the current study that support its conclusions, such as the randomised, double-blind, within-subjects design and array of experimental measures that demonstrate a consistent trend. Furthermore, the lifestyle intervention represents a 'real-life' scenario and, therefore, the findings are applicable to Western populations. However, due to the explorative nature of the study, the sample size was modest, especially in the cerebrovascular function data set, so the present cerebral data may not be an accurate representation of the cerebrovascular response to tea. Participant compliance to the lifestyle intervention was assessed via self-reported food and activity diaries and, therefore, it is not possible to guarantee 100% compliance. However, physical activity was also monitored via accelerometers and the data downloaded supported full participant compliance for the inactivity element of the intervention. Furthermore, the dysfunction observed for the post-intervention measures suggests that participants had adopted an altered lifestyle. Importantly, the translation of the present findings to longitudinal changes in vascular and metabolic function is unknown, given that the present lifestyle intervention was only for 7-days. A further limitation is that the present study does not provide any biochemical or biomolecular insight into the mechanisms underlying the observed improvements in peripheral vascular function and metabolic function following the tea intervention. However, plasma concentrations of flavonoids and NO compounds were not obtained as they were beyond the scope of the present study.

In conclusion, data from the present study suggests that adopting a Western lifestyle for 7-days is associated with a deleterious impact upon peripheral vascular function, with detrimental vascular changes observed in both microvascular and macrovascular beds, but also in peripheral and extracranial arteries. Furthermore, the Western lifestyle was associated with the promotion of insulin resistance in healthy, male adults. Importantly, the present study observed that daily consumption of green tea prevented the detrimental impact of a Western lifestyle on peripheral vascular function and insulin sensitivity. Such findings suggest that regular green tea consumption exerts a cardioprotective effect that may provide a simple, inexpensive, non-pharmacological therapeutic approach to help combat inactivity and dietary CVD risk factors associated with an unhealthy Western lifestyle.

CHAPTER 7: ACUTE EPICATECHIN INGESTION DOES NOT AFFECT CEREBROVASCULAR FUNCTION IN HEALTHY MEN

7.1 Introduction

Optimal cerebrovascular function requires maintenance of relatively constant perfusion, which is controlled via multiple integrated regulatory mechanisms, including perfusion pressure (BP; e.g., haemodynamics), the concentration of arterial blood gases (CO_2 and O_2), sympathetic nerve activity and cerebral metabolism (Willie *et al.*, 2014). The cerebrovasculature also has an inherent ability to self-regulate (termed autoregulation); static regulation responds to gradual changes in perfusion pressure, whereas dynamic regulation occurs in response to an acute stimulus affecting perfusion lasting for a few seconds, such as coughing (Zhang *et al.*, 1998). Subsequent reflex adjustments in cerebrovascular resistance ensure that adequate blood flow is maintained. Compromised steady-state CBF and blunted task-specific CBF increases, both indicative of impaired cerebrovascular health, are considered key causal factors in the development of cerebrovascular disease associated with ageing, such as stroke, impaired cognitive function and neurodegenerative diseases (Farkas *et al.*, 2002; Parkes *et al.*, 2004; Ruitenberg *et al.*, 2005b; Sorond *et al.*, 2008b; Bertsch *et al.*, 2009; Schuff *et al.*, 2009). Unsurprisingly, CVD risk factors such as poor diet, physical inactivity and obesity that are present earlier in life are associated with an increased risk of stroke, cognitive decline and dementia in later life (Gorelick *et al.*, 2011; Sharp *et al.*, 2011) through endothelial dysfunction impairing cerebrovascular reactivity and overall CBF regulation (Lavi *et al.*, 2006; Stephan *et al.*, 2017).

Dietary flavonoid intake is inversely correlated with CVD risk and incidence of stroke (Keli *et al.*, 1996; Hollman *et al.*, 2010), with a meta-analysis demonstrating a 21% reduction in stroke risk following consumption of ≥3 cups of black or green tea per day (Arab *et al.*, 2009). Tea and its polyphenolic compounds have a potent impact on peripheral vascular function, such as observed in previous chapters (*Chapters 5* and *6*), which may contribute to the cardioprotective effects of regular dietary flavonoids. Possibly, in line with these observations on cardiovascular health, flavonoids may protect against cerebrovascular disease through a direct effect on cerebrovascular function. There is scant

research into the effects of flavonoids on cerebrovascular function however. Currently, only one study has examined the effect (acute) of tea on CBF, concluding that tea had no effect on cerebrovascular reactivity to hypercapnia (Vidyasagar *et al.*, 2013). However, others found that cocoa flavanols, a product that also contains (-)-epicatechin such as that found in green tea, increased CBF 2-hours post-ingestion in a pilot study of four young, healthy females (Francis *et al.*, 2006) and in healthy older (55-65-years) adults (Lamport *et al.*, 2015). Furthermore, a 3-month high-flavanol dietary intervention containing (-)-epicatechin demonstrated enhanced dentate gyrus (hippocampus) function in healthy older adults compared to a low-flavanol diet (Brickman *et al.*, 2014). Caffeine, another compound found in tea, has also demonstrated improved dynamic cerebral autoregulation 30-minutes after consumption (Sasaki *et al.*, 2016), yet a further study observed a 20% global reduction in CBF post caffeine using positron emission tomography (Vidyasagar *et al.*, 2013).

Taken together, the conflicting limited results on the impact of tea on cerebrovascular health may relate to differences in intervention dosage, study population and/or experimental design, as well as the various compounds found in tea, for example, (-)-epigallocatechin-3-gallate (EGCG) and (-)-epicatechin. More specifically, acute low-dose (135 mg) EGCG reduced CBF (measured by NIRS) but a higher dose (270 mg) demonstrated no effect relative to placebo (Wightman *et al.*, 2012). (-)-Epicatechin, present in both tea and cocoa, has exhibited improved peripheral vascular function (Dower *et al.*, 2016b) and a protective effect on transient ischaemia-induced brain injury in mice (Shah *et al.*, 2010). These findings, alongside the beneficial effects of flavanols that contain (-)-epicatechin on cerebrovascular function outlined above (Francis *et al.*, 2006; Brickman *et al.*, 2014; Lamport *et al.*, 2015) suggest that (-)-epicatechin may represent a compound with potential beneficial effects on the cerebrovasculature. However, no study has directly examined the isolated impact of (-)-epicatechin on cerebrovascular function in humans. The aim of this study was, therefore, to examine the effect of acute oral (-)-epicatechin ingestion on cerebrovascular function in healthy adults. It was hypothesised

that acute (-)-epicatechin ingestion would be associated with increased cerebrovascular perfusion and function.

7.2 Methods

7.2.1 Participants

Seven healthy male participants were recruited through local advertisement. All participants were healthy and non-smokers (32 ± 13 yrs, height 1.78 ± 0.04 m, weight 78 ± 7 kg, BMI 25 ± 1 kg/m^2, baseline MAP 84 ± 7 mmHg). For details of the inclusion and exclusion criteria, please refer to *Chapter 3, General Methods.* After being fully informed of the methods verbally and in writing, written informed consent was obtained from all participants. The study conformed to the *Declaration of Helsinki* and was approved by the local research ethics committee.

7.2.2 Experimental Design

Participants attended a familiarisation session <7-days prior to their first experimental trial, when they were fully briefed on the experimental protocols. Subsequently, all participants attended two experimental trials ((-)-epicatechin and placebo) in a randomised, controlled, double-blind, crossover design, each trial being separated by a 6-day washout period to avoid any carry-over effects. The crossover design was chosen to eliminate between-participant variability and computer-generated randomisation was used to reduce potential selection bias. In the week preceding each laboratory visit, participants were instructed to refrain from consuming dietary sources high in polyphenols, particularly tea, dark chocolate, apples, berries and red wine. Participants were asked to adhere to all pre-test instructions regarding fasting and avoiding caffeine, as outlined in *Chapter 3, General Methods* (section 3.1). Anthropometric measurements were recorded at visit 1 (see *Chapter 3, General Methods,* section 3.2). All assessments were conducted in a quiet, temperature-controlled laboratory (22-24°C) (Cracowski *et al.*, 2006; Thijssen *et al.*, 2011) and at the same time of day to reduce any circadian influences on vascular function (Jones *et al.*, 2010; Thijssen *et al.*, 2011).

During each trial, participants underwent assessment of baseline cerebrovascular function using TCD ultrasonography, comprising cerebrovascular CO_2 reactivity and dynamic cerebral autoregulation via squat-stand manoeuvres, performed at 0.10 Hz (5-seconds squatting, followed by 5-seconds standing) and 0.05 Hz (10-seconds squatting, followed by 10-seconds standing) for 5-minutes each (see *Chapter 3, General Methods,* section 3.5). Following completion of the baseline measures, participants immediately consumed an oral dose of the test product (2 x 50 mg capsules of (-)-epicatechin or 2 capsules of colour-matched placebo) together with a glass of water, following which participants relaxed in the laboratory. 2-hours post-ingestion repeat measures of cerebrovascular function were performed (Figure 7.1).

The 100 mg (-)-epicatechin dose was in line with previous intervention studies (46-150 mg) that observed the effects of pure (-)-epicatechin and cocoa flavanols (containing (-)-epicatechin) on the cerebral (Francis *et al.*, 2006; Brickman *et al.*, 2014; Lamport *et al.*, 2015) and conduit (Engler *et al.*, 2004; Farouque *et al.*, 2006; Faridi *et al.*, 2008; Grassi *et al.*, 2008b; Dower *et al.*, 2015; Dower *et al.*, 2016b) vasculature. The (-)-epicatechin was isolated and purified according to procedures developed by ChromaDex (Irvine, CA, USA), whereby (-)-epicatechin was extracted from *Acacia* heart wood with aqueous alcohol and the crude extract was subsequently purified by preparative chromatography (Dower *et al.*, 2015). This was followed by repeated fractional crystallisation from water. All solvents and equipment used were food grade. Identity was confirmed using nuclear magnetic resonance spectroscopy and mass spectrometry. High performance liquid chromatography was used to check purity and was 96.2% for (-)-epicatechin (water 0.3%). Non-transparent capsules were used to encapsulate the supplements, which were matched for size and colour, using microcrystalline cellulose as an excipient and 1% colloidal silicium dioxide. Variation in contents was within 3.7% (SD/mean*100%) for epicatechin. The placebo capsules contained microcrystalline cellulose (Dower *et al.*, 2015).

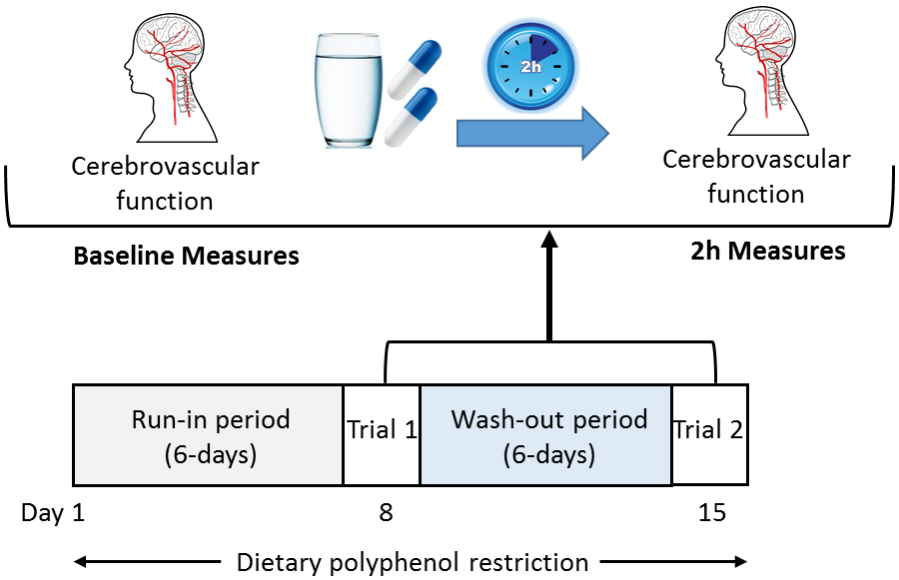

Figure 7.1 Timeline of study. Participants received the treatments (pure epicatechin and placebo) in a randomised, crossover design, each treatment being separated by a 6-day washout period. The treatments were consumed immediately after the baseline measurements were completed and measurements were repeated 2-hours following ingestion.

7.2.3 Statistical Analysis

Data were expressed as mean ± SD and statistical significance was set at $P \leq 0.05$. Linear mixed models (main effects of condition and time) were used to examine the differences between (-)-epicatechin and placebo interventions on cerebrovascular function. The repeated covariance type was Unstructured and Condition (placebo vs (-)-epicatechin), Time (baseline vs +2-hours) and Condition*Time were specified as Fixed Effects (intercept was included) and as Estimated Marginal Means. Significant main effects of Time or Condition or a Time*Condition interaction were followed up with a simple main effects analysis and the least significant difference (LSD) approach to multiple comparisons (Perneger, 1998). Data were stored and transformed within Microsoft Excel (Microsoft Office 2010, Microsoft Corporation), and statistical analyses were performed using SPSS 22.0 (SPSS, Chicago, IL, USA).

7.3 Results

7.3.1 Resting values

No differences were observed in resting MAP between placebo or (-)-epicatechin and there was no main effect of time across the 2-hour intervention (*P*=0.06), condition (*P*=0.89) or interaction of time*condition (*P*=0.40, Table 7.1). Resting MCAv demonstrated no differences between placebo or (-)-epicatechin, and no main effects of time, condition or time*condition interactions for MCAv (all *P*>0.05, Table 7.1). Resting common carotid artery (CCA) diameter demonstrated no differences across time, condition or time*condition (all *P*>0.05, Table 7.1).

7.3.2 Hypercapnia

MCAv increased during hypercapnia for all conditions (placebo and (-)-epicatechin). The peak changes in MCAv demonstrated no main effect of time (*P*=0.56) or condition (*P*=0.87) and there was no time*condition interaction (*P*=0.77, Table 7.1). MCAv vs $P_{ET}CO_2$ reactivity slopes were not different between placebo and (-)-epicatechin, with no main effect of time (*P*=0.10), condition (*P*=0.75) or time*condition interaction (*P*=0.75). When expressed as relative changes, MCAv vs $P_{ET}CO_2$ reactivity slopes demonstrated no main effect of time (*P*=0.08), condition (*P*=0.92) or a time*condition interaction (*P*=0.80, Table 7.1).

MAP increased during hypercapnia with no main effect of time (*P*=0.36) or between placebo and (-)-epicatechin (*P*=0.34). The peak changes in MAP demonstrated no differences across time, condition or time*condition (all *P*>0.05, Table 7.1). No differences were observed for resting CBVC and the peak changes in CBVC demonstrated no differences across time, condition or time*condition (all *P*>0.05, Table 7.1). Absolute CBVC vs $P_{ET}CO_2$ reactivity slopes demonstrated a main effect of time (*P*=0.01), with a subtle reduction after 2-hours (placebo; 0.02 ± 0.01 vs 0.02 ± 0.02 $cm.s^{-1}.mmHg:mmHg^{-1}$; (-)-epicatechin; 0.03 ± 0.02 vs 0.02 ± 0.02 $cm.s^{-1}.mmHg:mmHg^{-1}$). However, there was no main effect of condition (*P*=0.46) nor a time*condition interaction (*P*=0.43, Table 7.1). When expressed as relative

changes, a main effect of time was confirmed (*P*=0.02), whereby 2-hours post ingestion, a reduction was demonstrated for both placebo and (-)-epicatechin (Figure 7.2). However, there was no main effect of condition (*P*=0.26) and no time*condition interaction (*P*=0.31, Table 7.1).

The peak change in CCA diameter demonstrated no main effect of time (*P*=0.89), condition (*P*=0.10) and no time*condition interaction (*P*=0.79, Table 7.1). No significant differences were observed for the absolute change in carotid blood flow during hypercapnia between placebo (18 ± 20 % vs 35 ± 37 %) and (-)-epicatechin (14 ± 22 % vs 10 ± 20 %, *P*=0.29), with no main effect of time (*P*=0.28), and no time*condition interaction (*P*=0.16). The change in MAP during hypercapnia demonstrated no main effect of time (*P*=0.50) or condition (*P*=0.57), nor a time*condition interaction (*P*=0.68). No differences were evident for carotid artery vascular conductance (CarVC) at baseline or during hypercapnia across time, between conditions or time*condition (all *P*>0.05, Figure 7.3).

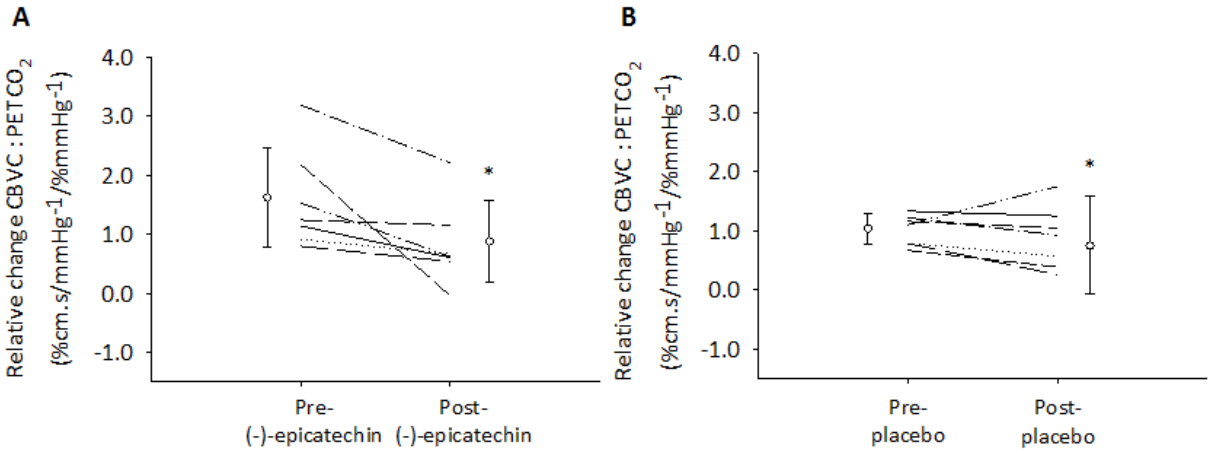

Figure 7.2 Cerebrovascular CO_2 reactivity as the relative change (%) between CBVC:$P_{ET}CO_2$ for **A.** (-)-epicatechin and **B.** placebo interventions. Data are presented as means, with error bars representing SD, and individual responses. *Main effect of time *P*≤0.05.

Table 7.1 Cerebrovascular and common carotid artery (CCA) variables during hypercapnia for placebo and (-)-epicatechin interventions.

	Intervention (mean ± SD)				Linear Mixed Model P Values		
	Pre-placebo	2-h placebo	Pre-epicatechin	2-h epicatechin	Time	Condition	T*C
Cerebrovascular Variables							
Baseline MCAv ($cm \cdot s^{-1}$)	68 ± 11	70 ± 6	66 ± 10	67 ± 12	0.29	0.39	0.73
Change in MCAv ($cm \cdot s^{-1}$)	16 ± 8	14 ± 9	15 ± 7	14 ± 11	0.56	0.87	0.77
Baseline $P_{ET}CO_2$ (mmHg)	37 ± 5	38 ± 4	39 ± 6	37 ± 7	0.47	0.94	0.27
Change in $P_{ET}CO_2$ (mmHg)	7 ± 2	6 ± 2	6 ± 3	7 ± 5	0.85	0.94	0.24
MCAv:$P_{ET}CO_2$ absolute ($cm \cdot s/mmHg^{-1}$)	2.80 ± 1.21	2.32 ± 1.64	2.88 ± 1.62	2.03 ± 1.42	0.10	0.75	0.75
MCAv:$P_{ET}CO_2$ relative (% $cm \cdot s/\%\ mmHg^{-1}$)	4.18 ± 1.84	3.31 ± 2.20	4.32 ± 2.18	3.24 ± 2.52	0.08	0.92	0.89
Baseline MAP (mmHg)	77 ± 8	87 ± 11	79 ± 10	85 ± 7	0.06	0.89	0.40
Change in MAP (mmHg)	1 ± 3	6 ± 6	3 ± 3	1 ± 4	0.36	0.34	0.14
Baseline CBVC ($cm \cdot s/mmHg^{-1}$)	0.88 ± 0.14	0.82 ± 0.13	0.85 ± 0.14	0.80 ± 0.19	0.11	0.40	0.82
Change in CBVC (%)	21 ± 7	16 ± 13	25 ± 11	22 ± 18	0.27	0.34	0.86
CBVC:$P_{ET}CO_2$ absolute ($cm \cdot s/mmHg^{-1}/mmHg^{-1}$)	0.02 ± 0.01	0.02 ± 0.02	0.03 ± 0.02	0.02 ± 0.02	0.01*	0.46	0.43
CBVC:$P_{ET}CO_2$ relative ($\%\ cm \cdot s/mmHg^{-1}/\%mmHg^{-1}$)	1.01 ± 0.26	0.72 ± 0.83	1.57 ± 0.85	0.82 ± 0.70	0.02*	0.26	0.31
Common Carotid Artery Variables							
Baseline diameter (mm)	0.68 ± 0.02	0.68 ± 0.03	0.67 ± 0.02	0.68 ± 0.02	0.13	0.65	0.71
Change in diameter (%)	0 ± 2	1 ± 2	-1 ± 3	-1 ± 1	0.89	0.10	0.79
Baseline blood flow (mL/min^{-1})	10.34 ± 2.15	8.84 ± 2.14	10.19 ± 2.76	9.66 ± 4.60	0.29	0.72	0.61
Change in blood flow (%)	18 ± 20	35 ± 37	14 ± 22	10 ± 20	0.28	0.29	0.16
Baseline CarVC ($mL \cdot min/mmHg^{-1}$)	0.14 ± 0.04	0.10 ± 0.03	0.13 ± 0.04	0.12 ± 0.07	0.12	0.62	0.55
Change in CarVC ($\%\ mL \cdot min/mmHg^{-1}$)	10 ± 20	27 ± 33	7 ± 24	6 ± 17	0.30	0.29	0.09

Data are mean ± SD. T*C, time*condition interaction; MCAv, middle cerebral artery velocity; $P_{ET}CO_2$, end-tidal carbon dioxide; MAP, mean arterial pressure; CBVC, cerebrovascular conductance; CarVC, carotid artery vascular conductance. *Main effect of time $P \leq 0.05$.

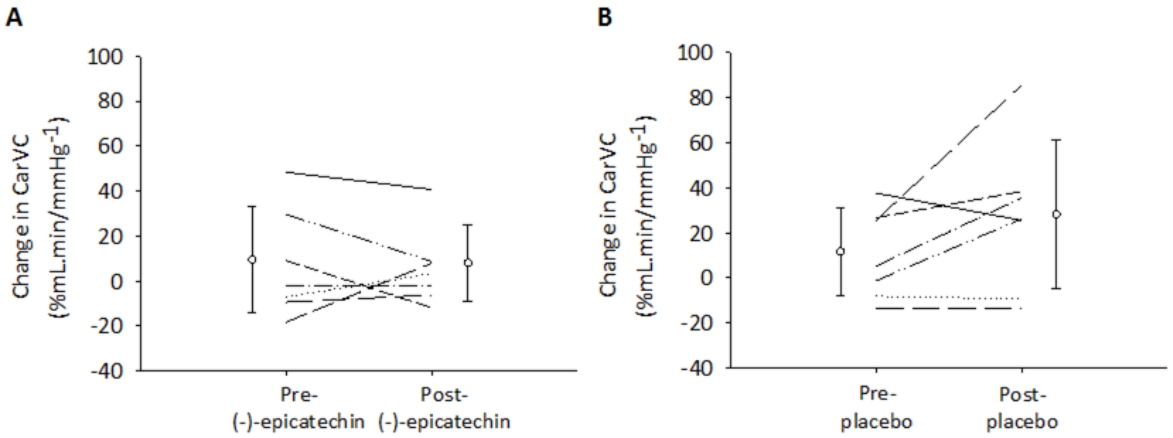

Figure 7.3 Carotid artery vascular conductance (CarVC) reactivity to 5% CO_2 expressed as the relative change (%) in CBVC for **A.** (-)-epicatechin and **B.** placebo interventions. Data are presented as means, with error bars representing SD, and individual responses.

7.3.3 Cerebral Autoregulation: Squat-Stand Manoeuvres

7.3.3.1 Squat-Stands at 0.05 Hz

Gain: At the VLF, a main effect of time (*P*=0.04) was demonstrated with a subtle increase at 2-hours (placebo; 0.7 ± 0.2 vs 0.8 ± 0.2 cm·s^{-1}·mmHg^{-1}; (-)-epicatechin; 0.7 ± 0.1 vs 0.7 ± 0.1 cm·s^{-1}·mmHg^{-1}). However, no effect of condition (*P*=0.70) was observed and there was no time*condition interaction (*P*=0.33). At the low and high frequencies, there were no main effects of time, condition or interactions of time*condition (all *P*>0.05, Table 7.2).

Phase: At the very low, low and high frequencies there were no main effects of time (all *P*>0.05), condition or interactions of time*condition (Table 7.2).

Coherence: At the very low, low and high frequencies there were no main effects of time (all *P*>0.05), condition or interactions of time*condition (Table 7.2).

7.3.3.2 Squat-Stands at 0.10 Hz

Gain: At the VLF, no main effect of time (*P*=0.62) was demonstrated, but a main effect of condition (*P*=0.03) was observed, whereby Gain was slightly higher for placebo (baseline; 0.7 ± 0.2; 2 hr; 0.8 ± 0.2 cm·s^{-1}·mmHg^{-1}) compared to (-)-epicatechin (baseline; 0.6 ± 0.2; 2 hr; 0.6 ± 0.2 cm·s^{-1}·mmHg^{-1}).

There was no time*condition interaction (*P*=0.20). At the low and high frequencies, there were no main effects of time, condition or interactions of time*condition (all *P*>0.05, Table 7.2).

Phase: For the VLF, there was no main effect of time (*P*=0.65), but a main effect of condition (*P*=0.04) was demonstrated. However, there was no time*condition interaction (*P*=0.34, Table 7.2). At the low and high frequencies there were no main effects of time, condition or interactions of time*condition (all *P*>0.05, Table 7.2).

Coherence: At the very low, low and high frequencies there were no main effects of time, condition or interactions of time*condition (all *P*>0.05, Table 7.2).

Table 7.2 Gain, phase and coherence determined by transfer function analysis at very low (VLF), low (LF) and high frequency (HF) ranges during repeated squat-stand manoeuvres at 0.05Hz and 0.10 Hz for placebo and (-)-epicatechin interventions.

	Intervention (mean ± SD)				Linear Mixed Model P Values		
0.05 Hz Squat-stands	**Pre-placebo**	**2-h placebo**	**Pre-epicatechin**	**2-h epicatechin**	**Time**	**Condition**	**T*C**
Gain (cm·s^{-1}·mmHg^{-1})							
VLF	0.7 ± 0.2	0.8 ± 0.2	0.7 ± 0.1	0.7 ± 0.1	0.04*	0.70	0.33
LF	0.8 ± 0.2	0.9 ± 0.3	0.9 ± 0.2	0.9 ± 0.1	0.54	0.82	0.36
HF	0.8 ± 0.2	0.9 ± 0.3	0.8 ± 0.1	0.9 ± 0.2	0.25	0.93	0.61
Phase (radians)							
VLF	55.7 ± 18.9	53.4 ± 23.7	52.9 ± 16.9	47.5 ± 13.7	0.52	0.33	0.59
LF	19.7 ± 12.1	24.6 ± 14.9	21.5 ± 13.5	12.9 ± 16.2	0.68	0.16	0.13
HF	16.8 ± 12.2	22.0 ± 24.2	13.9 ± 11.1	14.1 ± 18.0	0.65	0.47	0.74
Coherence							
VLF	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.83	0.06	0.46
LF	0.6 ± 0.2	0.7 ± 0.2	0.6 ± 0.1	0.6 ± 0.1	0.61	0.65	0.39
HF	0.4 ± 0.2	0.4 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.67	0.42	0.20
0.10 Hz Squat-stands							
Gain (cm·s^{-1}·mmHg^{-1})							
VLF	0.7 ± 0.2	0.8 ± 0.2	0.6 ± 0.2	0.6 ± 0.2	0.62	0.03^	0.20
LF	1.0 ± 0.4	1.0 ± 0.2	0.9 ± 0.2	0.9 ± 0.2	0.58	0.19	0.92
HF	0.9 ± 0.3	1.1 ± 0.3	1.1 ± 0.4	0.9 ± 0.2	0.98	0.72	0.29
Phase (radians)							
VLF	56.9 ± 20.1	47.7 ± 10.1	54.2 ± 13.9	62.4 ± 24.6	0.65	0.04^	0.34
LF	31.2 ± 18.4	33.6 ± 17.5	31.9 ± 19.9	29.2 ± 16.9	0.98	0.69	0.43
HF	21.3 ± 19.2	20.1 ± 27.0	32.0 ± 52.0	11.3 ± 49.0	0.31	0.95	0.35
Coherence							
VLF	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.2	0.40	0.60	0.77
LF	0.6 ± 0.1	0.5 ± 0.2	0.6 ± 0.1	0.6 ± 0.1	0.70	0.09	0.67
HF	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.40	0.37	0.77

Data are mean ± SD. T*C, time*condition interaction. *Main effect of time *P*≤0.05; ^main effect of condition *P*≤0.05.

7.4 Discussion

The primary aim of this exploratory study was to test the hypothesis that acute (-)-epicatechin ingestion improves cerebrovascular function in healthy adults. The study found no differences in cerebrovascular function 2-hours following ingestion of a single dose of (-)-epicatechin compared to placebo, with no effect of (-)-epicatechin on cerebrovascular responses to CO_2 or dynamic autoregulation, assessed via squat-stand manoeuvres. This is the first study to examine the acute impact of (-)-epicatechin on cerebrovascular function in humans using a range of functional tests. Current findings suggest that acute ingestion of (-)-epicatechin does not induce any immediate functional changes in the cerebrovasculature in healthy male individuals.

Despite previous studies observing encouraging effects of acute tea consumption on peripheral vascular function, there is a distinct lack of research examining the impact of tea and its isolated compounds on the cerebrovasculature that have used a variety of methods to interrogate cerebrovascular function. It is, therefore, difficult to compare current findings against the existing research base. This topic is of particular interest since cerebrovascular disease and stroke account for the greatest proportion of cardiovascular related deaths globally (Roger *et al.*, 2012) and dietary behaviours present a relatively easy opportunity to modify cerebrovascular disease risk. Although examining cerebrovascular function is challenging, CO_2 reactivity and cerebral autoregulation provide useful methods of assessment, given that the principle regulators of cerebral perfusion are arterial blood gases, such as CO_2, and blood pressure (Willie *et al.*, 2014). The present study found no effect of a single dose of (-)-epicatechin 2-hours after ingestion compared to placebo, either on cerebrovascular responses to CO_2 or dynamic autoregulation. CO_2 reactivity may act as a surrogate of cerebrovascular endothelial function (Lavi *et al.*, 2006), similar to the role of FMD in assessing endothelial function in the peripheral vasculature. NO is suggested to contribute towards the regulation of the cerebral circulation (Iadecola *et al.*, 1994; Lavi *et al.*, 2003), although it is unclear

whether the NO is derived from the endothelium or neuronal pathways (Lavi *et al.*, 2006). Nevertheless, impaired CO_2 reactivity has been observed in individuals with peripheral endothelial dysfunction, with no apparent effect on pressure-dependent CBF autoregulation (mechanoregulation) (Lavi *et al.*, 2006), thereby suggesting a role for the vascular endothelium in cerebrovascular function. Furthermore, adequate cerebral reactivity to hypercapnia appears to be dependent upon the integrity of the vascular endothelium (Silvestrini *et al.*, 2000; Lavi *et al.*, 2003). Given that (-)-epicatechin is the most abundant flavan-3-ol in cocoa (Dower *et al.*, 2016a; Dower *et al.*, 2016b), in addition to its presence in green tea, and is suggested to be (partly) responsible for mediating cocoa-induced improvements in peripheral vascular function (Schroeter *et al.*, 2006), it was reasonable to surmise that (-)-epicatechin may induce similar changes in the cerebrovasculature. However, the present study observed no differences in cerebrovascular function in response to hypercapnia, with no changes evident for autoregulation, suggesting that (-)-epicatechin does not affect the cerebrovasculature presumably via a direct effect on the endothelium.

In keeping with the concept that (-)-epicatechin does not influence cerebrovascular endothelial function, previous studies also demonstrated no significant improvements in peripheral vascular function (FMD) following acute (2-hours) (Dower *et al.*, 2016b) or regular (4-weeks) (Dower *et al.*, 2015) ingestion of (100mg (-)-epicatechin) in healthy adult males. As an alternative explanation, the cardioprotective effects of (-)-epicatechin may relate to improved insulin resistance rather than a direct effect on the vasculature (Dower *et al.*, 2015). (-)-Epicatechin and insulin are considered to exert similar protective effects on erythrocyte osmotic fragility and in increasing glucose uptake, but act by different mechanisms according to their different binding sites (Rizvi *et al.*, 1995). NO appears to be the primary molecule responsible for the protective effects of (-)-epicatechin, with increased bioavailability suggested to modulate inflammation and reduce the formation of reactive oxygen species (Grassi *et al.*, 2013b), although the precise mechanisms remain unclear. Insulin acts as a

neuroregulatory peptide and plays an important role in brain metabolism, memory and cognition, but is not considered a regulator of cerebrovascular function (Gray *et al.*, 2014). Therefore, it is perhaps unsurprising that the present study observed no differences in cerebrovascular function, and that previous effects in the peripheral vasculature may relate to an insulin-like action, given that these vessels are more dependent upon the liberation of NO by insulin-activated eNOS.

Regular tea consumption is associated with modest 1-2 mmHg reductions in BP (Greyling *et al.*, 2014) that may have implied an effect of (-)-epicatechin on the cerebrovasculature through pressure-dependent CBF regulation (mechanoregulation). However, the present study observed no significant differences in MAP over time or between conditions, and no changes in the findings when CBF was expressed relative to MAP (CBVC). It is likely that any potentially small changes in MAP induced by (-)-epicatechin ingestion were unlikely to be apparent, particularly given the small sample size. Similarly, if the beneficial effects of tea are due to a direct effect on the vascular endothelium arising from NO-mediated mechanisms, no changes in MAP may be observed (Lavi *et al.*, 2006).

Cerebral autoregulation assessed via squat-stand manoeuvres at 0.05 Hz and 0.10 Hz (i.e. 3- and 6-squats per minute, respectively), did not demonstrate any changes in the oscillations between CBF and MAP (transfer function determined gain and phase) following acute (-)-epicatechin ingestion. The mechanisms of cerebral autoregulation include mechanoregulation (see above), NO pathways (see above), myogenic (vascular smooth muscle), neurogenic (α-adrenergic sympathetic nerves) and metabolic (local blood gases) regulation (see above). The present chapter's results suggest that acute (-)-epicatechin ingestion does not affect dynamic cerebral autoregulation. These, as well as the CO_2 reactivity, findings are consistent with findings of the previous chapter (*Chapter 6*) in that ingestion of a dietary flavonoid has demonstrated no direct impact on the cerebrovasculature. As the brain is a highly complex organ and is tightly regulated through multiple interrelated mechanisms, it is possible

that the brain's plasticity or redundancy is able to cope with subtle changes and that a much greater stimulus is required to induce any functional variations. Furthermore, the regulatory mechanisms underpinning cerebrovascular function are incompletely understood so as our collective understanding continues to advance, future studies may be able to identify subtle differences that are possibly currently undetected.

The present study findings are in keeping with the only study to date that has directly investigated the cerebrovascular effects of tea, whereby acute black tea consumption demonstrated no effect on cerebrovascular reactivity to hypercapnia (ASL-MRI) in healthy male adults (Vidyasagar *et al.*, 2013). However, no previous study has specifically examined the effects of green tea or (-)-epicatechin on cerebrovascular function. EGCG, another catechin that is abundant in green tea (Murray *et al.*, 2015), demonstrated decreased CBF (measured by near-infrared spectroscopy; NIRS) during computerised cognitive task performance in healthy humans following a single low dose (135 mg), whereas a higher dose (270 mg) did not change CBF relative to placebo (Wightman *et al.*, 2012). Improved cerebrovascular function has been observed following the ingestion of cocoa flavanols (containing (-)-epicatechin), whereby increased regional CBF (ASL) was demonstrated 2-hours post-ingestion (cocoa flavanols containing 89mg of (-)-epicatechin) in the healthy elderly (Lamport *et al.*, 2015). Similarly, an acute dose (450mg) of flavanols ((-)-epicatechin content unknown) increased grey matter CBF (fMRI) 2-hours post-ingestion in healthy young females (Francis *et al.*, 2006) and increased CBF was observed following regular (1-12 weeks) ingestion in older individuals (Fisher *et al.*, 2006; Sorond *et al.*, 2008a; Brickman *et al.*, 2014). It is possible that the differences in the test product (tea, (-)-epicatechin, EGCG, cocoa flavanols) may be responsible for the variable observations of the aforementioned studies. However, given the paucity of studies to date, it is difficult to establish whether there are distinct differences between the effects of tea and tea-derived flavonoids. Furthermore, it is unlikely that the timing of administration was a factor in the present study, as 2-hours was consistent with suggestions

regarding peak bioavailability (Clifford *et al.*, 2013) and previous studies (Francis *et al.*, 2006; Lamport *et al.*, 2015; Dower *et al.*, 2016b).

It is possible that the acute dose of (-)-epicatechin in the present study was insufficient to affect the cerebrovasculature, particularly since it is difficult to ascertain the exact (-)-epicatechin content of some previous cocoa flavanol studies. In keeping with the present study, peripheral vascular function (FMD) demonstrated no significant effect of 100 mg (-)-epicatechin, yet dark chocolate containing 150 mg (-)-epicatechin significantly improved FMD (Dower *et al.*, 2016b), although a smaller 70 mg (-)-epicatechin dose acutely increased FMD and NO in a pilot study in only three individuals (Schroeter *et al.*, 2006). However, the lack of cerebrovascular (-)-epicatechin studies makes accurate comparison of dose administration difficult. Variations in the cerebrovascular assessment methods between the present and previous cerebral studies likely also contribute to differences in findings. Previously, differences in CBF were demonstrated using techniques such as ASL or fMRI, that are more sensitive in detecting changes in global and regional perfusion rather than using MCAv via TCD, as in the present study. It is, therefore, possible that subtle changes in cerebrovascular function were not detected in the present study, but may have been observed using alternative techniques that assess regional perfusion.

Older individuals are likely to exhibit compromised cerebrovascular function associated with ageing (Parkes *et al.*, 2004; Bertsch *et al.*, 2009), which may partly explain the improved (-)-epicatechin-induced cerebrovascular function found in previous studies (Fisher *et al.*, 2006; Sorond *et al.*, 2008a; Brickman *et al.*, 2014; Lamport *et al.*, 2015) and no differences observed in the healthy male adults examined in the present study. The present cohort likely had near-optimal cerebrovascular function and individuals with chronic cerebrovascular dysfunction *a priori* (such as the elderly or individuals with CVD risk factors) may be more likely to exhibit changes following ingestion of tea or tea-derived

flavonoids. Consistent with this, (-)-epicatechin intake was associated with a 46% lower risk of CVD related mortality in men with prevalent CVD, compared to no such risk reduction in healthy men in a prospective cohort study (Dower *et al.*, 2016a). However, further studies in at-risk and elderly populations are required to corroborate this suggestion.

7.4.1 Clinical Relevance and Perspectives

Findings from the present study suggest that acute (-)-epicatechin ingestion is not associated with improved cerebral CO_2 reactivity or dynamic autoregulation in healthy younger individuals. However, the long-term effects remain unclear and, furthermore, its effects in a population exhibiting impaired cerebrovascular function are unknown. This remains of special importance given previous observations in population studies that found that (-)-epicatechin intake is associated with lower risk for CVD-related mortality. Further studies are therefore warranted to explore the longitudinal effects of (-)-epicatechin and whether it has an impact upon individuals with existing (endothelial) dysfunction.

7.4.2 Limitations

Given that regulation of the cerebrovasculature is highly complex and involves multiple mechanisms, subtle changes in function following (-)-epicatechin ingestion may not have been detected in the current cohort given the small sample size. Inherent methodological considerations of TCD may have influenced the findings of the present study, as it is not possible to ensure complete accuracy in insonating the same part of the MCA for each set of measures. However, every effort was made to document Doppler probe positions and to record velocity and depth values for subsequent laboratory measures. It is assumed that during TCD the vessel diameter does not change which may have influenced the data obtained during hypercapnia. Nevertheless, the absolute CO_2 reactivity data was

indicative of similar cerebrovascular responses, suggesting that the MCA likely dilated to a similar extent following both (-)-epicatechin and placebo.

In conclusion, the present exploratory study suggests that acute ingestion of (-)-epicatechin does not affect cerebrovascular function, as assessed by CO_2 reactivity and dynamic autoregulation. The present findings are consistent with those of *Chapter 6*, in that the cerebrovasculature does not exhibit any changes in function following the ingestion of either the isolated compound (-)-epicatechin or tea *per se.* However, further investigation is warranted to examine the longitudinal effects of (-)-epicatechin on the cerebrovasculature, particularly in cohorts that may be more susceptible to changes in the cerebrovasculature.

CHAPTER 8: SYNTHESIS OF FINDINGS

8.1 Aims and Objectives

The research described within the present thesis was designed to explore the impact of tea ingestion on peripheral, central and cerebral micro- and macrovascular function in humans. Furthermore, the studies specifically examined NO-mediated endothelial function in both the cutaneous microvessels and conduit arteries and, for the first time, explored the impact of an unhealthy lifestyle intervention on multiple measures of vascular health and insulin resistance, along with the impact of regular green tea consumption on these measures of vascular health and insulin resistance. Similarly, work contained within this thesis provides the first observations of regular green tea consumption on the cerebrovasculature and the acute effect of the tea-derived catechin (-)-epicatechin on cerebrovascular function.

8.2 Major Findings

8.2.1 Simultaneous use of Local Heating Protocols in Examining Cutaneous Microvascular Function

The reproducibility study detailed in *Chapter 4* found that the *Rapid 42°C* and *Gradual 42°C* local heating protocols provide superior reproducibility to other local heating protocols, such as *Rapid 39°C,* when assessing cutaneous microvascular function. Furthermore, the data of *Chapter 4* provides useful sample size estimations to detect meaningful differences in future studies using local thermal hyperaemia. Combined with the observations of *Chapters 5* and *6*, whereby acute and regular consumption of black and green tea, respectively, demonstrated enhanced microvascular function, these findings collectively support the simultaneous use of multiple local heating protocols to interrogate the microvasculature and achieve better mechanistic insight for use in future epidemiological studies and clinical trials. In addition, current findings suggest that differences in cutaneous microvascular function are more likely to be detected with the local heating protocols that have the smallest variation (*Rapid 42°C* and *Gradual 42°C*).

8.2.2 Tea and Cutaneous Microvessel Endothelial Function

Chapter 5 demonstrated that acute (2-hour) black tea consumption enhanced cutaneous vascular function in healthy, middle-aged individuals compared to placebo. Two distinctly different techniques (LDF and FLPI) detected these effects using gradual local heating, thereby reinforcing the observed microvascular effects of acute black tea consumption. However, no differences were detected following rapid local heating using LDF. Furthermore, in *Chapter 6,* green tea also demonstrated a cardioprotective role in that daily consumption for 7-days largely attenuated the reduction in cutaneous blood flow in response to gradual, but not rapid, heating following an unhealthy lifestyle. As gradual local heating is largely NO-mediated (Black *et al.*, 2008b), current findings suggest that the microvascular benefits of tea consumption are likely achieved through a mechanism related to activation of endothelium-derived vasodilators, such as NO. Given that the rapid local heating protocols are predominantly NO-mediated, but to a lesser extent than gradual local heating, and that tea attenuated the unhealthy lifestyle-induced impairment in microvascular function in *Chapter 6,* reinforces the concept that NO plays an important protective role in the microvasculature.

8.2.3 Short-term Lifestyle Intervention and its Effects on Vascular and Metabolic Function

Chapter 6 demonstrated the deleterious impact of an unhealthy Western lifestyle, characterised by a high energy diet and physical inactivity, inducing peripheral vascular dysfunction and insulin resistance after a period of only 7-days. Importantly, as such sedentary lifestyles reflect the habitual behaviour of an ever-increasing number of individuals in developed countries, and others experience such a lifestyle during holidays and festivities, these observations are widely applicable. Furthermore, as the profound impact of excess calorie consumption and physical inactivity was observed after only a few days in a cohort of previously healthy male adults, current findings demonstrate the significant negative impact of this lifestyle on cardiovascular health. The ability of the lifestyle model adopted in *Chapter 6* to induce immediate changes in peripheral vascular function and metabolic function highlights its value and relevance as a tool in evaluating lifestyle-induced changes and the impact of interventions to enable the development of strategies to combat such negative consequences.

8.2.4 Tea and Conduit Artery Endothelial Function

Consistent with the findings in the microvasculature, *Chapter 6* found that tea was also associated with a cardioprotective role in conduit vessels, as daily green tea consumption attenuated the decline in conduit artery endothelial-dependent function following a 7-day lifestyle intervention in healthy males. These changes were present in the femoral artery which was likely to have been impacted by the physical activity reduction to a greater extent than the brachial artery. However, the brachial artery did demonstrate a non-significant increase in FMD following daily green tea consumption, again suggesting a role for tea in improving conduit artery endothelial function. As direct measures of endothelial function in the micro- and macrovasculature provide independent prognostic value in evaluating overall cardiovascular health and CVD risk (Green *et al.*, 2011), current findings support the role of tea as a cardioprotective strategy in reducing CVD risk amongst the general population.

Chapter 6 also demonstrated that extra-cranial artery function, assessed by carotid artery reactivity to the CPT, was impaired following the unhealthy lifestyle, with a concomitant increase in both the basal and peak BP during the CPT. These findings suggest that the unhealthy lifestyle induces systemic maladaptation, as the deleterious impact is not confined to the peripheral vasculature. Importantly, daily green tea consumption attenuated the decline in carotid artery reactivity, thus supporting the cardioprotective role of tea throughout the vascular tree.

8.2.5 Tea and Cerebrovascular Function

Contrary to the beneficial effects of tea observed on peripheral vascular function, *Chapter 6* observed no differences in cerebral reactivity or dynamic autoregulation in response to daily green tea consumption during a 7-day lifestyle intervention. Furthermore, acute (2-hour) ingestion of the tea-derived catechin (-)-epicatechin demonstrated no impact upon the cerebrovasculature in response to similar measures in *Chapter 7*. The combined observations on cerebrovascular function suggest that tea may not impact the cerebral vessels in the same way as the micro- and macro-vessels, or that the

brain requires much greater stimuli (e.g., inactivity, diet and/or polyphenols) to induce functional changes.

8.2.6 Tea and Insulin Resistance

Chapter 6 demonstrated that green tea was associated with a protective role on metabolic function in healthy male adults. Following short-term (7-day) lifestyle-induced insulin resistance, daily green tea ingestion not only attenuated elevated blood glucose levels, but also improved glucose handling and concomitantly enhanced insulin sensitivity. This study, therefore, highlights the ability of green tea to mitigate impairments in metabolic function and its use as a potential adjunct in combating the deleterious impact of high energy, sedentary lifestyles.

8.3 General Discussion

The skin is the human body's largest organ and represents an easily accessible vascular bed to assess peripheral microvascular reactivity, also providing a model to investigate underlying mechanisms in various diseased states (Levy *et al.*, 2001; Sokolnicki *et al.*, 2007; Levy *et al.*, 2008). The findings from *Chapters 4*, *5* and *6* support this notion, but further demonstrate the value of using multiple local heating protocols simultaneously. Interrogating the microvessels using a combination of local heating protocols provides a means of discovering the underlying mechanisms that are responsible for changes in function following intervention studies. Specifically, use of rapid and gradual local heating protocols exhibited different cutaneous responses to local heating in *Chapters 5* and *6* that investigated the effects of acute and regular tea consumption, respectively. The data presented in *Chapter 5* revealed that middle-aged healthy individuals exhibited increased cutaneous microvascular function 2-hours following black tea ingestion compared to placebo, as demonstrated following gradual local heating that is largely NO-mediated. Given that rapid local heating did not exhibit any such differences between tea and placebo, these findings highlight the importance of interrogating the cutaneous microvessels via multiple protocols. Similarly, the data presented in *Chapter 6* further

supports this approach, as gradual local heating again demonstrated tea-induced increases in cutaneous vasodilation, whereas rapid local heating observed no changes between pre- and post-tea, compared to a reduction in vasodilation following placebo. It is possible that the studies in *Chapters 5* and *6* were statistically underpowered to detect differences pre- vs post-tea with rapid local heating, according to the findings and sample size estimations contained within *Chapter 4*. However, the observed differences between rapid and gradual local heating protocols may also be due to essential differences in the underlying mechanisms. Despite rapid local heating protocols also being NO-mediated, but to a lesser extent than gradual heating, they also activate axon-reflexes and are partly mediated by EDHFs (Johnson *et al.*, 2014). Consequently, rapid local heating provides complementary mechanistic insight when used in conjunction with gradual heating.

Assessing cutaneous microvascular function is logical when examining the cardiovascular impact of interventions, since the skin represents a valid model of generalised microvascular (dys)function (Holowatz *et al.*, 2008) and is regarded as an earlier sentinel of CVD than the macrovasculature (Roustit & Cracowski, 2012). *Chapter 5* demonstrated acute (2-hour) improvements in cutaneous microvascular function in response to black tea consumption that is encouraging, given that this vascular bed is associated with some of the earliest manifestations of CVD and diabetes in multiple organ systems (Cade, 2008). Similarly, the lifestyle intervention adopted in *Chapter 6* demonstrated a deleterious impact in the microvasculature after only 7-days that was not present in all of the examined conduit arteries, again suggesting that detrimental changes first manifest in the cutaneous microvasculature which is, therefore, a crucial component of the vascular tree.

Chapter 6 demonstrated that a short lifestyle intervention had a significant deleterious impact on several markers of cardiovascular health. Previously, no research has examined the impact of such a lifestyle intervention on vascular health in a range of vascular beds simultaneously. The

comprehensive vascular assessment and observations in *Chapter* 6 are indicative of lifestyle-induced systemic changes in vascular function. Many individuals adopt such a lifestyle for much more prolonged durations than in *Chapter 6* and others who are generally active and consume eucaloric diets, adopt short-term unhealthy lifestyles during periods of celebration or festivities. It is, therefore, both surprising and concerning that such detrimental changes in vascular function and insulin sensitivity were observed in healthy adult males after only 7-days. The data presented in *Chapter 6* are, therefore, an important indication of the negative consequences such lifestyles have on cardiovascular health and furthermore, are suggestive of the early manifestation of changes throughout the vascular tree that may subsequently progress to overt CVD.

Given that *Chapter 6* observed impaired insulin signalling and altered glucose metabolism that are characteristic of insulin resistance, these findings demonstrate the development of insulin resistant mechanisms after only 7-days of an altered lifestyle. Under healthy conditions, NO production in the endothelial cells is stimulated by insulin activating NOS via the PI-3K pathway (Sena *et al.*, 2013), but this process is impaired in an insulin resistant state, thereby diminishing NO production and subsequently impairing the ability of vessels to dilate (Kim *et al.*, 2006). That changes in insulin sensitivity were detectable after only 7-days suggests that the inflammatory cascade leading to atherosclerosis manifests early following changes in lifestyle. However, it is unknown whether such rapid changes translate to long-term changes in the vasculature and metabolic pathways. Furthermore, the precise mechanisms responsible for tea-induced changes in glucose control and insulin sensitivity are uncertain. Animal models suggest several mechanisms, including a reduction in glucose production in the intestinal tract due to inhibition of carbohydrate digestive enzymes (Kobayashi *et al.*, 2000; Oh *et al.*, 2015). Enhanced insulin binding to adipocytes and improved glucose uptake by myocytes are also suggested to be responsible for improved insulin sensitivity following tea and catechin consumption (Wu *et al.*, 2004a; Wu *et al.*, 2004b; Ueda *et al.*, 2010; Ueda-Wakagi *et al.*,

2015). However, the metabolic pathways in humans have yet to be determined and *Chapter 6* did not investigate the gut microbiota so the mechanisms underlying the improved glucose handling and enhanced insulin sensitivity remain uncertain. It is perhaps interesting to consider the possibility that improved vascular function (as outlined in *Chapter 6*) could have also contributed to the improved glucose control and insulin sensitivity through improved delivery/disposal for example (Wagenmakers *et al.*, 2016).

The observations of previous research suggesting a cardioprotective role of tea on the vasculature were both supported and further extended by the findings of *Chapter 6,* in that daily green tea consumption attenuated the negative consequences of the short-term lifestyle intervention in previously healthy male adults. Previous studies have generally assessed the acute impact of tea on conduit artery endothelium-dependent vasodilation (Hodgson *et al.*, 2005; Schreuder *et al.*, 2014), with no prior work examining the vascular effects of tea on lifestyle changes. The ability of green tea to not only attenuate, but improve vascular function throughout the vascular tree in the presence of an unhealthy lifestyle, suggests that tea exerts a potent effect at the level of the endothelium, likely related to activation/upregulation of endothelium-derived vasodilators, such as NO. The observed metabolic changes are also consistent with this theory, given that green tea enhanced insulin sensitivity which is partly responsible for stimulating NO production in endothelial cells via the catalytic conversion of L-arginine to L-citrulline. Whilst the precise mechanisms underlying the tea-induced improvements in vascular and metabolic function remain unclear, the cumulative findings of *Chapters 5* and *6* are consistent with a NO-mediated mechanism.

Despite the beneficial effects of tea consumption observed in the peripheral vasculature in *Chapters 5* and *6*, based on current work contained within this thesis, tea and the tea-derived catechin (-)-epicatechin, do not appear to exert any effect upon cerebrovascular function in healthy adult males.

Such findings are largely consistent with the few studies previously undertaken concerning the cerebrovascular effects of tea consumption (Vidyasagar *et al.*, 2013). Given that the brain is a large organ and requires a large stimulus to induce functional variations, it is feasible that the duration and/or dosage of tea/(-)-epicatechin were insufficient to influence the cerebrovasculature. Similarly, the lifestyle intervention may not have been severe enough to generate an impairment in cerebrovascular function, particularly since the control mechanisms of the cerebrovasculature are different to those in the peripheral vessels. Furthermore, it is acknowledged that the sample sizes for the cerebral data of *Chapters 6* and *7* were small and unlikely to detect any subtle changes in function that may have been present. Likewise, current findings were observed in healthy adult males with optimal cerebral endothelial function. The impact in individuals with compromised endothelial function may exhibit different changes, particularly an ageing population. Further research is, therefore, required to determine the effects of tea in such populations who are at greater risk of cerebrovascular disease.

The work contained within this thesis provides compelling evidence for a cardioprotective role of tea on peripheral vascular function. The experimental studies detailed herein have contributed to the collective understanding of the role of tea in mitigating CVD risk and being a useful therapeutic approach in improving overall cardiovascular health. Furthermore, the experimental work of this thesis has contributed to the understanding of underlying mechanisms likely responsible for the observed vascular improvements and this thesis highlights the value of performing simultaneous local thermal hyperaemic assessment to interrogate the microvasculature for mechanistic insight.

8.4 Implications

Cardiovascular disease remains the leading cause of global mortality (WHO, 2016) and an economic burden for public health. Management of modifiable CVD risk factors remains an important strategy

in combating the progression of CVD risk towards overt disease. The research work undertaken in this thesis provides important insight into the effects of tea consumption on peripheral vascular function and insulin sensitivity, particularly its beneficial effects on lifestyle-induced vascular impairments. *Chapters 5* and *6* are the first studies to evidence improved cutaneous microvascular function following both black and green tea, respectively, suggesting that the benefits of tea may not be confined to one type and, therefore, its effects may be experienced by a greater percentage of the population. Moreover, the beneficial impact in the cutaneous microvasculature is encouraging given that it is an important vascular bed in the pathogenesis of diabetic microangiopathy (Jaap *et al.*, 1994; Tooke, 1995). Together with the generalised improvements in peripheral vascular function and insulin sensitivity observed in response to a short-term lifestyle intervention in *Chapter 6*, regular tea consumption may present a viable means of mitigating the deleterious impact of an unhealthy lifestyle. However, importantly, regular tea consumption should not be seen as a "quick fix" to temper the detrimental effects of an unhealthy lifestyle, or reduce the importance of regular exercise and a balanced diet in maintaining a healthy lifestyle and overall cardiovascular health. Nevertheless, regular tea consumption may represent a suitable intervention during forced periods of inactivity and/or excess calorie intake, such as illness or holiday periods, respectively. Longitudinal randomised controlled trials are required to determine whether such tea-induced vasoprotective effects are apparent following more prolonged lifestyle behaviours/changes. As higher levels of NO provide anti-atherogenic benefits and the data presented within this thesis support the role of tea in improving NO bioavailability, these findings provide potentially significant implications for the maintenance of cardiovascular health, in addition to the future prevention and management of CVD. Tea consumption presents a simple, inexpensive, non-pharmacological cardioprotective strategy to help combat the ever-increasing global burden of CVD.

8.5 Methodological Considerations and Limitations

There are several strengths in the methodology of this thesis. Firstly, determining the reproducibility of local heating protocols used to assess microvascular function (*Chapter* 4) enabled appropriate protocols to be selected for use in intervention studies in subsequent chapters of this thesis (*Chapters 5* and *6*). Furthermore, the simultaneous use of such local heating protocols within this thesis highlights that it is a simple, applicable and feasible approach to assess cutaneous microvascular function and may provide further insight into the skin vasodilatory mechanisms. The studies contained within this thesis were some of the first to adopt use of multiple local heating protocols. As the skin is a complicated organ, it is sensible to use a multiple protocol approach rather than focus on a single technique. It is, therefore, recommended that future microvascular studies should adopt a similar approach to the work contained within this thesis and simultaneously use multiple local heating protocols to interrogate the skin vasculature. As FMD assessment was undertaken according to the latest peer-reviewed consensus guidelines (Thijssen *et al.*, 2011), together with the use of custom-designed edge-detection and wall-tracking analysis software (*Chapter 6*), the accuracy, validity and prognostic index of FMD measures was maximised. Further methodological rigor was adhered to throughout the experimental chapters, with strict inclusion and exclusion criteria, in addition to the control of dietary polyphenols prior to and during laboratory measures. Finally, compliance to the (in)activity levels and dietary intake was monitored during the lifestyle intervention (*Chapter 6*) .

Despite the methodological strengths, there are apparent limitations within this thesis. The modest sample sizes of the intervention studies mean that the findings cannot be generalised towards the wider populace. Furthermore, the studies were undertaken in healthy individuals so the findings cannot simply be extrapolated to patient groups. The research contained within this thesis does not provide any biochemical or biomolecular insight into the mechanisms underlying the vascular and

metabolic improvements following tea consumption, although it is important to emphasise that this was beyond the aims of the thesis. A further limitation is the method used to assess insulin resistance in *Chapter 6*, whereby a mixed-meal tolerance test was used; a two-stage hyperinsulinaemic-euglycaemic clamp with infusion of deuterated glucose (Shojaee-Moradie *et al.*, 2007) would have provided a more comprehensive assessment of hepatic and peripheral insulin sensitivity. However, given that the participants arrived to the laboratory fasted and underwent a series of vascular assessments prior to the insulin and glucose measures, a mixed-meal was deemed more appropriate and ethical.

8.6 Future Direction

Several potential areas of future research have emerged following the studies detailed within this thesis. Firstly, whilst the effect of acute black tea consumption on microvascular function was established in *Chapter 5*, the long-term effects of tea on the microvasculature remain unknown which could potentially have wider implications for cardiovascular health in the general population. Furthermore, the tea-induced, likely NO-mediated, improvements in cutaneous vascular function were observed in healthy individuals and given that reduced NO bioavailability and endothelial dysfunction are early markers of atherosclerosis and type 2 diabetes (Sena *et al.*, 2013), further research is warranted to determine whether such improvements are seen in a population with compromised endothelial function, or whether they are confined to healthy individuals. Likewise, the mitigating effect of tea on the peripheral vasculature and metabolic function demonstrated in combination with the lifestyle intervention in *Chapter 6,* requires longitudinal study to determine whether tea exhibits such a protective role following an unhealthy, or even a 'normal' or healthy, lifestyle of a much greater duration. Further longitudinal randomised controlled trials will provide greater insight into whether regular tea consumption may also benefit both healthy individuals and patient groups. Given the increasing economic burden of CVD together with an ageing population, it

is important to establish whether the apparent cardioprotective properties of tea may be exploited for consideration as a potentially inexpensive, non-pharmacological alternative for improving cardiovascular health.

An important area requiring further study relates to the sequence of physiological events by which tea improves both vascular and metabolic function, as observed in *Chapters 5* and *6.* Whilst it is likely that tea exerts its beneficial effects via a mechanism related to upregulation of chemical mediators such as NO, it is currently unclear whether the observed improvements occur firstly in the vasculature, or whether tea consumption exerts an insulin-sensitising effect and stimulation of the PI-3K pathway to enhance NO production from the vascular endothelium. Determining this sequence will further our collective understanding of the mechanistic processes underpinning the beneficial effects of tea and any additional signalling pathways that may be involved.

It remains unclear whether tea and its isolated constituents such as (-)-epicatechin, influence cerebrovascular function based upon the work detailed within this thesis and the overall paucity of studies performed to date. Future research should, therefore, endeavour to further explore both the acute and chronic effects of tea and tea-derived catechins on the cerebrovasculature. The present findings were observed in healthy individuals whereby the brain may require a much greater stimulus than the studies herein to demonstrate any functional change. It would, therefore, be prudent to examine the effects of tea in a population at risk of cerebrovascular disease, particularly older individuals. Furthermore, it would be valuable to investigate the effects of tea and isolated tea-derived compounds on cognitive function, particularly since cerebral hypoperfusion is considered to be a potential trigger of cognitive decline and the development of neurodegenerative disorders, such as dementia (Farkas *et al.*, 2002).

Given the tea-induced improvements on endothelial function, it would be worthwhile examining the impact of tea consumption upon athletic performance, particularly since blood flow and maximum cardiac output are critical determinants of overall cardiovascular performance (Somerville *et al.*, 2017). Polyphenols, such as quercetin, have demonstrated improved aerobic performance in double-blind randomised controlled trials linked to mechanisms related to upregulation of mitochondrial biogenesis (MacRae & Mefferd, 2006; Nieman *et al.*, 2010). However, tea and tea-derived catechins have demonstrated equivocal outcomes and warrant further investigation.

CHAPTER 9: REFERENCES

Aadland E & Ylvisåker E (2015). Reliability of the Actigraph GT3X+ Accelerometer in adults under free-living conditions. *PLoS ONE* **10,** e0134606.

Aaslid R, Markwalder TM & Nornes H (1982). Noninvasive transcranial Doppler ultrasound recording of flow velocity in basal cerebral arteries. *Journal of Neurosurgery* **57,** 769-774.

Adochio RL, Leitner JW, Gray K, Draznin B & Cornier MA (2009). Early responses of insulin signaling to high-carbohydrate and high-fat overfeeding. *Nutrition and Metabolism* **6,** 37.

Agarwal SC, Allen J, Murray A & Purcell IF (2010). Comparative reproducibility of dermal microvascular blood flow changes in response to acetylcholine iontophoresis, hyperthermia and reactive hyperaemia. *Physiological Measurement* **31,** 1-11.

Ainslie PN, Ashmead JC, Ide K, Morgan BJ & Poulin MJ (2005). Differential responses to CO_2 and sympathetic stimulation in the cerebral and femoral circulations in humans. *The Journal of Physiology* **566,** 613-624.

Ainslie PN, Cotter JD, George KP, Lucas S, Murrell C, Shave R, Thomas KN, Williams MJA & Atkinson G (2008). Elevation in cerebral blood flow velocity with aerobic fitness throughout healthy human ageing. *The Journal of Physiology* **586,** 4005-4010.

Ainslie PN & Duffin J (2009). Integration of cerebrovascular CO_2 reactivity and chemoreflex control of breathing: Mechanisms of regulation, measurement, and interpretation. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* **296,** R1473-R1495.

Alexopoulos N, Vlachopoulos C, Aznaouridis K, Baou K, Vasiliadou C, Pietri P, Xaplanteris P, Stefanadi E & Stefanadis C (2008). The acute effect of green tea consumption on endothelial function in healthy individuals. *European Journal of Cardiovascular Prevention and Rehabilitation* **15,** 300-305.

Amiot MJ, Riva C & Vinet A (2016). Effects of dietary polyphenols on metabolic syndrome features in humans: A systematic review. *Obesity Reviews* **17,** 573-586.

Arab L, Liu W & Elashoff D (2009). Green and black tea consumption and risk of stroke: A meta-analysis. *Stroke* **40,** 1786-1792.

Arts IC & Hollman PC (2005). Polyphenols and disease risk in epidemiologic studies. *The American Journal of Clinical Nutrition* **81,** 317S-325S.

Arts IC, Hollman PC, Feskens EJ, Bueno de Mesquita HB & Kromhout D (2001). Catechin intake might explain the inverse relation between tea consumption and ischemic heart disease: The Zutphen Elderly Study. *The American Journal of Clinical Nutrition* **74,** 227-232.

Avogaro A, Albiero M, Menegazzo L, de Kreutzenberg S & Fadini GP (2011). Endothelial dysfunction in diabetes: The role of reparatory mechanisms. *Diabetes Care* **34,** S285-S290.

Bae J-H, Bassenge E, Kim K-B, Kim Y-N, Kim K-S, Lee H-J, Moon K-C, Lee M-S, Park K-Y & Schwemmer M (2001). Postprandial hypertriglyceridemia impairs endothelial function by enhanced oxidant stress. *Atherosclerosis* **155,** 517-523.

Baer DJ, Novotny JA, Harris GK, Stote K, Clevidence B & Rumpler WV (2011). Oolong tea does not improve glucose metabolism in non-diabetic adults. *European Journal of Clinical Nutrition* **65,** 87-93.

Bain AR, Morrison SA & Ainslie PN (2014). Cerebral oxygenation and hyperthermia. *Frontiers in Physiology* **5,** 92.

Baker C (2017). Obesity statistics ed. Library HOC. UK Parliament

Beecher GR (2003). Overview of dietary flavonoids: Nomenclature, occurrence and intake. *The Journal of Nutrition* **133,** 3248S-3254S.

Bertsch K, Hagemann D, Hermes M, Walter C, Khan R & Naumann E (2009). Resting cerebral blood flow, attention, and aging. *Brain Research* **1267,** 77-88.

Betik AC, Luckham VB & Hughson RL (2004). Flow-mediated dilation in human brachial artery after different circulatory occlusion conditions. *American Journal of Physiology - Heart and Circulatory Physiology* **286,** H442-H448.

Bhatnagar P, Wickramasinghe K, Wilkins E & Townsend N (2016). Trends in the epidemiology of cardiovascular disease in the UK. *Heart* **102,** 1945-1952.

BHF (2017). Physical inactivity and sedentary behaviour. From https://www.bhf.org.uk/publications/statistics/physical-inactivity-report-2017.

Black MA, Cable NT, Thijssen DHJ & Green DJ (2008a). Importance of measuring the time course of flow-mediated dilatation in humans. *Hypertension* **51,** 203-210.

Black MA, Green DJ & Cable NT (2008b). Exercise prevents age-related decline in nitric-oxide-mediated vasodilator function in cutaneous microvessels. *The Journal of Physiology* **586,** 3511-3524.

Bleeker MW, De Groot PC, Poelkens F, Rongen GA, Smits P & Hopman MT (2005a). Vascular adaptation to 4 wk of deconditioning by unilateral lower limb suspension. *American Journal of Physiology - Heart and Circulatory Physiology* **288,** H1747-1755.

Bleeker MW, Kooijman M, Rongen GA, Hopman MT & Smits P (2005b). Preserved contribution of nitric oxide to baseline vascular tone in deconditioned human skeletal muscle. *The Journal of Physiology* **565,** 685-694.

Bleeker MWP, De Groot PCE, Rongen GA, Rittweger J, Felsenberg D, Smits P & Hopman MTE (2005c). Vascular adaptation to deconditioning and the effect of an exercise countermeasure: Results of the Berlin Bed Rest study. *Journal of Applied Physiology* **99,** 1293-1300.

Bleys RL, Cowen T, Groen GJ, Hillen B & Ibrahim NB (1996). Perivascular nerves of the human basal cerebral arteries: I. Topographical distribution. *Journal of Cerebral Blood Flow and Metabolism* **16,** 1034-1047.

Bogdanski P, Suliburska J, Szulinska M, Stepien M, Pupek-Musialik D & Jablecka A (2012). Green tea extract reduces blood pressure, inflammatory biomarkers, and oxidative stress and improves parameters associated with insulin resistance in obese, hypertensive patients. *Nutrition Research* **32,** 421-427.

Bogert LWJ & van Lieshout JJ (2005). Non-invasive pulsatile arterial pressure and stroke volume changes from the human finger. *Experimental Physiology* **90,** 437-446.

Bonetti PO, Lerman LO & Lerman A (2003). Endothelial dysfunction - A marker of atherosclerotic risk. *Arteriosclerosis, Thrombosis and Vascular Biology* **23,** 168-175.

Bonnin P, Ben Driss A, Benessiano J, Maillet A, Pavy le Traon A & Levy BI (2001). Enhanced flow-dependent vasodilatation after bed rest, a possible mechanism for orthostatic intolerance in humans. *European Journal of Applied Physiology* **85,** 420-426.

Booth FW, Roberts CK & Laye MJ (2012). Lack of exercise is a major cause of chronic diseases. *Comprehensive Physiology* **2,** 1143-1211.

Borodulya AV & Pletchkova EK (1973). Distribution of cholinergic and adrenergic nerves in the internal carotid artery. A histochemical study. *Acta Anatomica* **86,** 410-425.

Borodulya AV & Pletchkova EK (1976). Cholinergic innervation of vessels of the base of the brain. *Acta Anatomica* **96,** 135-147.

Bots ML, Hofman A & Grobbee DE (1994). Common carotid intima-media thickness and lower extremity arterial atherosclerosis: The Rotterdam Study. *Arteriosclerosis and Thrombosis* **14,** 1885-1891.

Bouzat P, Oddo M & Payen JF (2014). Transcranial Doppler after traumatic brain injury: Is there a role? *Current Opinion in Critical Care* **20,** 153-160.

Boyle LJ, Credeur DP, Jenkins NT, Padilla J, Leidy HJ, Thyfault JP & Fadel PJ (2013). Impact of reduced daily physical activity on conduit artery flow-mediated dilation and circulating endothelial microparticles. *Journal of Applied Physiology* **115,** 1519-1525.

Bravo L (1998). Polyphenols: Chemistry, dietary sources, metabolism, and nutritional significance. *Nutrition Reviews* **56,** 317-333.

Bray GA, Redman LM, de Jonge L, Rood J & Smith SR (2016). Effect of three levels of dietary protein on metabolic phenotype of healthy individuals with 8 weeks of overfeeding. *The Journal of Clinical Endocrinology & Metabolism* **101,** 2836-2843.

Brickman AM, Khan UA, Provenzano FA, Yeung L-K, Suzuki W, Schroeter H, Wall M, Sloan RP & Small SA (2014). Enhancing dentate gyrus function with dietary flavanols improves cognition in older adults. *Nature Neuroscience* **17,** 1798-1803.

Briers D, Duncan DD, Hirst E, Kirkpatrick SJ, Larsson M, Steenbergen W, Stromberg T & Thompson OB (2013). Laser speckle contrast imaging: theoretical and practical limitations. *Journal of Biomedical Optics* **18**.

Brons C, Jensen CB, Storgaard H, Hiscock NJ, White A, Appel JS, Jacobsen S, Nilsson E, Larsen CM, Astrup A, Quistorff B & Vaag A (2009). Impact of short-term high-fat feeding on glucose and insulin metabolism in young healthy men. *The Journal of Physiology* **587,** 2387-2397.

Brown AL, Lane J, Coverly J, Stocks J, Jackson S, Stephen A, Bluck L, Coward A & Hendrickx H (2009). Effects of dietary supplementation with the green tea polyphenol epigallocatechin-3-gallate on insulin resistance and associated metabolic risk factors: randomized controlled trial. *British Journal of Nutrition* **101,** 886-894.

Brown AL, Lane J, Holyoak C, Nicol B, Mayes AE & Dadd T (2011). Health effects of green tea catechins in overweight and obese men: A randomised controlled cross-over trial. *British Journal of Nutrition* **106,** 1880-1889.

Brown AM & Ransom BR (2007). Astrocyte glycogen and brain energy metabolism. *Glia* **55,** 1263-1271.

Bruckner V & Szent-Györgyi A (1936). Chemical nature of citrin. *Nature* **138,** 1057.

Brunt VE & Minson CT (2011). Cutaneous thermal hyperemia: More than skin deep. *Journal of Applied Physiology* **111,** 5-7.

Bryans JA, Judd PA & Ellis PR (2007). The effect of consuming instant black tea on postprandial plasma glucose and insulin concentrations in healthy humans. *Journal of the American College of Nutrition* **26,** 471-477.

Butacnum A, Chongsuwat R & Bumrungpert A (2017). Black tea consumption improves postprandial glycemic control in normal and pre-diabetic subjects: A randomized, double-blind, placebo-controlled crossover study. *Asia Pacific Journal of Clinical Nutrition* **26,** 59-64.

Cable NT (2006). Unlocking the secrets of skin blood flow. *The Journal of Physiology* **572,** 613-613.

Cabrera C, Artacho R & Gimenez R (2006). Beneficial Effects of Green Tea - A Review. *Journal of the American College of Nutrition* **25,** 79-99.

Cade WT (2008). Diabetes-related microvascular and macrovascular diseases in the physical therapy setting. *Physical Therapy* **88,** 1322-1335.

Carter HH, Spence AL, Pugh CJA, Ainslie PN, Naylor LH & Green DJ (2014). Cardiovascular responses to water immersion in humans: Impact on cerebral perfusion. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*.

Carter SJ & Hodges GJ (2011). Sensory and sympathetic nerve contributions to the cutaneous vasodilator response from a noxious heat stimulus. *Experimental Physiology* **96,** 1208-1217.

Cassidy A, Mukamal KJ, Liu L, Franz M, Eliassen AH & Rimm EB (2013). A high anthocyanin intake is associated with a reduced risk of myocardial infarction in young and middle-aged women. *Circulation* **127,** 188-196.

Chan CC, Koo MW, Ng EH, Tang OS, Yeung WS & Ho PC (2006). Effects of Chinese green tea on weight, and hormonal and biochemical profiles in obese patients with polycystic ovary syndrome-a randomized placebo-controlled trial. *Journal of the Society for Gynecologic Investigation* **13,** 63-68.

Chang J, Rimando A, Pallas M, Camins A, Porquet D, Reeves J, Shukitt-Hale B, Smith MA, Joseph JA & Casadesus G (2012). Low-dose pterostilbene, but not resveratrol, is a potent neuromodulator in aging and Alzheimer's disease. *Neurobiology of Aging* **33,** 2062-2071.

Charkoudian N (2001). Influences of female reproductive hormones on sympathetic control of the circulation in humans. *Clinical Autonomic Research* **11,** 295-301.

Charkoudian N (2003). Skin blood flow in adult human thermoregulation: How it works, when it does not, and why. *Mayo Clinic Proceedings* **78,** 603-612.

Charkoudian N & Johnson JM (2000). Female reproductive hormones and thermoregulatory control of skin blood flow. *Exercise and Sport Sciences Reviews* **28,** 108-112.

Charkoudian N & Stachenfeld NS (2014). Reproductive hormone influences on thermoregulation in women. *Comprehensive Physiology* **4,** 793-804.

Charkoudian N, Stephens DP, Pirkle KC, Kosiba WA & Johnson JM (1999). Influence of female reproductive hormones on local thermal control of skin blood flow. *Journal of Applied Physiology* **87,** 1719-1723.

Choi PJ, Brunt VE, Fujii N & Minson CT (2014). New approach to measure cutaneous microvascular function: an improved test of NO-mediated vasodilation by thermal hyperemia. *Journal of Applied Physiology* **117,** 277-283.

Chong MF, Macdonald R & Lovegrove JA (2010). Fruit polyphenols and CVD risk: A review of human intervention studies. *British Journal of Nutrition* **104, Suppl 3,** S28-39.

Claassen JA, Levine BD & Zhang R (2009). Cerebral vasomotor reactivity before and after blood pressure reduction in hypertensive patients. *American Journal of Hypertension* **22,** 384-391.

Claassen JA, Meel-van den Abeelen AS, Simpson DM & Panerai RB (2016). Transfer function analysis of dynamic cerebral autoregulation: A white paper from the International Cerebral Autoregulation Research Network. *Journal of Cerebral Blood Flow & Metabolism* **36,** 665-680.

Clerk LH, Vincent MA, Jahn LA, Liu Z, Lindner JR & Barrett EJ (2006). Obesity blunts insulin-mediated microvascular recruitment in human forearm muscle. *Diabetes* **55,** 1436-1442.

Clifford MN, van der Hooft JJ & Crozier A (2013). Human studies on the absorption, distribution, metabolism, and excretion of tea polyphenols. *The American Journal of Clinical Nutrition* **98,** 1619S-1630S.

Commenges D, Scotet V, Renaud S, Jacqmin-Gadda H, Barberger-Gateau P & Dartigues JF (2000). Intake of flavonoids and risk of dementia. *European Journal of Epidemiology* **16,** 357-363.

Convertino VA, Goldwater DJ & Sandler H (1986). Bedrest-induced peak VO_2 reduction associated with age, gender, and aerobic capacity. *Aviation, Space, and Environmental Medicine* **57,** 17-22.

Cornier MA, Bergman BC & Bessesen DH (2006). The effects of short-term overfeeding on insulin action in lean and reduced-obese individuals. *Metabolism* **55,** 1207-1214.

Corretti MC, DeLeon YW, Mangano C, Vogel RA & Plotnick GD (2002). The acute effect of tea consumption with a high fat meal on flow-mediated brachial artery vasodilation in healthy individuals. *Journal of the American College of Cardiology* **39, Suppl 1,** 218.

Cracowski J-L & Roustit M (2015). Current methods to assess human cutaneous blood flow. An updated focus on laser based-techniques. *Microcirculation* **23,** 337-344.

Cracowski JL, Minson CT, Salvat-Melis M & Halliwill JR (2006). Methodological issues in the assessment of skin microvascular endothelial function in humans. *Trends in Pharmacological Sciences* **27,** 503-508.

Cuthbertson DJ, Steele T, Wilding JP, Halford JC, Harrold JA, Hamer M & Karpe F (2017). What have human experimental overfeeding studies taught us about adipose tissue expansion and susceptibility to obesity and metabolic complications. *International Journal of Obesity*.

D'Andrea A, Conte M, Scarafile R, Riegler L, Cocchia R, Pezzullo E, Cavallaro M, Carbone A, Natale F, Russo MG, Gregorio G & Calabrò R (2016). Transcranial Doppler ultrasound: physical principles and principal applications in neurocritical care unit. *Journal of Cardiovascular Echography* **26,** 28-41.

Davignon J & Ganz P (2004). Role of endothelial dysfunction in atherosclerosis. *Circulation* **109,** 27-32.

Dawson EA, Low DA, Meeuwis IH, Kerstens FG, Atkinson CL, Cable NT, Green DJ & Thijssen DH (2015). Reproducibility of cutaneous vascular conductance responses to slow local heating assessed using seven-laser array probes. *Microcirculation* **22,** 276-284.

de Boer MP, Meijer RI, Wijnstok NJ, Jonk AM, Houben AJ, Stehouwer CD, Smulders YM, Eringa EC & SernÉ EH (2012). Microvascular dysfunction: A potential mechanism in the pathogenesis of obesity-associated insulin resistance and hypertension. *Microcirculation* **19,** 5-18.

de Groot P, Crozier J, Rakobowchuk M, Hopman M & MacDonald M (2005). Electrical stimulation alters FMD and arterial compliance in extremely inactive legs. *Medicine and Science in Sports and Exercise* **37,** 1356-1364.

de Groot PC, Bleeker MW, van Kuppevelt DH, van der Woude LH & Hopman MT (2006). Rapid and extensive arterial adaptations after spinal cord injury. *Archives of Physical Medicine and Rehabilitation* **87,** 688-696.

de Groot PCE, Poelkens F, Kooijman M & Hopman MTE (2004). Preserved flow-mediated dilation in the inactive legs of spinal cord-injured individuals. *American Journal of Physiology - Heart and Circulatory Physiology* **287,** H374-H380.

de Jongh RT, Serné EH, IJzerman RG, de Vries G & Stehouwer CDA (2004). Impaired microvascular function in obesity: Implications for obesity-associated microangiopathy, hypertension, and insulin resistance. *Circulation* **109,** 2529-2535.

de Souza RJ, Mente A, Maroleanu A, Cozma AI, Ha V, Kishibe T, Uleryk E, Budylowski P, Schünemann H, Beyene J & Anand SS (2015). Intake of saturated and trans unsaturated fatty acids and risk of all cause mortality, cardiovascular disease, and type 2 diabetes: systematic review and meta-analysis of observational studies. *British Medical Journal* **351**.

de Vriese AS, Verbeuren TJ, Van de Voorde J, Lameire NH & Vanhoutte PM (2000). Endothelial dysfunction in diabetes. *British Journal of Pharmacology* **130,** 963-974.

Deanfield JE, Halcox JP & Rabelink TJ (2007). Endothelial function and dysfunction - Testing and clinical relevance. *Circulation* **115,** 1285-1295.

Deka A & Vita JA (2011). Tea and cardiovascular disease. *Pharmacological Research* **64,** 136-145.

Del Pozzi AT & Hodges GJ (2015). Comparison of the noradrenergic sympathetic nerve contribution during local skin heating at forearm and leg sites in humans. *European Journal of Applied Physiology* **115,** 1155-1164.

Demiot C, Dignat-George F, Fortrat J-O, Sabatier F, Gharib C, Larina I, Gauquelin-Koch G, Hughson R & Custaud M-A (2007). WISE 2005: Chronic bed rest impairs microcirculatory endothelium in women. *American Journal of Physiology - Heart and Circulatory Physiology* **293,** H3159-H3164.

DeWitt LD & Wechsler LR (1988). Transcranial Doppler. *Stroke* **19,** 915-921.

Di Francescomarino S, Sciartilli A, Di Valerio V, Di Baldassarre A & Gallina S (2009). The effect of physical exercise on endothelial function. *Sports Medicine* **39,** 797-812.

Dow CA, Stauffer BL, Greiner JJ & DeSouza CA (2015). Influence of habitual high dietary fat intake on endothelium-dependent vasodilation. *Applied Physiology, Nutrition & Metabolism* **40,** 711-715.

Dower JI, Geleijnse JM, Gijsbers L, Zock PL, Kromhout D & Hollman PC (2015). Effects of the pure flavonoids epicatechin and quercetin on vascular function and cardiometabolic health: a randomized, double-blind, placebo-controlled, crossover trial. *The American Journal of Clinical Nutrition* **101,** 914-921.

Dower JI, Geleijnse JM, Hollman PC, Soedamah-Muthu SS & Kromhout D (2016a). Dietary epicatechin intake and 25-y risk of cardiovascular mortality: The Zutphen Elderly Study. *The American Journal of Clinical Nutrition* **104,** 58-64.

Dower JI, Geleijnse JM, Kroon PA, Philo M, Mensink M, Kromhout D & Hollman PCH (2016b). Does epicatechin contribute to the acute vascular function effects of dark chocolate? A randomized, crossover study. *Molecular Nutrition & Food Research* **60,** 2379-2386.

Draijer R & Duchateau GS (2015). Capsule formats may hamper green tea catechin bioavailability. *Journal of Nutrition* **145,** 2797-2798.

Duffy SJ, Keaney Jr JF, Holbrook M, Gokce N, Swerdloff PL, Frei B & Vita JA (2001). Short- and long-term black tea consumption reverses endothelial dysfunction in patients with coronary artery disease. *Circulation* **104,** 151-156.

Dull RO & Davies PF (1991). Flow modulation of agonist (ATP)-response (Ca2+) coupling in vascular endothelial cells. *American Journal of Physiology - Heart and Circulatory Physiology* **261,** H149-H154.

Durel LA, Kus LA, Anderson NB, McNeilly M, Llabre MM, Spitzer S, Saab PG, Efland JAY, Williams R & Schneiderman N (1993). Patterns and stability of cardiovascular responses to variations of the cold pressor test. *Psychophysiology* **30,** 39-46.

Dwyer JT & Peterson JJ (2013). Tea and flavonoids: where we are, where to go next. *American Journal of Clinical Nutrition* **98,** 1611S-1618S.

Engelhart MJ, Geerlings MI, Ruitenberg A & et al. (2002). Dietary intake of antioxidants and risk of Alzheimer disease. *JAMA* **287,** 3223-3229.

Engler MB, Engler MM, Chen CY, Malloy MJ, Browne A, Chiu EY, Kwak H-K, Milbury P, Paul SM, Blumberg J & Mietus-Snyder ML (2004). Flavonoid-rich dark chocolate improves endothelial function

and increases plasma epicatechin concentrations in healthy adults. *Journal of the American College of Nutrition* **23,** 197-204.

Fahrenberg J, Schneider HJ & Safian P (1987). Psychophysiological assessments in a repeated-measurement design extending over a one-year interval: trends and stability. *Biological Psychology* **24,** 49-66.

Faridi Z, Njike VY, Dutta S, Ali A & Katz DL (2008). Acute dark chocolate and cocoa ingestion and endothelial function: a randomized controlled crossover trial. *The American Journal of Clinical Nutrition* **88,** 58-63.

Farkas E, de Wilde MC, Kiliaan AJ & Luiten PG (2002). Chronic cerebral hypoperfusion-related neuropathologic changes and compromised cognitive status: Window of treatment. *Drugs of Today* **38,** 365-376.

Farkas E & Luiten PGM (2001). Cerebral microvascular pathology in aging and Alzheimer's disease. *Progress in Neurobiology* **64,** 575-611.

Farouque HM, Leung M, Hope SA, Baldi M, Schechter C, Cameron JD & Meredith IT (2006). Acute and chronic effects of flavanol-rich cocoa on vascular function in subjects with coronary artery disease: A randomized double-blind placebo-controlled study. *Clinical Science* **111,** 71-80.

Fasano ML, Sand T, Brubakk AO, Kruszewski P, Bordini C & Sjaastad O (1996). Reproducibility of the cold pressor test: Studies in normal subjects. *Clinical Autonomic Research* **6,** 249-253.

Ferrières J (2004). The French paradox: Lessons for other countries. *Heart* **90,** 107-111.

Feyh A, Bracero L, Lakhani HV, Santhanam P, Shapiro JI, Khitan Z & Sodhi K (2016). Role of dietary components in modulating hypertension. *Journal of Clinical and Experimental Cardiology* **7**.

Fierstra J, Sobczyk O, Battisti-Charbonney A, Mandell DM, Poublanc J, Crawley AP, Mikulis DJ, Duffin J & Fisher JA (2013). Measuring cerebrovascular reactivity: What stimulus to use? *Journal of Physiology* **591,** 5809-5821.

Fisher ND, Sorond FA & Hollenberg NK (2006). Cocoa flavanols and brain perfusion. *Journal of Cardiovascular Pharmacology* **47, Suppl 2,** S210-214.

Fortney SM, Hyatt KH, Davis JE & Vogel JM (1991). Changes in body fluid compartments during a 28-day bed rest. *Aviation, Space, and Environmental Medicine* **62,** 97-104.

Francis J (2016). ECG monitoring leads and special leads. *Indian Pacing and Electrophysiology Journal* **16,** 92-95.

Francis ST, Head K, Morris PG & Macdonald IA (2006). The effect of flavanol-rich cocoa on the fMRI response to a cognitive task in healthy young people. *Journal of Cardiovascular Pharmacology* **47,** S215-S220.

Frank J, George TW, Lodge JK, Rodriguez-Mateos AM, Spencer JP, Minihane AM & Rimbach G (2009). Daily consumption of an aqueous green tea extract supplement does not impair liver function or alter cardiovascular disease risk biomarkers in healthy men. *Journal of Nutrition* **139,** 58-62.

Fuchs D, Nyakayiru J, Draijer R, Mulder TPJ, Hopman MTE, Eijsvogels TMH & Thijssen DH (2016). Impact of flavonoid-rich black tea and beetroot juice on postprandial peripheral vascular resistance and glucose homeostasis in obese, insulin-resistant men: a randomized controlled trial. *Nutrition and Metabolism* **13,** 1-10.

Fujii N, McNeely BD & Kenny GP (2017). Administration of prostacyclin mediates cutaneous vasodilation but not sweating in young and older females: roles for nitric oxide and KCa channels. *The FASEB Journal* **31,** 681.686.

Fujii N, Meade RD, Minson CT, Brunt VE, Boulay P, Sigal RJ & Kenny GP (2016). Cutaneous blood flow during intradermal NO administration in young and older adults: Roles for calcium-activated potassium channels and cyclooxygenase? *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* **310,** R1081-R1087.

Fukino Y, Ikeda A, Maruyama K, Aoki N, Okubo T & Iso H (2008). Randomized controlled trial for an effect of green tea-extract powder supplementation on glucose abnormalities. *European Journal of Clinical Nutrition* **62,** 953-960.

Fukino Y, Shimbo M, Aoki N, Okubo T & Iso H (2005). Randomized controlled trial for an effect of green tea consumption on insulin resistance and inflammation markers. *Journal of Nutritional Science and Vitaminology* **51,** 335-342.

Furchgott RF & Jothianandan D (1991). Endothelium-dependent and -independent vasodilation involving cyclic GMP: Relaxation induced by nitric oxide, carbon monoxide and light. *Blood Vessels* **28,** 52-61.

Gaffney FA, Nixon JV, Karlsson ES, Campbell W, Dowdey AB & Blomqvist CG (1985). Cardiovascular deconditioning produced by 20 hours of bedrest with head-down tilt (-5 degrees) in middle-aged healthy men. *The American Journal of Cardiology* **56,** 634-638.

Garland CJ & Dora KA (2017). EDH: endothelium-dependent hyperpolarization and microvascular signalling. *Acta Physiologica* **219,** 152-161.

Geleijnse JM & Hollman PC (2008). Flavonoids and cardiovascular health: Which compounds, what mechanisms? *The American Journal of Clinical Nutrition* **88,** 12-13.

Geleijnse JM, Launer LJ, van der Kuip DA, Hofman A & Witteman JC (2002). Inverse association of tea and flavonoid intakes with incident myocardial infarction: The Rotterdam Study. *The American Journal of Clinical Nutrition* **75,** 880-886.

Giller CA, Hatab MR & Giller AM (1998). Estimation of vessel flow and diameter during cerebral vasospasm using transcranial Doppler indices. *Neurosurgery* **42,** 1076-1081; discussion 1081-1072.

Godden JO, Roth GM, Hines EA & Schlegel JF (1955). The changes in the intra-arterial pressure during immersion of the hand in ice-cold water. *Circulation* **12,** 963-973.

Gokce N, Keaney JF, Hunter LM, Watkins MT, Nedeljkovic ZS, Menzoian JO & Vita JA (2003). Predictive value of noninvasivelydetermined endothelial dysfunction for long-term cardiovascular events inpatients with peripheral vascular disease. *Journal of the American College of Cardiology* **41,** 1769-1775.

Gooding KM, Hannemann MM, Tooke JE, Clough GF & Shore AC (2006). Maximum skin hyperaemia induced by local heating: Possible mechanisms. *Journal of Vascular Research* **43,** 270-277.

Gorelick PB, Scuteri A, Black SE, DeCarli C, Greenberg SM, Iadecola C, Launer LJ, Laurent S, Lopez OL, Nyenhuis D, Petersen RC, Schneider JA, Tzourio C, Arnett DK, Bennett DA, Chui HC, Higashida RT, Lindquist R, Nilsson PM, Roman GC, Sellke FW & Seshadri S (2011). Vascular contributions to cognitive impairment and dementia. A Statement for Healthcare Professionals From the American Heart Association/American Stroke Association. *Stroke* **42,** 2672-2713.

Gosmanov AR, Smiley DD, Robalino G, Siquiera J, Khan B, Le N-A, Patel RS, Quyyumi AA, Peng L, Kitabchi AE & Umpierrez GE (2010). Effects of oral and intravenous fat load on blood pressure, endothelial function, sympathetic activity, and oxidative stress in obese healthy subjects. *American Journal of Physiology - Endocrinology and Metabolism* **299,** E953-E958.

Grabowski E, Naus G & Weksler B (1985). Prostacyclin production in vitro by rabbit aortic endothelium: Correction for unstirred diffusional layers. *Blood* **66,** 1047-1052.

Grassi D, Aggio A, Onori L, Croce G, Tiberti S, Ferri C, Ferri L & Desideri G (2008a). Tea, flavonoids, and nitric oxide-mediated vascular reactivity. *Journal of Nutrition* **138,** 1554S-1560S.

Grassi D, Desideri G, Croce G, Tiberti S, Aggio A & Ferri C (2009a). Flavonoids, vascular function and cardiovascular protection. *Current Pharmaceutical Design* **15,** 1072-1084.

Grassi D, Desideri G, Di Giosia P, De Feo M, Fellini E, Cheli P, Ferri L & Ferri C (2013a). Tea, flavonoids, and cardiovascular health: Endothelial protection. *American Journal of Clinical Nutrition* **98,** 1660s-1666s.

Grassi D, Desideri G & Ferri C (2013b). Protective effects of dark chocolate on endothelial function and diabetes. *Current Opinion in Clinical Nutrition and Metabolic Care* **16,** 662-668.

Grassi D, Desideri G, Necozione S, Lippi C, Casale R, Properzi G, Blumberg JB & Ferri C (2008b). Blood pressure is reduced and insulin sensitivity increased in glucose-intolerant, hypertensive subjects after 15 days of consuming high-polyphenol dark chocolate. *The Journal of Nutrition* **138,** 1671-1676.

Grassi D, Desideri G, Necozione S, Ruggieri F, Blumberg JB, Stornello M & Ferri C (2012a). Protective effects of flavanol-rich dark chocolate on endothelial function and wave reflection during acute hyperglycemia. *Hypertension* **60,** 827-832.

Grassi D, Draijer R, Desideri G, Mulder T & Ferri C (2015). Black tea lowers blood pressure and wave reflections in fasted and postprandial conditions in hypertensive patients: A randomised study. *Nutrients* **7,** 1037.

Grassi D, Draijer R, Desideri G, Mulder TPJ & Ferri C (2012b). Abstract P061: Effects of black tea with and without a fat load on vascular function in mildly hypertensive subjects. *Circulation* **125**.

Grassi D, Draijer R, Schalkwijk C, Desideri G, D'Angeli A, Francavilla S, Mulder T & Ferri C (2016). Black tea increases circulating endothelial progenitor cells and improves flow mediated dilatation counteracting deleterious effects from a fat load in hypertensive patients: A randomized controlled study. *Nutrients* **8,** 727.

Grassi D, Mulder TPJ, Draijer R, Desideri G, Molhuizen HOF & Ferri C (2009b). Black tea consumption dose-dependently improves flow-mediated dilation in healthy males. *Journal of Hypertension* **27,** 774-781.

Gray SM, Meijer RI & Barrett EJ (2014). Insulin regulates brain function, but how does it get there? *Diabetes* **63,** 3992-3997.

Greaves DK, Arbeille P & Hughson RL (2007). WISE 2005: Altered cerebrovascular autoregulation after 60 day head-down bed rest. *Journal of Gravitational Physiology* **14,** P61-62.

Green DJ, Jones H, Thijssen D, Cable NT & Atkinson G (2011). Flow-mediated dilation and cardiovascular event prediction. Does nitric oxide matter? *Hypertension* **57,** 363-369.

Green DJ, Maiorana A, O'Driscoll G & Taylor R (2004). Effect of exercise training on endothelium-derived nitric oxide function in humans. *Journal of Physiology* **561,** 1-25.

Greyling A, Ras RT, Zock PL, Lorenz M, Hopman MT, Thijssen DHJ & Draijer R (2014). The effect of black tea on blood pressure: A systematic review with meta-analysis of randomized controlled trials. *PLoS ONE* **9,** 1-9.

Greyling A, van Mil ACCM, Zock PL, Green DJ, Ghiadoni L & Thijssen DH (2016). Adherence to guidelines strongly improves reproducibility of brachial artery flow-mediated dilation. *Atherosclerosis* **248,** 196-202.

Groves P, Kurz S, Just H & Drexler H (1995). Role of endogenous bradykinin in human coronary vasomotor control. *Circulation* **92,** 3424-3430.

Gupta AK, Johnson WD, Johannsen D & Ravussin E (2013). Cardiovascular risk escalation with caloric excess: a prospective demonstration of the mechanics in healthy adults. *Cardiovascular Diabetology* **12,** 23-23.

Gur AY, Bova I & Bornstein NM (1996). Is impaired cerebral vasomotor reactivity a predictive factor of stroke in asymptomatic patients? *Stroke* **27,** 2188-2190.

Habauzit V & Morand C (2012). Evidence for a protective effect of polyphenols-containing foods on cardiovascular health: an update for clinicians. *Therapeutic Advances in Chronic Disease* **3,** 87-106.

Hagobian TA & Braun B (2006). Interactions between energy surplus and short-term exercise on glucose and insulin responses in healthy people with induced, mild insulin insensitivity. *Metabolism* **55,** 402-408.

Hallal PC, Andersen LB, Bull FC, Guthold R, Haskell W & Ekelund U Global physical activity levels: surveillance progress, pitfalls, and prospects. *The Lancet* **380,** 247-257.

Hamburg NM, McMackin CJ, Huang AL, Shenouda SM, Widlansky ME, Schulz E, Gokce N, Ruderman NB, Keaney JF & Vita JA (2007). Physical inactivity rapidly induces insulin resistance and microvascular dysfunction in healthy volunteers. *Arteriosclerosis, Thrombosis, and Vascular Biology* **27,** 2650-2656.

Harper AM & Bell RA (1963). The effect of metabolic acidosis and alkalosis on the blood flow through the cerebral cortex. *Journal of Neurology, Neurosurgery & Psychiatry* **26,** 341-344.

Hassellund SS, Flaa A, Sandvik L, Kjeldsen SE & Rostrup M (2010). Long-term stability of cardiovascular and catecholamine responses to stress tests: An 18-year follow-up study. *Hypertension* **55,** 131-136.

Heilbronn LK, Coster ACF, Campbell LV, Greenfield JR, Lange K, Christopher MJ, Meikle PJ & Samocha-Bonet D (2013). The effect of short-term overfeeding on serum lipids in healthy humans. *Obesity* **21,** E649-E659.

Heinrich U, Tronnier H, Moore CE, De Spirt S & Stahl W (2011). Green tea polyphenols provide photoprotection, increase microcirculation, and modulate skin properties of women. *Journal of Nutrition* **141,** 1202-1208.

Heitzer T, Just H & Munzel T (1996). Antioxidant vitamin C improves endothelial dysfunction in chronic smokers. *Circulation* **94,** 6-9.

Hennig B, Toborek M & McClain CJ (2001). High-energy diets, fatty acids and endothelial cell function: Implications for atherosclerosis. *Journal of the American College of Nutrition* **20,** 97-105.

Hertog MGL, Feskens EJM, Kromhout D, Hertog MGL, Hollman PCH, Hertog MGL & Katan MB (1993). Dietary antioxidant flavonoids and risk of coronary heart disease: The Zutphen Elderly Study. *Lancet* **342,** 1007-1011.

Hesse C, Siedler H, Luntz SP, Arendt BM, Goerlich R, Fricker R, Heer M & Haefeli WE (2005). Modulation of endothelial and smooth muscle function by bed rest and hypoenergetic, low-fat nutrition. *Journal of Applied Physiology* **99,** 2196-2203.

Hewett PW (2009). Vascular endothelial cells from human micro- and macrovessels: Isolation, characterisation and culture. *Methods in Molecular Biology* **467,** 95-111.

Hill AM, Coates AM, Buckley JD, Ross R, Thielecke F & Howe PR (2007). Can EGCG reduce abdominal fat in obese subjects? *Journal of the American College of Nutrition* **26,** 396s-402s.

Hodges GJ, Kosiba WA, Zhao K & Johnson JM (2008). The involvement of norepinephrine, neuropeptide Y, and nitric oxide in the cutaneous vasodilator response to local heating in humans. *Journal of Applied Physiology* **105,** 233-240.

Hodges GJ, Kosiba WA, Zhao K & Johnson JM (2009). The involvement of heating rate and vasoconstrictor nerves in the cutaneous vasodilator response to skin warming. *American Journal of Physiology - Heart and Circulatory Physiology* **296,** H51-H56.

Hodges GJ & Sparks PA (2014). Noradrenaline and neuropeptide Y contribute to initial, but not sustained, vasodilatation in response to local skin warming in humans. *Experimental Physiology* **99,** 381-392.

Hodgson JM, Burke V & Puddey IB (2005). Acute effects of tea on fasting and postprandial vascular function and blood pressure in humans. *Journal of Hypertension* **23,** 47-54.

Hodgson JM & Croft KD (2010). Tea flavonoids and cardiovascular health. *Molecular Aspects of Medicine* **31,** 495-502.

Hodgson JM, Croft KD, Woodman RJ, Puddey IB, Fuchs D, Draijer R, Lukoshkova E & Head GA (2013). Black tea lowers the rate of blood pressure variation: A randomized controlled trial. *The American Journal of Clinical Nutrition* **97,** 943-950.

Hodgson JM, Puddey IB, Burke V, Watts GF & Beilin LJ (2002). Regular ingestion of black tea improves brachial artery vasodilator function. *Clinical Science* **102,** 195-201.

Hodgson JM, Puddey IB, Woodman RJ, Mulder TP, Fuchs D, Scott K & Croft KD (2012). Effects of black tea on blood pressure: a randomized controlled trial. *Archives of Internal Medicine* **172,** 186-188.

Hollman PCH, Geelen A & Kromhout D (2010). Dietary flavonol intake may lower stroke risk in men and women. *The Journal of Nutrition* **140,** 600-604.

Holowatz LA, Thompson-Torgerson CS & Kenney WL (2007). Altered mechanisms of vasodilation in aged human skin. *Exercise and Sport Sciences Reviews* **35,** 119-125.

Holowatz LA, Thompson-Torgerson CS & Kenney WL (2008). The human cutaneous circulation as a model of generalized microvascular function. *Journal of Applied Physiology* **105,** 370-372.

Holwerda SW, Reynolds LJ, Restaino RM, Credeur DP, Leidy HJ, Thyfault JP & Fadel PJ (2015). The influence of reduced insulin sensitivity via short-term reductions in physical activity on cardiac baroreflex sensitivity during acute hyperglycemia. *Journal of Applied Physiology* **119,** 1383-1392.

Hooper L, Kroon PA, Rimm EB, Cohn JS, Harvey I, Le Cornu KA, Ryder JJ, Hall WL & Cassidy A (2008). Flavonoids, flavonoid-rich foods, and cardiovascular risk: A meta-analysis of randomized controlled trials. *The American Journal of Clinical Nutrition* **88,** 38-50.

Hosoda K, Wang M-F, Liao M-L, Chuang C-K, Iha M, Clevidence B & Yamamoto S (2003). Antihyperglycemic effect of Oolong tea in type 2 diabetes. *Diabetes Care* **26,** 1714-1718.

Houben AJ, Eringa EC, Jonk AM, Serne EH, Smulders YM & Stehouwer CD (2012). Perivascular fat and the microcirculation: Relevance to insulin resistance, diabetes, and cardiovascular disease. *Current Cardiovascular Risk Reports* **6,** 80-90.

Houghton BL, Meendering JR, Wong BJ & Minson CT (2006). Nitric oxide and noradrenaline contribute to the temperature threshold of the axon reflex response to gradual local heating in human skin. *Journal of Physiology* **572,** 811-820.

Hsu CH, Liao YL, Lin SC, Tsai TH, Huang CJ & Chou P (2011). Does supplementation with green tea extract improve insulin resistance in obese type 2 diabetics? A randomized, double-blind, and placebo-controlled clinical trial. *Alternative Medicine Review* **16,** 157-163.

Huang C-S, Wang S-F & Tsai Y-F (2013). Increasing acclimation period improves the reproducibility of short-heating local thermal hyperemia. *Microvascular Research* **85,** 93-98.

Hughes TM & Craft S (2016). The role of insulin in the vascular contributions to age-related dementia. *Biochimica et Biophysica Acta - Molecular Basis of Disease* **1862,** 983-991.

Iadecola C, Pelligrino DA, Moskowitz MA & Lassen NA (1994). Nitric oxide synthase inhibition and cerebrovascular regulation. *Journal of Cerebral Blood Flow & Metabolism* **14,** 175-192.

Ignarro LJ, Harbison RG, Wood KS & Kadowitz PJ (1986). Activation of purified soluble guanylate cyclase by endothelium-derived relaxing factor from intrapulmonary artery and vein: stimulation by acetylcholine, bradykinin and arachidonic acid. *Journal of Pharmacology and Experimental Therapeutics* **237,** 893-900.

Ijzerman RG, De Jongh RT, Beijk MAM, Van Weissenbruch MM, Delemarre-van de Waal HA, Serné EH & Stehouwer CDA (2003). Individuals at increased coronary heart disease risk are characterized by an impaired microvascular function in skin. *European Journal of Clinical Investigation* **33,** 536-542.

Imholz BP, Parati G, Mancia G & Wesseling KH (1992). Effects of graded vasoconstriction upon the measurement of finger arterial pressure. *Journal of Hypertension* **10,** 979-984.

Imholz BP, van Montfrans GA, Settels JJ, van der Hoeven GM, Karemaker JM & Wieling W (1988). Continuous non-invasive blood pressure monitoring: Reliability of Finapres device during the Valsalva manoeuvre. *Cardiovascular Research* **22,** 390-397.

Imholz BPM, Wieling W, Langewouters GJ & van Montfrans GA (1991). Continuous finger arterial pressure: Utility in the cardiovascular laboratory. *Clinical Autonomic Research* **1,** 43-53.

Imholz BPM, Wieling W, van Montfrans GA & Wesseling KH (1998). Fifteen years experience with finger arterial pressure monitoring: Assessment of the technology. *Cardiovascular Research* **38,** 605-616.

Inaba Y, Chen JA & Bergmann SR (2010). Prediction of future cardiovascular outcomes by flow-mediated vasodilatation of brachial artery: A meta-analysis. *The International Journal of Cardiovascular Imaging* **26,** 631-640.

Iso H, Date C, Wakai K, Fukui M & Tamakoshi A (2006). The relationship between green tea and total caffeine intake and risk for self-reported type 2 diabetes among Japanese adults. *Annals of Internal Medicine* **144,** 554-562.

Jaap AJ, Hammersley MS, Shore AC & Tooke JE (1994). Reduced microvascular hyperaemia in subjects at risk of developing Type 2 (non-insulin-dependent) diabetes mellitus. *Diabetologia* **37,** 214-216.

Jellema WT, Imholz BP, van Goudoever J, Wesseling KH & van Lieshout JJ (1996). Finger arterial versus intrabrachial pressure and continuous cardiac output during head-up tilt testing in healthy subjects. *Clinical Science* **91,** 193-200.

Jennersjo P, Ludvigsson J, Lanne T, Nystrom FH, Ernerudh J & Ostgren CJ (2012). Pedometer-determined physical activity is linked to low systemic inflammation and low arterial stiffness in Type 2 diabetes. *Diabetic Medicine* **29,** 1119-1125.

Jeong SM, Hwang GS, Kim SO, Levine BD & Zhang R (2014). Dynamic cerebral autoregulation after bed rest: Effects of volume loading and exercise countermeasures. *Journal of Applied Physiology* **116,** 24-31.

Jing Y, Han G, Hu Y, Bi Y, Li L & Zhu D (2009). Tea consumption and risk of type 2 diabetes: A meta-analysis of cohort studies. *Journal of General Internal Medicine* **24,** 557-562.

Jochmann N, Lorenz M, Krosigk Av, Martus P, Böhm V, Baumann G, Stangl K & Stangl V (2008). The efficacy of black tea in ameliorating endothelial function is equivalent to that of green tea. *British Journal of Nutrition* **99,** 863-868.

Johnson JM, Minson CT & Kellogg DL, Jr. (2014). Cutaneous vasodilator and vasoconstrictor mechanisms in temperature regulation. *Comprehensive Physiology* **4,** 33-89.

Johnson PC (2011). Overview of the microcirculation. In: *Comprehensive Physiology*. John Wiley & Sons, Inc.

Jones H, Green DJ, George K & Atkinson G (2010). Intermittent exercise abolishes the diurnal variation in endothelial-dependent flow-mediated dilation in humans. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology* **298,** R427-R432.

Jonk AM, Houben AJHM, de Jongh RT, Serné EH, Schaper NC & Stehouwer CDA (2007). Microvascular dysfunction in obesity: A potential mechanism in the pathogenesis of obesity-associated insulin resistance and hypertension. *Physiology* **22,** 252-260.

Jordan J, Shannon JR, Diedrich A, Black B, Costa F, Robertson D & Biaggioni I (2000). Interaction of carbon dioxide and sympathetic nervous system activity in the regulation of cerebral perfusion in humans. *Hypertension* **36,** 383-388.

Josic J, Olsson AT, Wickeberg J, Lindstedt S & Hlebowicz J (2010). Does green tea affect postprandial glucose, insulin and satiety in healthy subjects: A randomized controlled trial. *Nutrition Journal* **9,** 63.

Kasagi F, Akahoshi M & Shimaoka K (1995). Relation between cold pressor test and development of hypertension based on 28-year follow-up. *Hypertension* **25,** 71-76.

Keli SO, Hertog MG, Feskens EJ & Kromhout D (1996). Dietary flavonoids, antioxidant vitamins, and incidence of stroke: The Zutphen study. *Archives of Internal Medicine* **156,** 637-642.

Kellogg DL (2006). In vivo mechanisms of cutaneous vasodilation and vasoconstriction in humans during thermoregulatory challenges. *Journal of Applied Physiology* **100,** 1709-1718.

Kellogg DL, Johnson JM, Kenney WL, Pergola PE & Kosiba WA (1993). Mechanisms of control of skin blood flow during prolonged exercise in humans. *American Journal of Physiology - Heart and Circulatory Physiology* **265,** H562-H568.

Kellogg DL, Liu Y, Kosiba IF & O'Donnell D (1999). Role of nitric oxide in the vascular effects of local warming of the skin in humans. *Journal of Applied Physiology* **86,** 1185-1190.

Kety SS & Schmidt CF (1948). The effects of altered arterial tensions of carbon dioxide and oxygen on cerebral blood flow and cerebral oxygen consumption of normal young men. *Journal of Clinical Investigation* **27,** 484-492.

Kim JA, Montagnani M, Koh KK & Quon MJ (2006). Reciprocal relationships between insulin resistance and endothelial dysfunction: Molecular and pathophysiological mechanisms. *Circulation* **113**.

Kim K, Vance TM & Chun OK (2016). Greater flavonoid intake is associated with improved CVD risk factors in US adults. *British Journal of Nutrition* **115,** 1481-1488.

Knudsen SH, Hansen LS, Pedersen M, Dejgaard T, Hansen J, Hall GV, Thomsen C, Solomon TPJ, Pedersen BK & Krogh-Madsen R (2012). Changes in insulin sensitivity precede changes in body composition during 14 days of step reduction combined with overfeeding in healthy young men. *Journal of Applied Physiology* **113,** 7-15.

Kobayashi Y, Suzuki M, Satsu H, Arai S, Hara Y, Suzuki K, Miyamoto Y & Shimizu M (2000). Green tea polyphenols inhibit the sodium-dependent glucose transporter of intestinal epithelial cells by a competitive mechanism. *Journal of Agricultural and Food Chemistry* **48,** 5618-5623.

Kontos HA, Raper AJ & Patterson JL (1977). Analysis of vasoactivity of local pH, PCO2 and bicarbonate on pial vessels. *Stroke* **8,** 358-360.

Krentz AJ, Clough G & Byrne CD (2007). Interactions between microvascular and macrovascular disease in diabetes: Pathophysiology and therapeutic implications. *Diabetes, Obesity and Metabolism* **9,** 781-791.

Krogh-Madsen R, Thyfault JP, Broholm C, Mortensen OH, Olsen RH, Mounier R, Plomgaard P, van Hall G, Booth FW & Pedersen BK (2010). A 2-wk reduction of ambulatory activity attenuates peripheral insulin sensitivity. *Journal of Applied Physiology* **108,** 1034-1040.

Kuga T, Mohri M, Egashira K, Hirakawa Y, Tagawa T, Shimokawa H & Takeshita A (1997). Bradykinin-induced vasodilation of human coronary arteries in vivo: Role of nitric oxide and angiotensin-converting enzyme. *Journal of the American College of Cardiology* **30,** 108-112.

Laakso M, Edelman SV, Brechtel G & Baron AD (1990). Decreased effect of insulin to stimulate skeletal muscle blood flow in obese man. A novel mechanism for insulin resistance. *Journal of Clinical Investigation* **85,** 1844-1852.

Lambertsen CJ, Semple SJ, Smyth MG & Gelfand R (1961). H and pCO_2 as chemical factors in respiratory and cerebral circulatory control. *Journal of Applied Physiology* **16,** 473-484.

Lamport D, Pal D, Moutsiana C, Field D, Williams C, Spencer JE & Butler L (2015). The effect of flavanol-rich cocoa on cerebral perfusion in healthy older adults during conscious resting state: A placebo controlled, crossover, acute trial. *Psychopharmacology* **232,** 3227-3234.

Lantorno M, Campia U, Di Daniele N, Nistico S, Forleo GB, Cardillo C & Tesauro M (2014). Obesity, inflammation and endothelial dysfunction. *Journal of Biological Regulators and Homeostatic Agents* **28,** 169-176.

Lassen NA (1959). Cerebral blood flow and oxygen consumption in man. *Physiological Reviews* **39,** 183-238.

Lassen NA (1968). Brain extracellular pH: The main factor controlling cerebral blood flow. *Scandinavian Journal of Clinical and Laboratory Investigation* **22,** 247-251.

Lavi S, Egbarya R, Lavi R & Jacob G (2003). Role of nitric oxide in the regulation of cerebral blood flow in humans. Chemoregulation versus mechanoregulation. *Circulation*.

Lavi S, Gaitini D, Milloul V & Jacob G (2006). Impaired cerebral CO2 vasoreactivity: Association with endothelial dysfunction. *American Journal of Physiology - Heart and Circulatory Physiology* **291,** H1856-1861.

Lavie CJ, Milani RV & Ventura HO (2009). Obesity and cardiovascular disease. *Journal of the American College of Cardiology* **53,** 1925-1932.

Le Stunff C & Bougneres P (1994). Early changes in postprandial insulin secretion, not in insulin sensitivity, characterize juvenile obesity. *Diabetes* **43,** 696-702.

Lee PH, Macfarlane DJ, Lam TH & Stewart SM (2011). Validity of the international physical activity questionnaire short form (IPAQ-SF): A systematic review. *International Journal of Behavioral Nutrition and Physical Activity* **8**.

LeMarbre G, Stauber S, Khayat RN, Puleo DS, Skatrud JB & Morgan BJ (2003). Baroreflex-induced sympathetic activation does not alter cerebrovascular CO_2 responsiveness in humans. *Journal of Physiology* **551,** 609-616.

Levy BI, Ambrosio G, Pries AR & Struijker-Boudier HAJ (2001). Microcirculation in hypertension: A new target for treatment? *Circulation* **104,** 735-740.

Levy BI, Schiffrin EL, Mourad J-J, Agostini D, Vicaut E, Safar ME & Struijker-Boudier HAJ (2008). Impaired tissue perfusion. A pathology common to hypertension, obesity, and diabetes mellitus. *Circulation* **118,** 968-976.

Lindegaard KF, Lundar T, Wiberg J, Sjøberg D, Aaslid R & Nornes H (1987). Variations in middle cerebral artery blood flow investigated with non-invasive transcranial blood velocity measurements. *Stroke* **18,** 1025-1030.

Liu CY, Huang CJ, Huang LH, Chen IJ, Chiu JP & Hsu CH (2014). Effects of green tea extract on insulin resistance and glucagon-like peptide 1 in patients with type 2 diabetes and lipid abnormalities: a randomized, double-blinded, and placebo-controlled trial. *PLoS ONE* **9,** e91163.

Liu SM & Manson JE (2001). Dietary carbohydrates, physical inactivity, obesity, and the 'metabolic syndrome' as predictors of coronary heart disease. *Current Opinion in Lipidology* **12,** 395-404.

Loke WM, Proudfoot JM, Hodgson JM, McKinley AJ, Hime N, Magat M, Stocker R & Croft KD (2010). Specific dietary polyphenols attenuate atherosclerosis in apolipoprotein e–knockout mice by alleviating inflammation and endothelial dysfunction. *Arteriosclerosis, Thrombosis, and Vascular Biology* **30,** 749-757.

Lorenz MW, Markus HS, Bots ML, Rosvall M & Sitzer M (2007). Prediction of clinical cardiovascular events with carotid intima-media thickness: A systematic review and meta-analysis. *Circulation* **115,** 459-467.

Low DA, Bailey TG, Timothy Cable N & Jones H (2016). Thermoregulatory responses to combined moderate heat stress and hypoxia. *Microcirculation* **23,** 487-494.

Łuczaj W & Skrzydlewska E (2005). Antioxidative properties of black tea. *Preventive Medicine* **40,** 910-918.

Lunt MJ, Ragab S, Birch AA, Schley D & Jenkinson DF (2004). Comparison of caffeine-induced changes in cerebral blood flow and middle cerebral artery blood velocity shows that caffeine reduces middle cerebral artery diameter. *Physiological Measurement* **25,** 467.

MacRae HS & Mefferd KM (2006). Dietary antioxidant supplementation combined with quercetin improves cycling time trial performance. *International Journal of Sport Nutrition and Exercise Metabolism* **16,** 405-419.

Mahé G, Haj-Yassin F, Rousseau P, Humeau A, Durand S, Leftheriotis G & Abraham P (2011). Distance between laser head and skin does not influence skin blood flow values recorded by laser speckle imaging. *Microvascular Research* **82,** 439-442.

Maiorana A, O'Driscoll G, Cheetham C, Dembo L, Stanton K & al. e (2001). The effect of combined aerobic and resistance training on vascular function in type 2 diabetes. *Journal of American College of Cardiology.* **38,** 860-866.

Malar DS & Devi KP (2014). Dietary polyphenols for treatment of Alzheimer's disease - future research and development. *Current Pharmaceutical Biotechnology* **15,** 330-342.

Manach C, Scalbert A, Morand C, Rémésy C & Jiménez L (2004). Polyphenols: Food sources and bioavailability. *The American Journal of Clinical Nutrition* **79,** 727-747.

Mangels DR & Mohler ER (2017). Catechins as potential mediators of cardiovascular health. *Arteriosclerosis, Thrombosis, and Vascular Biology* **37,** 757-763.

Maria Assunta P, Sara G, Carmela N, Maria Rosaria C & Monica M (2009). Endothelial dysfunction in diabetes: From mechanisms to therapeutic targets. *Current Medicinal Chemistry* **16,** 94-112.

Marinoni M, Ginanneschi A, Forleo P & Amaducci L (1997). Technical limits in transcranial Doppler recording: Inadquate acoustic windows. *Ultrasound in Medicine & Biology* **23,** 1275-1277.

Mastroiacovo D, Kwik-Uribe C, Grassi D, Necozione S, Raffaele A, Pistacchio L, Righetti R, Bocale R, Lechiara MC, Marini C, Ferri C & Desideri G (2015). Cocoa flavanol consumption improves cognitive function, blood pressure control, and metabolic profile in elderly subjects: the Cocoa, Cognition, and Aging (CoCoA) Study - a randomized controlled trial. *The American Journal of Clinical Nutrition*.

McCord GR & Minson CT (2005). Cutaneous vascular responses to isometric handgrip exercise during local heating and hyperthermia. *Journal of Applied Physiology* **98,** 2011-2018.

McCullough ML, Peterson JJ, Patel R, Jacques PF, Shah R & Dwyer JT (2012). Flavonoid intake and cardiovascular disease mortality in a prospective cohort of US adults. *The American Journal of Clinical Nutrition* **95,** 454-464.

Melkumyants AM, Balashov SA & Khayutin VM (1989). Endothelium dependent control of arterial diameter by blood viscosity. *Cardiovascular Research* **23,** 741-747.

Michas G, Micha R & Zampelas A (2014). Dietary fats and cardiovascular disease: Putting together the pieces of a complicated puzzle. *Atherosclerosis* **234,** 320-328.

Mikus CR, Oberlin DJ, Libla JL, Taylor AM, Booth FW & Thyfault JP (2012). Lowering physical activity impairs glycemic control in healthy volunteers. *Medicine and Science in Sports and Exercise* **44,** 225-231.

Miller RJ, Jackson KG, Dadd T, Mayes AE, Brown AL & Minihane AM (2011). The impact of the catechol-O-methyltransferase genotype on the acute responsiveness of vascular reactivity to a green tea extract. *British Journal of Nutrition* **105,** 1138-1144.

Mink PJ, Scrafford CG, Barraj LM, Harnack L, Hong C-P, Nettleton JA & Jacobs DR (2007). Flavonoid intake and cardiovascular disease mortality: A prospective study in postmenopausal women. *The American Journal of Clinical Nutrition* **85,** 895-909.

Minson CT (2010). Thermal provocation to evaluate microvascular reactivity in human skin. *Journal of Applied Physiology* **109,** 1239-1246.

Minson CT, Berry LT & Joyner MJ (2001). Nitric oxide and neurally mediated regulation of skin blood flow during local heating. *Journal of Applied Physiology* **91,** 1619-1626.

Minson CT, Holowatz LA, Wong BJ, Kenney WL & Wilkins BW (2002). Decreased nitric oxide- and axon reflex-mediated cutaneous vasodilation with age during local heating. *Journal of Applied Physiology* **93,** 1644-1649.

Moppett IK & Mahajan RP (2004). Transcranial Doppler ultrasonography in anaesthesia and intensive care. *British Journal of Anaesthesia* **93,** 710-724.

Mourot L, Bouhaddi M & Regnard J (2009). Effects of the cold pressor test on cardiac autonomic control in normal subjects. *Physiological Research* **58,** 83-91.

Muniyappa R, Iantorno M & Quon MJ (2008). An integrated view of insulin resistance and endothelial dysfunction. *Endocrinology and Metabolism Clinics of North America* **37,** 685-711.

Muniyappa R, Montagnani M, Koh KK & Quon MJ (2007). Cardiovascular actions of insulin. *Endocrine Reviews* **28,** 463-491.

Murray M, Walchuk C, Suh M & Jones PJ (2015). Green tea catechins and cardiovascular disease risk factors: Should a health claim be made by the United States Food and Drug Administration? *Trends in Food Science & Technology* **41,** 188-197.

Mutlu B, Tigen K, Gurel E, Ozben B, Karaahmet T & Basaran Y (2011). The predictive value of flow-mediated dilation and carotid artery intima-media thickness for occult coronary artery disease. *Echocardiography* **28,** 1141-1147.

Nagao T, Meguro S, Hase T, Otsuka K, Komikado M, Tokimitsu I, Yamamoto T & Yamamoto K (2009). A catechin-rich beverage improves obesity and blood glucose control in patients with type 2 diabetes. *Obesity* **17,** 310-317.

Nathan DM, Davidson MB, DeFronzo RA, Heine RJ, Henry RR, Pratley R & Zinman B (2007). Impaired fasting glucose and impaired glucose tolerance: Implications for care. *Diabetes Care* **30,** 753-759.

Neyestani TR, Shariatzade N, Kalayi A, Gharavi A, Khalaji N, Dadkhah M, Zowghi T, Haidari H & Shab-bidar S (2010). Regular daily intake of black tea improves oxidative stress biomarkers and decreases serum c-reactive protein levels in type 2 diabetic patients. *Annals of Nutrition and Metabolism* **57,** 40-49.

Nguyen TT, Wang JJ & Wong TY (2007). Retinal vascular changes in pre-diabetes and prehypertension. *New findings and their research and clinical implications* **30,** 2708-2715.

NICE (2011). Hypertension: quick reference guide: Clinical management of primary hypertension in adults., London: National Institute for Health and Clinical Excellence.

Nieman DC, Williams AS, Shanely RA, Jin F, McAnulty SR, Triplett NT, Austin MD & Henson DA (2010). Quercetin's influence on exercise performance and muscle mitochondrial biogenesis. *Medicine and Science in Sports and Exercise* **42,** 338-345.

Nishimura N & Schaffer CB (2013). Big effects from tiny vessels: Imaging the impact of microvascular clots and hemorrhages on the brain. *Stroke* **44,** S90-92.

Nosova EV, Yen P, Chong KC, Alley HF, Stock EO, Quinn A, Hellmann J, Conte MS, Owens CD, Spite M & Grenon SM (2014). Short-term physical inactivity impairs vascular function. *Journal of Surgical Research* **190,** 672-682.

Oh J, Jo SH, Kim JS, Ha KS, Lee JY, Choi HY, Yu SY, Kwon YI & Kim YC (2015). Selected tea and tea pomace extracts inhibit intestinal alpha-glucosidase activity in vitro and postprandial hyperglycemia in vivo. *International Journal of Molecular Sciences* **16,** 8811-8825.

Okon EB, Chung AWY, Rauniyar P, Padilla E, Tejerina T, McManus BM, Luo H & van Breemen C (2005). Compromised arterial function in human type 2 diabetic patients. *Diabetes* **54,** 2415-2423.

Oleson DR & Johnson DR (1988). Regulation of human natural cytotoxicity by enkephalins and selective opiate agonists. *Brain Behav Immun* **2,** 171-186.

Olver TD, Mattar L, Grisé KN, Twynstra J, Noble EG, Lacefield JC & Shoemaker JK (2013). Glucose-stimulated insulin secretion causes an insulin-dependent nitric oxide-mediated vasodilation in the blood supply of the rat sciatic nerve. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* **305,** R157-163.

Oyama J, Maeda T, Kouzuma K, Ochiai R, Tokimitsu I & Higuchi Y (2010). Green tea catechins improve human forearm endothelial dysfunction and have antiatherosclerotic effects in smokers. *Circulation Journal* **74**.

Padilla J, Johnson BD, Newcomer SC, Wilhite DP, Mickleborough TD, Fly AD, Mather KJ & Wallace JP (2009). Adjusting flow-mediated dilation for shear stress stimulus allows demonstration of endothelial dysfunction in a population with moderate cardiovascular risk. *Journal of Vascular Research* **46,** 592-600.

Palmer RM, Ashton DS & Moncada S (1988). Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature* **333,** 664-666.

Pandey KB & Rizvi SI (2009). Plant polyphenols as dietary antioxidants in human health and disease. *Oxidative Medicine and Cellular Longevity* **2,** 270-278.

Papamichael C, Karatzis E, Karatzi K, Aznaouridis K, Papaioannou T, Protogerou A, Stamatelopoulos K, Zampelas A, Lekakis J & Mavrikakis M (2004). Red wine's antioxidants counteract acute endothelial dysfunction caused by cigarette smoking in healthy nonsmokers. *American Heart Journal* **147,** E5.

Park CS, Kim W, Woo JS, Ha SJ, Kang WY, Hwang SH, Park YW, Kim YS, Ahn YK, Jeong MH & Kim W (2010). Green tea consumption improves endothelial function but not circulating endothelial progenitor cells in patients with chronic renal failure. *International Journal of Cardiology* **145,** 261-262.

Park J-H, Bae J-H, Im S-S & Song D-K (2014). Green tea and type 2 diabetes. *Integrative Medicine Research* **3,** 4-10.

Parkes LM, Rashid W, Chard DT & Tofts PS (2004). Normal cerebral perfusion measurements using arterial spin labeling: Reproducibility, stability, and age and gender effects. *Magnetic Resonance in Medicine* **51,** 736-743.

Parry SA, Smith JR, Corbett TRB, Woods RM & Hulston CJ (2017). Short-term, high-fat overfeeding impairs glycaemic control but does not alter gut hormone responses to a mixed meal tolerance test in healthy, normal-weight individuals. *British Journal of Nutrition* **117,** 48-55.

Paulson OB, Strandgaard S & Edvinsson L (1990). Cerebral autoregulation. *Cerebrovascular and Brain Metabolism Reviews* **2,** 161-192.

Penaz J (1973). Photoelectric measurement of blood pressure, volume and flow in the finger. *Digest of the International Conference on Medicine and Biological Engineering* **104**.

Perez-Jimenez J, Neveu V, Vos F & Scalbert A (2010). Identification of the 100 richest dietary sources of polyphenols: An application of the Phenol-Explorer database. *European Journal of Clinical Nutrition* **64,** S112-S120.

Perneger TV (1998). What's wrong with Bonferroni adjustments? *British Medical Journal* **316,** 1236.

Peterson JJ, Dwyer JT, Jacques PF & McCullough ML (2012). Associations between flavonoids and cardiovascular disease incidence or mortality in European and US populations. *Nutrition Reviews* **70,** 491-508.

Phillips AA, Chan FH, Zheng MMZ, Krassioukov AV & Ainslie PN (2016). Neurovascular coupling in humans: Physiology, methodological advances and clinical implications. *Journal of Cerebral Blood Flow & Metabolism* **36,** 647-664.

Plotnick GD, Corretti MC & Vogel RA (1997). Effect of antioxidant vitamins on the transient impairment of endothelium—dependent brachial artery vasoactivity following a single high-fat meal. *JAMA* **278,** 1682-1686.

Plotnick GD, Corretti MC, Vogel RA, Hesslink R & Wise JA (2003). Effect of supplemental phytonutrients on impairment of the flow-mediated brachialartery vasoactivity after a single high-fat meal. *Journal of the American College of Cardiology* **41,** 1744-1749.

Ponzo V, Goitre I, Fadda M, Gambino R, De Francesco A, Soldati L, Gentile L, Magistroni P, Cassader M & Bo S (2015). Dietary flavonoid intake and cardiovascular risk: A population-based cohort study. *Journal of Translational Medicine* **13,** 218.

Pugh CJA, Cuthbertson DJ, Sprung VS, Kemp GJ, Richardson P, Margot Umpleby A, Green DJ, Timothy Cable N & Jones H (2013). Exercise training improves cutaneous microvascular function in nonalcoholic fatty liver disease. *American Journal of Physiology - Endocrinology and Metabolism* **305,** E50-E58.

Qin CC, Hui RT & Liu ZH (2008). Aging-related cerebral microvascular degeneration is an important cause of essential hypertension. *Medical Hypotheses* **70,** 643-645.

Quine SD & Raghu PS (2005). Effects of (-)-epicatechin, a flavonoid on lipid peroxidation and antioxidants in streptozotocin-induced diabetic liver, kidney and heart. *Pharmacological Reports* **57,** 610-615.

Ras RT, Zock PL & Draijer R (2011). Tea consumption enhances endothelial-dependent vasodilation; A meta-analysis. *PLoS ONE* **6,** e16974.

Reiner Z, Catapano AL, De Backer G, Graham I, Taskinen MR, Wiklund O, Agewall S, Alegria E, Chapman MJ, Durrington P, Erdine S, Halcox J, Hobbs R, Kjekshus J, Filardi PP, Riccardi G, Storey RF & Wood D (2011). Clinical Practice Guidelines Committee of the Spanish Society of Cardiology [ESC/EAS Guidelines for the management of dyslipidaemias]. *Revista Espanola de Cardiologia* **64,** 1168 e1161-1168 e1160.

Reynolds LJ, Credeur DP, Holwerda SW, Leidy HJ, Fadel PJ & Thyfault JP (2015). Acute inactivity impairs glycemic control but not blood flow to glucose ingestion. *Medicine and Science in Sports and Exercise* **47,** 1087-1094.

Rizvi SI, Abu Zaid M & Suhail M (1995). Insulin-mimetic effect of (-) epicatechin on osmotic fragility of human erythrocytes. *Indian Journal of Experimental Biology* **33,** 791-792.

Roberts KA, van Gent T, Hopkins ND, Jones H, Dawson EA, Draijer R, Carter HH, Atkinson CL, Green DJ, Thijssen DHJ & Low DA (2017). Reproducibility of four frequently used local heating protocols to assess cutaneous microvascular function. *Microvascular Research* **112,** 65-71.

Roger VL, Go AS, Lloyd-Jones DM, Benjamin EJ, Berry JD, Borden WB, Bravata DM, Dai S, Ford ES, Fox CS, Fullerton HJ, Gillespie C, Hailpern SM, Heit JA, Howard VJ, Kissela BM, Kittner SJ, Lackland DT, Lichtman JH, Lisabeth LD, Makuc DM, Marcus GM, Marelli A, Matchar DB, Moy CS, Mozaffarian D, Mussolino ME, Nichol G, Paynter NP, Soliman EZ, Sorlie PD, Sotoodehnia N, Turan TN, Virani SS, Wong ND, Woo D & Turner MB (2012). Heart disease and stroke statistics 2012 update: a report from the American Heart Association. *Circulation* **125,** e2-e220.

Roghani M & Baluchnejadmojarad T (2010). Hypoglycemic and hypolipidemic effect and antioxidant activity of chronic epigallocatechin-gallate in streptozotocin-diabetic rats. *Pathophysiology* **17,** 55-59.

Roher AE, Debbins JP, Malek-Ahmadi M, Chen K, Pipe JG, Maze S, Belden C, Maarouf CL, Thiyyagura P, Mo H, Hunter JM, Kokjohn TA, Walker DG, Kruchowsky JC, Belohlavek M, Sabbagh MN & Beach TG (2012). Cerebral blood flow in Alzheimer's disease. *Vascular Health and Risk Management* **8,** 599-611.

Ros E, Martínez-González MA, Estruch R, Salas-Salvadó J, Fitó M, Martínez JA & Corella D (2014). Mediterranean diet and cardiovascular health: Teachings of the PREDIMED study. *Advances in Nutrition: An International Review Journal* **5,** 330S-336S.

Roustit M, Blaise S, Millet C & Cracowski JL (2010). Reproducibility and methodological issues of skin post-occlusive and thermal hyperemia assessed by single-point laser Doppler flowmetry. *Microvascular Research* **79,** 102-108.

Roustit M & Cracowski J-L (2013). Assessment of endothelial and neurovascular function in human skin microcirculation. *Trends in Pharmacological Sciences* **34,** 373-384.

Roustit M & Cracowski JL (2012). Non-invasive assessment of skin microvascular function in humans: An insight into methods. *Microcirculation* **19,** 47-64.

Rubenfire M, Rajagopalan S & Mosca L (2000). Carotid artery vasoreactivity in response to sympathetic stress correlates with coronary disease risk and is independent of wall thickness. *Journal of the American College of Cardiology* **36,** 2192-2197.

Rudolph TK, Ruempler K, Schwedhelm E, Tan-Andresen J, Riederer U, Böger RH & Maas R (2007). Acute effects of various fast-food meals on vascular function and cardiovascular disease risk markers: The Hamburg burger trial. *The American Journal of Clinical Nutrition* **86,** 334-340.

Ruitenberg A, den Heijer T, Bakker SL, van Swieten JC, Koudstaal PJ, Hofman A & Breteler MM (2005a). Cerebral hypoperfusion and clinical onset of dementia: the Rotterdam Study. *Annals of Neurology* **57,** 789-794.

Ruitenberg A, den Heijer T, Bakker SLM, van Swieten JC, Koudstaal PJ, Hofman A & Breteler MMB (2005b). Cerebral hypoperfusion and clinical onset of dementia: The Rotterdam study. *Annals of Neurology* **57,** 789-794.

Rusznyak S & Szent-Györgyi A (1936). Vitamin P: Flavonols as vitamins. *Nature* **138**.

Ryu OH, Lee J, Lee KW, Kim HY, Seo JA, Kim SG, Kim NH, Baik SH, Choi DS & Choi KM (2006). Effects of green tea consumption on inflammation, insulin resistance and pulse wave velocity in type 2 diabetes patients. *Diabetes Research in Clinical Practice* **71,** 356-358.

Saab PG, Llabre MM, Hurwitz BE, Schneiderman N, Wohlgemuth W, Durel LA, Massie C & Nagel J (1993). The cold pressor test: vascular and myocardial response patterns and their stability. *Psychophysiology* **30,** 366-373.

Samarghandian S, Azimi-Nezhad M & Farkhondeh T (2017). Catechin treatment ameliorates diabetes and its complications in streptozotocin-induced diabetic rats. *Dose Response* **15**.

Samocha-Bonet D, Campbell LV, Mori TA, Croft KD, Greenfield JR, Turner N & Heilbronn LK (2012). Overfeeding reduces insulin sensitivity and increases oxidative stress, without altering markers of mitochondrial content and function in humans. *PLoS ONE* **7,** e36320.

Sasaki H, Hirasawa A, Washio T & Ogoh S (2016). Acute effect of coffee drinking on dynamic cerebral autoregulation. *European Journal of Applied Physiology* **116,** 879-884.

Schmidt MD, Cleland VJ, Shaw K, Dwyer T & Venn AJ (2009). Cardiometabolic risk in younger and older adults across an index of ambulatory activity. *American Journal of Preventive Medicine* **37,** 278-284.

Schreiber SJ, Gottschalk S, Weih M, Villringer A & Valdueza JM (2000). Assessment of blood flow velocity and diameter of the middle cerebral artery during the acetazolamide provocation test by use of transcranial Doppler sonography and MR imaging. *American Journal of Neuroradiology* **21,** 1207-1211.

Schreuder THA, Eijsvogels TMH, Greyling A, Draijer R, Hopman MTE & Thijssen DHJ (2014). Effect of black tea consumption on brachial artery flow-mediated dilation and ischaemia-reperfusion in humans. *Applied Physiology, Nutrition & Metabolism* **39,** 145-151.

Schroeter H, Heiss C, Balzer J, Kleinbongard P, Keen CL, Hollenberg NK, Sies H, Kwik-Uribe C, Schmitz HH & Kelm M (2006). (–)-Epicatechin mediates beneficial effects of flavanol-rich cocoa on vascular function in humans. *Proceedings of the National Academy of Sciences of the United States of America* **103,** 1024-1029.

Schuff N, Matsumoto S, Kmiecik J, Studholme C, Du AT, Ezekiel F, Miller BL, Kramer JH, Jagust WJ, Chui HC & Weiner MW (2009). Cerebral blood flow in ischemic vascular dementia and Alzheimer's disease by arterial spin labeling MRI. *Alzheimer's & Dementia* **5,** 454-462.

Scott MJ, Randolph PH & Leier CV (1989). Reproducibility of systolic and diastolic time intervals in normal humans: An important issue in clinical cardiovascular pharmacology. *Journal of Cardiovascular Pharmacology* **13,** 125-130.

Sena CM, Pereira AM & Seica R (2013). Endothelial dysfunction - A major mediator of diabetic vascular disease. *Biochemica et Biophysica Acta - Molecular Basis of Disease* **1832,** 2216-2231.

Shah ZA, Li R-c, Ahmad AS, Kensler TW, Yamamoto M, Biswal S & Doré S (2010). The flavanol (−)-epicatechin prevents stroke damage through the Nrf2/HO1 pathway. *Journal of Cerebral Blood Flow & Metabolism* **30,** 1951-1961.

Sharp SI, Aarsland D, Day S, Sønnesyn H, Group AsSVDSR & Ballard C (2011). Hypertension is a potential risk factor for vascular dementia: Systematic review. *International Journal of Geriatric Psychiatry* **26,** 661-669.

Shechter M, Issachar A, Marai I, Koren-Morag N, Freinark D, Shahar Y, Shechter A & Feinberg MS (2009). Long-term association of brachial artery flow-mediated vasodilation and cardiovascular events in middle-aged subjects with no apparent heart disease. *International Journal of Cardiology* **134,** 52-58.

Shechter M, Shechter A, Koren-Morag N, Feinberg MS & Hiersch L (2014). Usefulness of brachial artery flow-mediated dilation to predict long-term cardiovascular events in subjects without heart disease. *The American Journal of Cardiology* **113,** 162-167.

Sherwood A, Girdler SS, Bragdon EE, West SG, Brownley KA, Hinderliter AL & Light KC (1997). Ten-year stability of cardiovascular responses to laboratory stressors. *Psychophysiology* **34,** 185-191.

Shimokawa H, Yasutake H, Fujii K, Owada MK, Nakaike R, Fukumoto Y, Takayanagi T, Nagao T, Egashira K, Fujishima M & Takeshita A (1996). The importance of the hyperpolarizing mechanism increases as the vessel size decreases in endothelium-dependent relaxations in rat mesenteric circulation. *Journal of Cardiovascular Pharmacology* **28,** 703-711.

Shoemaker JK, Hogeman CS, Silber DH, Gray K, Herr M & Sinoway LI (1998). Head-down-tilt bed rest alters forearm vasodilator and vasoconstrictor responses. *Journal of Applied Physiology* **84,** 1756-1762.

Shojaee-Moradie F, Baynes KC, Pentecost C, Bell JD, Thomas EL, Jackson NC, Stolinski M, Whyte M, Lovell D, Bowes SB, Gibney J, Jones RH & Umpleby AM (2007). Exercise training reduces fatty acid availability and improves the insulin sensitivity of glucose metabolism. *Diabetologia* **50,** 404-413.

Silke B, Spiers JP, Boyd S, Graham E, McParland G & Scott ME (1994). Evaluation of non-invasive blood pressure measurement by the Finapres method at rest and during dynamic exercise in subjects with cardiovascular insufficiency. *Clinical Autonomic Research* **4,** 49-56.

Silvestrini M, Vernieri F, Pasqualetti P & et al. (2000). Impaired cerebral vasoreactivity and risk of stroke in patients with asymptomatic carotid artery stenosis. *JAMA* **283,** 2122-2127.

Sisson SB, Camhi SM, Church TS, Tudor-Locke C, Johnson WD & Katzmarzyk PT (2010). Accelerometer-determined steps/day and metabolic syndrome. *American Journal of Preventive Medicine* **38,** 575-582.

Skeaff CM & Miller J (2009). Dietary fat and coronary heart disease: Summary of evidence from prospective cohort and randomised controlled trials. *Annals of Nutrition and Metabolism* **55,** 173-201.

Skow RJ, MacKay CM, Tymko MM, Willie CK, Smith KJ, Ainslie PN & Day TA (2013). Differential cerebrovascular CO_2 reactivity in anterior and posterior cerebral circulations. *Respiratory Physiology & Neurobiology* **189,** 76-86.

Smith BA, Clayton EW & Robertson D (2011). Experimental arrest of cerebral blood flow in human subjects: The red wing studies revisited. *Perspectives in Biology and Medicine* **54,** 121-131.

Smith DL & Fernhall B (2011). *Advanced Cardiovascular Exercise Physiology (Advanced Exercise Physiology)*. Human Kinetics Ltd, Champaign, IL.

Sokolnicki LA, Roberts SK, Wilkins BW, Basu A & Charkoudian N (2007). Contribution of nitric oxide to cutaneous microvascular dilation in individuals with type 2 diabetes mellitus. *American Journal of Physiology - Endocrinology and Metabolism* **292,** E314-E318.

Somerville V, Bringans C & Braakhuis A (2017). Polyphenols and performance: A systematic review and meta-analysis. *Sports Medicine* **47,** 1589-1599.

Sonne MP, Alibegovic AC, Højbjerre L, Vaag A, Stallknecht B & Dela F (2010). Effect of 10 days of bedrest on metabolic and vascular insulin action: A study in individuals at risk for type 2 diabetes. *Journal of Applied Physiology* **108,** 830-837.

Sorond FA, Lipsitz LA, Hollenberg NK & Fisher ND (2008a). Cerebral blood flow response to flavanol-rich cocoa in healthy elderly humans. *Neuropsychiatric Disease and Treatment* **4,** 433-440.

Sorond FA, Schnyer DM, Serrador JM, Milberg WP & Lipsitz LA (2008b). Cerebral blood flow regulation during cognitive tasks: Effects of healthy aging. *Cortex* **44,** 179-184.

Spencer JPE (2009). Flavonoids and brain health: Multiple effects underpinned by common mechanisms. *Genes & Nutrition* **4,** 243-250.

Spilk S, Herr MD, Sinoway LI & Leuenberger UA (2013). Endothelium-derived hyperpolarizing factor contributes to hypoxia-induced skeletal muscle vasodilation in humans. *American Journal of Physiology - Heart and Circulatory Physiology* **305,** H1639-H1645.

Sprague RS & Ellsworth ML (2010). Vascular disease in pre-diabetes: New insights derived from systems biology. *Missouri Medicine* **107,** 265-269.

Sprung VS, Cuthbertson DJ, Pugh CJA, Daousi C, Atkinson G, Aziz NF, Kemp GJ, Green DJ, Cable NT & Jones H (2013). Nitric oxide-mediated cutaneous microvascular function is impaired in polycystic ovary sydrome but can be improved by exercise training. *The Journal of Physiology* **591,** 1475-1487.

Stendell-Hollis NR, Thomson CA, Thompson PA, Bea JW, Cussler EC & Hakim IA (2010). Green tea improves metabolic biomarkers, not weight or body composition: a pilot study in overweight breast cancer survivors. *Journal of Human Nutrition and Dietetics* **23,** 590-600.

Stephan BCM, Harrison SL, Keage HAD, Babateen A, Robinson L & Siervo M (2017). Cardiovascular disease, the nitric oxide pathway and risk of cognitive impairment and dementia. *Current Cardiology Reports* **19,** 87.

Stettler R, Ith M, Acheson KJ, Décombaz J, Boesch C, Tappy L & Binnert C (2005). Interaction between dietary lipids and physical inactivity on insulin sensitivity and on intramyocellular lipids in healthy men. *Diabetes Care* **28,** 1404-1409.

Stote KS & Baer DJ (2008). Tea consumption may improve biomarkers of insulin sensitivity and risk factors for diabetes. *The Journal of Nutrition* **138,** 1584s-1588s.

Suganya N, Bhakkiyalakshmi E, Sarada DVL & Ramkumar KM (2016). Reversibility of endothelial dysfunction in diabetes: Role of polyphenols. *British Journal of Nutrition* **116,** 223-246.

Sulibursk a J, Bogdanski P, Szulinska M, Stepien M, Pupek-Musialik D & Jablecka A (2012). Effects of green tea supplementation on elements, total antioxidants, lipids, and glucose values in the serum of obese patients. *Biological Trace Element Research* **149,** 315-322.

Tam CS, Viardot A, Clément K, Tordjman J, Tonks K, Greenfield JR, Campbell LV, Samocha-Bonet D & Heilbronn LK (2010). Short-term overfeeding may induce peripheral insulin resistance without altering subcutaneous adipose tissue macrophages in humans. *Diabetes* **59,** 2164-2170.

Tew GA, Klonizakis M, Moss J, Ruddock AD, Saxton JM & Hodges GJ (2011a). Reproducibility of cutaneous thermal hyperaemia assessed by laser Doppler flowmetry in young and older adults. *Microvascular Research* **81,** 177-182.

Tew GA, Saxton JM, Klonizakis M, Moss J, Ruddock AD & Hodges GJ (2011b). Aging and aerobic fitness affect the contribution of noradrenergic sympathetic nerves to the rapid cutaneous vasodilator response to local heating. *Journal of Applied Physiology* **110,** 1264-1270.

Thijssen DH, Ellenkamp R, Kooijman M, Pickkers P, Rongen GA, Hopman MT & Smits P (2007). A causal role for endothelin-1 in the vascular adaptation to skeletal muscle deconditioning in spinal cord injury. *Arteriosclerosis, Thrombosis, and Vascular Biology* **27,** 325-331.

Thijssen DHJ, Black MA, Pyke KE, Padilla J, Atkinson G, Harris RA, Parker B, Widlansky ME, Tschakovsky ME & Green DJ (2011). Assessment of flow-mediated dilation in humans: A methodological and physiological guideline. *American Journal of Physiology - Heart and Circulatory Physiology* **300,** H2-H12.

Thijssen DHJ, Maiorana AJ, O'Driscoll G, Cable NT, Hopman MTE & Green DJ (2010). Impact of inactivity and exercise on the vasculature in humans. *European Journal of Applied Physiology* **108,** 845-875.

Thyfault JP & Krogh-Madsen R (2011). Metabolic disruptions induced by reduced ambulatory activity in free-living humans. *Journal of Applied Physiology* **111,** 1218-1224.

Tooke JE (1995). Microvascular function in human diabetes. A physiological perspective. *Diabetes* **44,** 721-726.

Triedman JK & Saul JP (1994). Comparison of intraarterial with continuous noninvasive blood pressure measurement in postoperative pediatric patients. *Journal of Clinical Monitoring* **10,** 11-20.

Tsai W-C, Lo Y-H, Lin C-C, Chao T-H & Chen J-H (2004). Effects of oxidative stress on endothelial function after a high-fat meal. *Clinical Science* **106,** 315-319.

Tzeng YC, Ainslie PN, Cooke WH, Peebles KC, Willie CK, MacRae BA, Smirl JD, Horsman HM & Rickards CA (2012). Assessment of cerebral autoregulation: The quandary of quantification. *American Journal of Physiology - Heart and Circulatory Physiology* **303,** H658-671.

Ueda-Wakagi M, Mukai R, Fuse N, Mizushina Y & Ashida H (2015). 3-O-Acyl-epicatechins increase glucose uptake activity and GLUT4 translocation through activation of PI3K signaling in skeletal muscle cells. *International Journal of Molecular Sciences* **16,** 16288.

Ueda M, Furuyashiki T, Yamada K, Aoki Y, Sakane I, Fukuda I, Yoshida K-i & Ashida H (2010). Tea catechins modulate the glucose transport system in 3T3-L1 adipocytes. *Food & Function* **1,** 167-173.

Vallance P, Collier J & Moncada S (1989). Effects of endothelium-derived nitric oxide on peripheral arteriolar tone in man. *Lancet* **2,** 997-1000.

van Beek AHEA, Claasen JAHR, Rikkert MGMO & Jansen RWMM (2008). Cerebral autoregulation: An overview of current concepts and methodology with special focus on the elderly. *Journal of Cerebral Blood Flow & Metabolism* **28,** 1071-1085.

van Duijnhoven NT, Green DJ, Felsenberg D, Belavy DL, Hopman MT & Thijssen DH (2010). Impact of bed rest on conduit artery remodeling: Effect of exercise countermeasures. *Hypertension* **56,** 240-246.

van Duijnhoven NT, Janssen TW, Green DJ, Minson CT, Hopman MT & Thijssen DH (2009). Effect of functional electrostimulation on impaired skin vasodilator responses to local heating in spinal cord injury. *Journal of Applied Physiology* **106,** 1065-1071.

van Lieshout JJ, Wieling W, Karemaker JM & Secher NH (2003). Syncope, cerebral perfusion, and oxygenation. *Journal of Applied Physiology* **94,** 833-848.

van Mil AC, Hartman Y, van Oorschot F, Heemels A, Bax N, Dawson EA, Hopkins N, Hopman MT, Green DJ, Oxborough DL & Thijssen DH (2017). Correlation of carotid artery reactivity with cardiovascular risk factors and coronary artery vasodilator responses in asymptomatic, healthy volunteers. *Journal of Hypertension* **35,** 1026-1034.

Victor RG, Leimbach WN, Seals DR, Wallin BG & Mark AL (1987). Effects of the cold pressor test on muscle sympathetic nerve activity in humans. *Hypertension* **9,** 429-436.

Vidyasagar R, Greyling A, Draijer R, Corfield DR & Parkes LM (2013). The effect of black tea and caffeine on regional cerebral blood flow measured with arterial spin labeling. *Journal of Cerebral Blood Flow & Metabolism* **33,** 963-968.

Virdis A (2016). Endothelial dysfunction in obesity: Role of inflammation. *High Blood Pressure & Cardiovascular Prevention* **23,** 83-85.

Vita JA (2003). Tea Consumption and Cardiovascular Disease: Effects on Endothelial Function. *The Journal of Nutrition* **133,** 3293S-3297S.

Vita JA (2005). Polyphenols and cardiovascular disease: Effects on endothelial and platelet function. *The American Journal of Clinical Nutrition* **81,** 292S-297S.

Vita JA & Keaney JF (2002). Endothelial function: A barometer for cardiovascular risk? *Circulation* **106,** 640-642.

Vogel RA, Corretti MC & Plotnick GD (1997). Effect of a single high-fat meal on endothelial function in healthy subjects. *The American Journal of Cardiology* **79,** 350-354.

Wagenmakers AJM, Strauss JA, Shepherd SO, Keske MA & Cocks M (2016). Increased muscle blood supply and transendothelial nutrient and insulin transport induced by food intake and exercise: Effect of obesity and ageing. *The Journal of Physiology* **594,** 2207-2222.

Wahl M, Deetjen P, Thurau K, Ingvar DH & Lassen NA (1970). Micropuncture evaluation of the importance of perivascular pH for the arteriolar diameter on the brain surface. *Pflügers Archiv* **316,** 152-163.

Walhin J-P, Richardson JD, Betts JA & Thompson D (2013). Exercise counteracts the effects of short-term overfeeding and reduced physical activity independent of energy imbalance in healthy young men. *The Journal of Physiology* **591,** 6231-6243.

Wallengren J (1997). Vasoactive peptides in the skin. *Journal of Investigative Dermatology Symposium Proceedings* **2,** 49-55.

Wasilewski R, Ubara EO & Klonizakis M (2016). Assessing the effects of a short-term green tea intervention in skin microvascular and oxygen tension in older and younger adults. *Microvascular Research* **107,** 65-71.

Wasserman AJ & Patterson JL, Jr. (1961). The cerebral vascular response to reduction in arterial carbon dioxide tension. *The Journal of Clinical Investigation* **40,** 1297-1303.

Wesseling KH, Dewit B, Van der Hoeven GMA, Van Goudoever J & Settels JJ (1995). Physiocal, calibrating finger vascular physiology for Finapres. *Homeostasis* **36,** 67-82.

Westerbacka J, Vehkavaara S, Bergholm R, Wilkinson I, Cockcroft J & Yki-Järvinen H (1999). Marked resistance of the ability of insulin to decrease arterial stiffness characterizes human obesity. *Diabetes* **48,** 821-827.

Wheatcroft SB, Williams IL, Shah AM & Kearney MT (2003). Pathophysiological implications of insulin resistance on vascular endothelial function. *Diabetic Medicine* **20,** 255-268 214p.

WHO (2015). Physical Inactivity: A Global Public Health Problem. http://www.who.int/dietphysicalactivity/factsheet_inactivity/en/.

WHO (2016). Cardiovascular Diseases (CVDs). http://www.who.int/mediacentre/factsheets/fs317/en/.

Wiernsperger N (2000). Defects in microvascular haemodynamics during prediabetes: Contributor or epiphenomenon? *Diabetologia* **43,** 1439-1448.

Wightman EL, Haskell CF, Forster JS, Veasey RC & Kennedy DO (2012). Epigallocatechin gallate, cerebral blood flow parameters, cognitive performance and mood in healthy humans: A double-blind, placebo-controlled, crossover investigation. *Human Psychopharmacology: Clinical and Experimental* **27,** 177-186.

Williamson G & Manach C (2005). Bioavailability and bioefficacy of polyphenols in humans. II. Review of 93 intervention studies. *The American Journal of Clinical Nutrition* **81,** 243S-255S.

Willie CK, Colino FL, Bailey DM, Tzeng YC, Binsted G, Jones LW, Haykowsky MJ, Bellapart J, Ogoh S, Smith KJ, Smirl JD, Day TA, Lucas SJ, Eller LK & Ainslie PN (2011). Utility of transcranial Doppler ultrasound for the integrative assessment of cerebrovascular function. *Journal of Neuroscience Methods* **196,** 221-237.

Willie CK, Macleod DB, Shaw AD, Smith KJ, Tzeng YC, Eves ND, Ikeda K, Graham J, Lewis NC, Day TA & Ainslie PN (2012). Regional brain blood flow in man during acute changes in arterial blood gases. *The Journal of Physiology* **590,** 3261-3275.

Willie CK, Tzeng Y-C, Fisher JA & Ainslie PN (2014). Integrative regulation of human brain blood flow. *The Journal of Physiology* **592,** 841-859.

Wilson MH, Edsell MEG, Davagnanam I, Hirani SP, Martin DS, Levett DZH, Thornton JS, Golay X, Strycharczuk L, Newman SP, Montgomery HE, Grocott MPW, Imray CHE & for the Caudwell Xtreme Everest Research G (2011). Cerebral artery dilatation maintains cerebral oxygenation at extreme altitude and in acute hypoxia—an ultrasound and MRI study. *Journal of Cerebral Blood Flow & Metabolism* **31,** 2019-2029.

Wong BJ & Fieger SM (2010). Transient receptor potential vanilloid type-1 (TRPV-1) channels contribute to cutaneous thermal hyperaemia in humans. *The Journal of Physiology* **588,** 4317-4326.

Wong BJ & Minson CT (2006). Neurokinin-1 receptor desensitization attenuates cutaneous active vasodilatation in humans. *The Journal of Physiology* **577,** 1043-1051.

Wong BJ, Tublitz NJ & Minson CT (2005). Neurokinin-1 receptor desensitization to consecutive microdialysis infusions of substance P in human skin. *The Journal of Physiology* **568,** 1047-1056.

Woodman RJ, Playford DA, Watts GF, Cheetham C, Reed C, Taylor RR, Puddey IB, Beilin LJ, Burke V, Mori TA & Green D (2001). Improved analysis of brachial artery ultrasound using a novel edge-detection software system. *Journal of Applied Physiology* **91,** 929-937.

Woodward KA, Draijer R, Thijssen DHJ & Low DA (2017). Polyphenols and microvascular function in humans: A systematic review. *Current Pharmaceutical Design* **23,** 1-24.

Woodward KA, Hopkins ND, Draijer R, de Graaf Y, Low DA & Thijssen DHJ (2016). Acute black tea consumption improves cutaneous vascular function in healthy middle-aged humans. *Clinical Nutrition. Advance online publication https://doi.org/10.1016/j.clnu.2016.12.013.*

Wu AH, Spicer D, Stanczyk FZ, Tseng CC, Yang CS & Pike MC (2012). Effect of 2-month controlled green tea intervention on lipoprotein cholesterol, glucose, and hormone levels in healthy postmenopausal women. *Cancer Prevention Research* **5,** 393-402.

Wu LY, Juan CC, Ho LT, Hsu YP & Hwang LS (2004a). Effect of green tea supplementation on insulin sensitivity in Sprague-Dawley rats. *Journal of Agricultural and Food Chemistry* **52,** 643-648.

Wu LY, Juan CC, Hwang LS, Hsu YP, Ho PH & Ho LT (2004b). Green tea supplementation ameliorates insulin resistance and increases glucose transporter IV content in a fructose-fed rat model. *European Journal of Nutrition* **43,** 116-124.

Xiang L, Mittwede PN & Clemmer JS (2011). Glucose homeostasis and cardiovascular alterations in diabetes. In: *Comprehensive Physiology*. John Wiley & Sons, Inc.

Yahya HM, Day A, Lawton C, Myrissa K, Croden F, Dye L & Williamson G (2016). Dietary intake of 20 polyphenol subclasses in a cohort of UK women. *European Journal of Nutrition* **55,** 1839-1847.

Yamabe N, Yokozawa T, Oya T & Kim M (2006). Therapeutic potential of (-)-epigallocatechin 3-O-gallate on renal damage in diabetic nephropathy model rats. *Journal of Pharmacology and Experimental Therapeutics* **319,** 228-236.

Yang W-S, Wang W-Y, Fan W-Y, Deng Q & Wang X (2014). Tea consumption and risk of type 2 diabetes: A dose–response meta-analysis of cohort studies. *British Journal of Nutrition* **111,** 1329-1339.

Yarmolinsky J, Gon G & Edwards P (2015). Effect of tea on blood pressure for secondary prevention of cardiovascular disease: A systematic review and meta-analysis of randomized controlled trials. *Nutrition Reviews* **73,** 236-246.

Zhang P, Huang G & Shi X (2011). Cerebral vasoreactivity during hypercapnia is reset by augmented sympathetic influence. *Journal of Applied Physiology* **110,** 352-358.

Zhang R, Crandall CG & Levine BD (2004). Cerebral hemodynamics during the valsalva maneuver: Insights from ganglionic blockade. *Stroke* **35,** 843-847.

Zhang R, Zuckerman JH, Giller CA & Levine BD (1998). Transfer function analysis of dynamic cerebral autoregulation in humans. *The American Journal of Physiology* **274,** H233-241.

Zhao Q, Bazzano LA, Cao J, Li J, Chen J, Huang J, Chen J, Kelly TN, Chen C-S, Hu D, Ma J, Rice TK, He J & Gu D (2012). Reproducibility of blood pressure response to the cold pressor test: The GenSalt study. *American Journal of Epidemiology* **176,** S91-S98.

Zimmermann C & Haberl RL (2003). L-arginine improves diminished cerebral CO2 reactivity in patients. *Stroke* **34,** 643-647.