THE IMPACT OF COCOA FLAVANOLS ON PERIPHERAL VASCULAR, CEREBROVASCULAR AND COGNITIVE FUNCTION IN YOUNGER AND OLDER HEALTHY ADULTS

by

ROSIE MAIR PRITCHARD

A thesis submitted to the University of Birmingham for the degree of MASTER OF SCIENCE

School of Sport, Exercise and Rehabilitation Sciences
College of Life & Environmental Sciences
University of Birmingham
September 2019

Abstract

Cocoa flavanols, have proven effective at ameliorating human peripheral vascular function. However, the extent to which peripheral vasculature effects translate to the cerebrovasculature have been less explored. The present study aimed to assess the acute impact of a flavanol-rich cocoa on peripheral and cerebrovascular function in young and older adults and investigate whether such vascular outcomes may underpin improvements in cognitive performance.

An acute trial was conducted in young (21 ± 4 yrs, n=20) and older adults (71 ± 5 yrs, n=14) to assess the impact of a High-Flavanol Cocoa (150 mg of (-)-epicatechin; 1000 mg of total polyphenols) and a Low-Flavanol Cocoa (4 mg of (-)-epicatechin; 140 mg of total polyphenols) on: i) Flow-mediated dilation of the brachial artery; ii) CO_2-cerebrovascular reactivity of the middle and posterior cerebral arteries; iii) cognitive performance. All vascular outcome measures were assessed at baseline and 2 hours post-ingestion.

FMD increased in both cohorts with high-flavanol cocoa but decreased within the younger cohort with low-flavanol cocoa. Cerebrovascular reactivity decreased with high-flavanol cocoa for the younger cohort. No changes in cognition were seen.

Findings from this study indicate potential opposing effects of high-flavanol cocoa ingestion between peripheral and cerebral vascular function.

Dedication

I would like to dedicate this thesis to a few people especially my family, who know how much of a struggle it was for me to get to this point. Thank you for everything you've done to help me get here, it means the world. I would also like to specially dedicate this to some of my Grandparents, Mims and Grandad Clint for allowing me to live with them during part of my time working on this. I would also like to say in writing to my Grandad Clint who has been a huge influence on me and has really motivated me to achieve this……an MSc is better than a M.A.!

Acknowledgements

I would like to acknowledge my supervisors, Dr. Samuel Lucas and Dr. Catarina Rendeiro, for helping me to set up and run my research, as well as helping me become proficient in new laboratory techniques. Although the data is not presented in this thesis due to time constraints, I'd like to acknowledge Dr. Aaron Philips and his laboratory at the University of Calgary for helping me analyse my neurovascular coupling data. An additional special acknowledgement to David Owen (MEng) for helping me in more ways than he knows, get to this point and allowing me somewhere to stay during my homeless time in Birmingham to finish this project!

Table of Contents

List of illustrations

List of tables

List of Equations

List of Abbreviations

Cerebrovascular Abbreviations:

Middle cerebral artery	MCA
Middle cerebral artery velocity	MCAv
Middle cerebral artery cerebrovascular reactivity	CVR_{MCA}
Middle cerebral artery cerebrovascular conductance	CVC_{MCA}
Middle cerebral artery cerebrovascular conductance reactivity	$CVC\text{-}CVR_{MCA}$
Posterior cerebral artery	PCA
Posterior cerebral artery velocity	PCAv
Posterior cerebral artery cerebrovascular reactivity	CVR_{PCA}
Posterior cerebral artery cerebrovascular conductance	CVC_{PCA}
Posterior cerebral artery cerebrovascular conductance reactivity	$CVC\text{-}CVR_{PCA}$

Other physiological abbreviations:

Partial pressure of end-tidal carbon dioxide	$P_{ET}CO_2$
Heart rate	HR
Blood pressure	BP
Systolic blood pressure	SBP
Diastolic blood pressure	DBP
Mean arterial pressure	MAP

Equipment and testing abbreviations:

Electrocardiogram	ECG
Transcranial Doppler Ultrasound	TCD
Cambridge Neuropsychological Test Automated Battery	CANTAB
Flow mediated dilation	FMD

Other abbreviations:

Nitric oxide	NO
Cardiovascular disease	CVD
Coronary heart disease	CHD
Flavanol-rich cocoa	FRC
Flavanol-poor cocoa	FPC

LITERATURE REVIEW: THE IMPACT OF COCOA FLAVANOLS ON VASCUALR AND COGNITVE FUNCTION

Introduction to Literature Review

As life expectancy continues to increase worldwide, the inherently positive aspects of living longer are also accompanied by a higher prevalence of age-related diseases. In particular, cardiovascular diseases (CVD), cerebrovascular diseases and neurological diseases have become increasingly ubiquitous in the last 30 years, with age being one of the main risk factors for the onset of such diseases (Xu et al., 2017). Therefore, it is of great importance to find lifestyle strategies that may delay or prevent the onset of age-associated diseases. Such lifestyle strategies can effectively improve quality of life for many individuals but can also reduce strain on healthcare services.

Both physical exercise and diet are well known to play key roles in the prevention of age-associated cardiovascular complications and cognitive impairments (Heyn et al., 2004; Letenneur et al., 2007; Estruch et al., 2013; Klonizakis et al., 2014; Jakovljevic, 2017). Plant-derived small molecules found ubiquitously within fruits and vegetables, widely known as polyphenols have been shown to effectively improve CVD risk factors (Quinones et al., 2013; Mokhtar et al., 2017). Furthermore, dietary polyphenols have also shown promise for reducing neurological diseases such as dementia, partly by reducing age-related cognitive decline (Sokolov et al., 2013; Molino et al., 2016). In particular, a subcategory of polyphenols known as flavonoids, which are particularly enriched in foods and beverages such as; cocoa, berries, tea, grapes and apples, have been widely researched over the last 20 years. Evidence from both epidemiological studies and human randomized controlled trials have consistently shown that plant-

derived flavonoid intake improves biomarkers of cardiovascular health, including blood pressure and endothelial function (Hooper et al., 2008; Galleano et al., 2009). Furthermore, positive improvements in various aspects of cognitive function have been demonstrated via plant-derived flavonoid intake (Macready et al., 2009). As a consequence, there has been an increasing interest within the scientific community to understand the impact of flavonoids on the central nervous system and human cognition.

In the following review we aim to explore evidence surrounding the impact of polyphenols, specifically flavonoids, on peripheral vascular function, cerebrovascular function and cognition. We will further discuss the specific impact of well-studied cocoa flavanols on vascular function in different population cohorts, including both younger and older cohorts and healthy and diseased populations as well as how changes in the peripheral vasculature might also manifest in the cerebral vasculature.

1. Impact of ageing on peripheral and cerebrovascular function

Vascular diseases, whether cardio or cerebral, affect millions of lives globally leading to increased mortality and disability rates. Whilst cardiovascular disease is the leading cause of death worldwide, being responsible for around 28% of all deaths in the UK alone (British Heart Foundation, 2019), stroke is a leading cause of disability and the 4th leading cause of death in the UK (Stroke Association, 2018). Vascular diseases directly cause premature death via embolism or haemorrhage but can also lead to other diseases such as Vascular Dementia and Alzheimer's Disease (Knopman, 2006). It is estimated that in 2017 50 million people globally lived with dementia, this

number is expected to double every 20 years due to the rapidly increasing elderly population (Alzheimer's Disease International, 2015).

Healthy ageing of the vasculature causes several molecular, morphological and physiological changes to the vessels, the nature of which can depend on variables such as location of the vessel, type of vessel and the target organs (Xu et al., 2017). Such age-related alterations to the vasculature tree can cause vascular wall stiffness, impaired endothelial function and lead to vascular dysfunction, plaque accumulation and atherosclerosis, all of which are linked to cardiovascular complications and disease (Bolton & Rajkumar, 2011). Traditionally these age-related modifications are characterised as 'healthy aging', therefore non-modifiable or preventable. However, in recent years research utilising lifestyle or pharmaceutical interventions have demonstrated an ability to alter this ageing trajectory (Jankovic et al., 2015; Gando et al., 2016), therefore changing what may be characterised as 'healthy ageing'.

Arterial wall stiffening is one of the key characteristics seen in ageing vessels and has been the focus of scientific research for many years. Several large longitudinal studies have been performed analysing arterial stiffening within ageing individuals (AlGhatrif et al., 2013; Gando et al., 2016). One such study conducted by AlGhatrif *et al.* (2013) examined arterial stiffness changes using carotid-femoral pulse wave velocity (PWV) in 354 men and 423 women (aged 21-94 years) between 1988-2013. They reported that arterial wall stiffness increases with age and further showed that dimorphism within this stiffening becomes greater with advancing age, as males have steeper longitudinal increases in PWV than females (AlGhatrif et al., 2013; Noon et al., 2008). Studies, such as the aforementioned, indicate that vascular arterial stiffening throughout the vascular tree seems to be uniform. However, other studies have

reported that conduit arteries display notably stiffer walls as opposed to peripheral or muscular arteries (Shirai, et al., 2011; Choi, et al., 2013). Recent findings have shown that in comparison to peripheral (muscular/distributing) arteries, conduit arteries, such as cerebral arteries, display notably stiffer walls with increasing ageing due to morphological changes (Xu et al., 2017). Age-related structural changes take place within the intima and media; these changes can be different depending on artery type. Peripheral arteries are characterised by hypertrophic (thickening) or eutrophic (invariant) structural remodelling whereas conduit artery ageing manifests its changes by an increase in arterial diameter and gradual hypertrophic remodelling (Xu et al., 2017). Within peripheral arteries this stiffness can also be accounted for by the reduction in elastin content, however, within cerebral arteries elastin content is not reduced, rather fragmentation of the elastin occurs (Xu et al., 2017). Arterial stiffness is linked to the ratio of collagen to elastin, a combination of collagen accumulation and elastin deterioration or fragmentation alongside thickening and dysfunction of the endothelium progresses arterial stiffening (Vaitkevicius et al., 1993). Age is noted to be a large independent risk factor leading to endothelial dysfunction, namely due to oxidative stress and inflammation, even without other risk factors being present. Endothelial dysfunction leads to impaired endothelial dilation and an imbalance between vasodilation and vasoconstriction, as well as being highly associated with cardiovascular disease risk (Bolton & Rajkumar 2011). Therefore, enhancing the vasodilatory ability of ageing vessels via improvements in endothelial function, will decrease risk of an initial cardiovascular event occurring. Promoting lifestyle interventions that will allow the elderly population to reduce their risk without need for

seeking pharmaceutical mediation or to work alongside required medication, is key for reducing reliance on pharmaceutical drugs and hospital care.

2. Plant-derived Flavonoids: structure and food sources

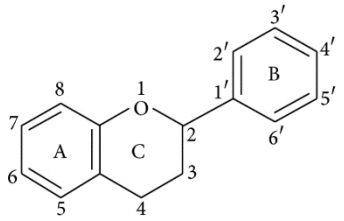
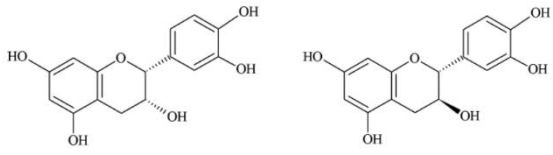
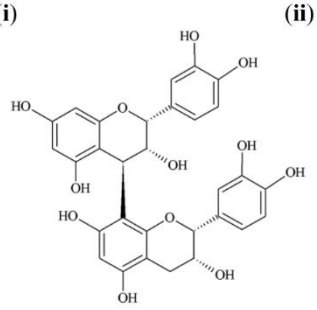
A vast array of organic compounds are synthesised by plants, broadly categorized as primary and secondary metabolites. Whereas primary metabolites are essential for short term health, secondary metabolites are suggested to impart long term health benefits, protecting against chronic illnesses such as cancers and cardiovascular disease. Secondary metabolites can be further subcategorized into three main classes: i) phenolics, ii) terpenes, and iii) nitrogen-containing compounds (Crozier et al., 2006).

Phenolics are distinguished by their chemical make-up, having one or more aromatic ring with single or multiple hydroxyl groups attached. A great variety of phenolic structures (over 8000 polyphenols) have been reported throughout the plant kingdom (Crozier et al., 2006). Polyphenols can be subdivided into categories including phenols, flavonoids and non-flavonoids each with varying structures ranging from a simple phenol core to higher levels of polymerisation constituting more complex molecules (Jalil & Ismail, 2008). The subject of our investigation, flavonoids, can also be further sub-categorised into flavanones, flavonols, flavones, flavan-3-ols, anthocyanins/anthocyanidins and isoflavones (Hooper et al., 2008).

The flavonoid family have a basic structure comprising of fifteen carbons with two aromatic rings linked by a three-carbon bridge; subtypes are characterized by a substitution pattern in the B and C rings (Crozier et al., 2006; Galleano et al., 2009) as shown in **Table 1**. Flavonoids can be found in a large abundance of plants, notably in

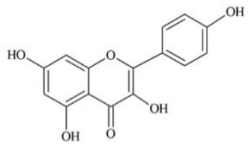
high concentrations within skins of fruits and in the epidermis of leaves and have a wide range of roles within the plant, including pigmentation and disease resistance. The different classes of flavonoids are presented in **Table 1**, with detailed biochemical structures, dietary sources and indication of typical amounts found in foods. Although flavonoids are found within most fruits and vegetables some food sources are particularly enriched, the most plentiful sources of the flavonoid subtypes are shown within the **Table 1**.

Table 1. Flavonoid group, common compounds, dietary sources and amounts, table adapted from Thilakarathna & Rupasinghe (2013).

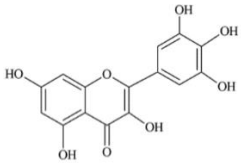
Flavanoid Group (Common Compounds)	**Common Food Sourcess and Amounts (mg/100g)**			
Basic Flavonoid Structure 				
Flavan-3-ols **i)** (+)-Catechin, **ii)** (-)-Epicatechin, **iii)** Procyandin B2 (dimer) example; food for all dimers present  (i) (ii)  (iii)	**Source**	**i)**	**ii)**	**iii)**
	Apples (Red with skin)	2.00	9.83	15.12
	Apricots (raw)	3.67	4.74	1.33
	Peaches (raw)	4.92	2.34	12.24
	Pears (raw)	0.27	3.76	2.73
	Strawberries (raw)	6.65	1.56	5.26
	Black tea (brewed)	1.51	2.13	3.74
	Blueberries (highbush, raw)	5.29	0.62	5.71
	Cranberries (raw)	0.39	4.37	25.93
	Cocoa (dry powder)	64.82	196.43	183.49
	Grapes (black)	0.82	0.96	2.38
	Red wine	7.12	3.76	20.49

Flavonols

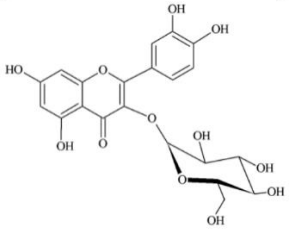
i) Kaempferol, **ii)** Myricetin,**iii)** Quercetin-3-*O*-glucoside, example; food values for quercetin



(i)



(ii)



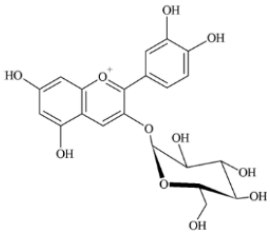
(iii)

Source	i)	ii)	iii)
Blueberries (highbush, raw)	1.66	1.26	7.67
Garlic	0.26	1.61	1.74
Onions	0.63	0.03	21.40
Kale	46.80	0.00	22.58
Broccoli	7.83	0.06	3.26
Spinach	15.75	0.00	5.75
Black tea (brewed)	1.31	0.45	1.99
Red wine	0.20	0.83	1.76
Cheery tomatoes	0.10	0.00	2.76

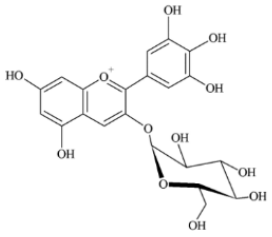
Found ubiquitous in plant families

Anthocyanins

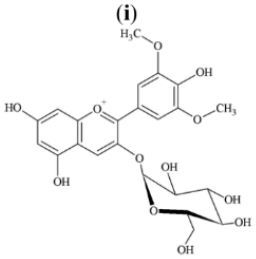
i) Cyanidin-3-*O*-glucoside, **ii)** Delphinidin-3-*O*-glucoside, **iii)** Malvidin-3-*O*-glucoside, **iv)** Pelargonidin-3-*O*-glucoside; food values are for anthocyanidins (without sugar)



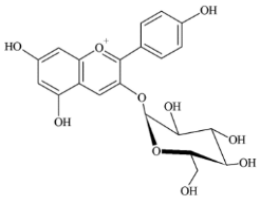
(i)



(ii)



(iii)



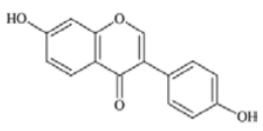
(iv)

Source	i)	ii)	iii)	iv)
Apple	1.27	0.00	0.00	0.00
Blueberries (lowbush)	17.92	34.00	54.00	2.65
Red wine	0.45	2.75	15.29	-
Strawberries	1.63	0.31	0.01	25.69

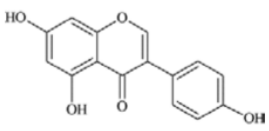
In most pink/purple fruit/vegtables execpt Chenopodiaceae family (beets, quinoa, spiach, Swiss chard, etc.)

Isoflavones

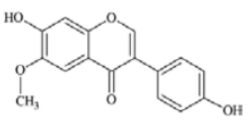
i) Daidzein, **ii)** Genistein, **iii)** Glycitein



(i)



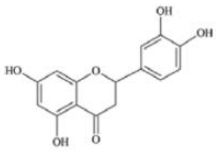
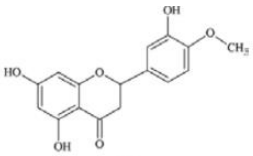
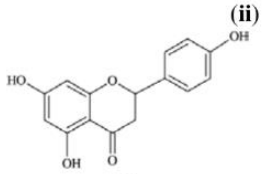
(ii)

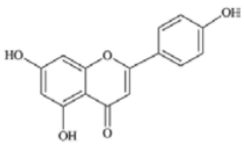
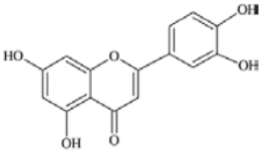


(iii)

Source	i)	ii)	iii)
Tofu (raw)	8.56	12.99	1.98
Tempeh	22.66	36.15	3.82
Soybean (mature seeds, raw – USA)	61.33	86.33	13.33
Peanuts (raw)	0.02	0.24	0.26
Beans (raw)	0.29	0.30	0.00

Within Fabaceae (legume) family, especially the genus Glycine (soy)

Flavanones **i)** Eriodictyol, **ii)** Hesperetin, **iii)** Naringenin	Source	i)	ii)	iii)
 (i)	Grapefruit (juice, white)	0.65	2.35	18.23
 (ii)	Lemon (juice)	4.88	14.47	1.38
 (iii)	Orange (juice)	0.17	20.39	3.27
	Peppermint	30.92	9.52	0.00

Flavones **i)** Apigenin, **ii)** Luteolin	Source	i)	ii)
 (i)	Celery	2.85	1.05
 (ii)	Celery seeds (spice)	83.70	811.41
	Parsley	215.46	1.09
	Green peppers	0.00	4.71
	Peppermint	8.71	11.33
	Common in leafy plant, particularly Apiaceae family		

3. Impact of plant-derived flavonoids on vascular and cognitive health

3.1 Human cardiovascular health: Epidemiological evidence

In the last 30 years, epidemiological evidence has linked intake of flavonoid-rich diets with reduction in cardiovascular risk and protection against age-related cognitive decline.

A study examining 5,133 Finnish men and women (30-69 years) assessing the correlation concerning dietary flavonoid intake and mortality, showed an inverse relationship between flavonoid consumption and coronary mortality, with a stronger association observed in females (Knekt et al., 1996). Similarly, results from an American study showed a 38% reduction in coronary heart disease (CHD) mortality

within post-menopausal women (N = 3,713; 55-69 years) who ingested higher levels of flavonoids within their diet than those whose intake was substantially lower; 4 mg/day for lowest quintile compared with 28.6 mg/day for the highest (Yochum et al., 1999). Keli and colleagues (1996) further reported that the relative risk of CVD (RR; probability of an event occurring) within people that consumed more than 4.7 cups of black tea per day versus < 2.6 cups per day was 0.31 RR, supporting an inverse relationship between flavonoid intake and stroke mortality. Hertog *et al.* (1997) also showed an inverse relationship, as well as a dose-dependent one between flavonoid intake and CHD mortality, with higher flavonoid consumption also predicting a decreased severity of first myocardial infarction, however this finding was only a trend ($p = 0.078$). In agreement with these observations, the Rotterdam study, further reported significant associations with incidents of myocardial infarction and flavonoid intake, in which the largest reductions were seen with fatal events (Geleijnse et al., 2002). The Rotterdam study also reported a stronger inverse relationship between atherosclerosis of the aorta and flavonoid ingestion within females than males, agreeing with Knekt and colleagues' findings (Knekt et al., 1996). Further studies such as the REGARDS study, which included 16,678 participants who ranged in age, race and sex, showed after a 6.0 ± 1.9 year follow up anthocyanidins and proathocyanidins were associated with a lower coronary heart disease incident risk by 27% and 34% retrospectively, regardless of age, sex or race (Goetz et al., 2015). A study conducted by Cassidy and colleagues (2012), demonstrated that out of 69,622 females (30-55 years) over a 14-year period, the participants with the higher flavanone intake had a 19% lower risk of ischemic stroke. A meta-analysis conducted by Huxley and Neil (2003), reported that the people consuming the highest levels of flavonoids had a 20%

lower risk of developing coronary heart disease. Within the studies included in this meta-analysis (n=7), tomatoes, broccoli, onions, apples and red wine appear to be the sources most closely linked with the reduction in disease risk. Another study that supports the association between flavonoid intake and stroke risk was conducted in middle-aged Finnish men (N = 1,950), showing that high flavonoid intake was associated with a decrease risk of ischemic stroke (Mursu et al., 2008). However, this study did not show an association between flavonoid intake and cardiovascular disease mortality, which contradicts the consensus within the majority of the field (Mursu et al., 2008). Their reasoning for this finding was that break away plague from atherosclerosis would block the cerebral arteries first due to their relatively small circumference size in comparison to arteries outside of the cerebrum, although plausible this has not been reported elsewhere within the literature.

Other studies have also reported contradictory findings, Yochum and colleagues saw no association between flavonoid intake and stroke mortality, however, this may be partly due to the low incidence of strokes seen throughout this study and their questionnaire not including baseline stroke history (Yochum et al., 1999). Sesso, and co-workers (2003) conducted a follow up study (~6.9 years) in 38,445 middle aged women and found only a weak trend between cardiovascular disease risk and flavonoid intake, however, this study only focussed on broccoli, apple, tea, onions and tofu, therefore may have missed associations with other food intake. A longitudinal 13-year study assessing the relationship between flavonoid intake and coronary heart disease within females (55-69 years), showed no association between (+)-catechin intake and coronary heart disease mortality. However, they did see a strong inverse association between (+)-catechin plus (-)-epicatechin intake and coronary heart

disease mortality, evidently indicating a role for (-)-epicatechin in cardiovascular disease prevention (Arts et al., 2001).

Although some studies failed to find associations between flavonoids and cardiovascular disease risk, the reasoning behind the failure may be due to; misrepresentation of self-reported dietary intake, misclassification of dietary exposure, lack of cardiovascular events within the study time frame and time frame of studies being too short (average for failed findings ~6 years versus ~9.5 years). Nevertheless, the majority of studies examining this link do see some relation between flavonoids and cardiovascular disease risk.

Reviewing the epidemiological evidence collected so far, most of the literature supports the hypothesis that higher flavonoid intake reduces the incidence of fatal cardiovascular events, with this association appearing to be strongest within post-menopausal females. This may be due to hormonal changes within women who have undergone the menopause, causing an increased risk of atherosclerosis and cardiovascular disease, due to loss of the vascular protective effects imparted by oestrogen (Cagnacci et al., 2012). Flavonoids may help to partly counteract these negative effects by increasing nitric oxide production allowing for greater vasodilation (Duarte et al., 2014), whereas prior to the menopause circulating estrogen allowed for robust vasodilation via similar pathways (Chambliss & Shaul 2002). This may explain the more robust association between flavonoids and CVD seen within post-menopausal women than within other groups. Although most of the data collected does not allow for specific foods and flavonoid sub-groups to be directly linked to these positive cardiovascular effects, some studies give us an insight. Indeed, the meta-analysis conducted by Huxley and Neil (2003) indicate that fruit (apples and tomatoes),

vegetables (broccoli and onions) and red wine may be major contributors to this observed relationship between flavonoids and cardiovascular events. However, this may just be due to these sources contained abundance of flavonoids and the fact they are sources most highly consumed within the studies included within the analysis. Collectively, these studies support the hypothesis that a high flavonoid containing diet can protect against cardiovascular disease risk factors and decrease mortality rates within the general population.

3.2. Human cognitive function: Epidemiological evidence

Epidemiological studies have indicated that high dietary flavonoid intake might further protect against age-related cognitive decline. One study (N = 1,791) conducted within an ageing French cohort (~76 years old) across 8 years observed a significant reduction in the risk of developing dementia with the highest dietary intake of flavonoids. In this study, 32.5% flavonoids derived from fruit accounted for the highest source of intake, vegetables accounted for 19.1%, wine for 16.9% and tea was 16.0% (Commenges et al., 2000). In 2007, Letenneur and colleagues showed that over a 10-year period, non-demented, healthy aged individuals (65+ years; N = 1,640) within the highest quartiles of flavonoid intake scored higher on follow-up cognitive evaluation tests than those within the lower quartiles (shown by Mini-Mental State Examination (MMSE) score in **Fig. 1**).This indicates that flavonoids may be associated with better sustained cognitive ability and therefore might have the potential to protect against

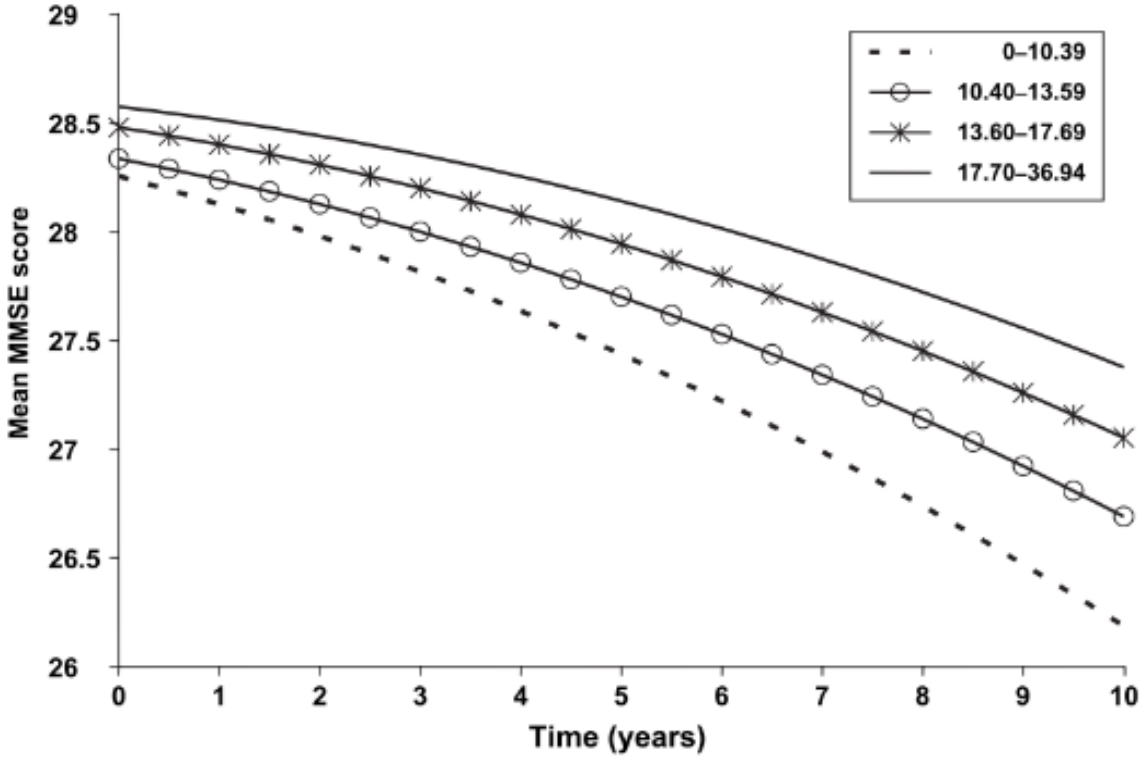


Figure 1. Figure taken from Letenneur *et al.* (2007) showing mean MMSE score for French flavonoid study, 1991-2001.

age-related cognitive decline. In agreement with this, a study conducted within the older Chinese population (66 ± 7 years old) across a 2-year time span examined the influence of regular tea consumption on development of cognitive impairments and dementia (Ng et al., 2008). They utilised the MMSE to assess cognition after baseline tests at 1 and 2 years, with their cohort being divided into groups that consumed differing quantities of tea (low, medium and high), the groups were not divided into types of tea. Of the studied cohort, 38.1% drank tea rarely or not at all, 14.3% drank only English black tea, 13.8% Chinese black/oolong tea only, 13.8% drank green tea only and the rest drank a combination of either two or three of the tea types. Results showed that higher tea consumption of any kind reduced risk of cognitive impairment and decline, with this association being greater for consumers of black or oolong tea;

although it should be noted that these were the most highly consumed teas. Overall, the epidemiological evidence examining the influence of flavonoids on ageing cognitive ability and development of neurological diseases such as dementia shows promise as an effective dietary strategy to delay the on-set on cognitive decline in ageing.

4. Cocoa flavanols

Among flavonoids, flavanols found in cocoa have been one of the most extensively studied in regard to their health effects on the human vasculature. The main flavanols present in cocoa are the monomers (+)-catechin and (-)-epicatechin (5-10%), with their oligomeric and polymeric forms (e.g Procyanidins B1, B2, B5 and C1) comprising close to 90% of the polyphenol content in cocoa (Sánchez-Rebaneda et al., 2003). Cocoa is also made up of smaller quantities of flavan-3-ol, anthocyanins, flavonols and flavones. For example, flavonol glycosides quercetin, quercetin-3-gluronide, quercetin-3-arabinoside, quercetin-3-glucodise (isoquercitrin), quercetin-3-*O*-galactoside (hyperoside), quercetin-3-*O*-glucuronide and flavones, apigenin, apigenin-6-*C*-glucoside, apigenin-8-*C*-glucoside (vitexin), apigenin-6-*C*-glucoside (isovitexin), luteolin and luteolin-7-*O*-glucoside, have all been found within cocoa (Sánchez-Rebaneda et al., 2003; Andres-Lacueva et al., 2008).

4.1 Metabolism and bioavailability of cocoa flavanols

In the last 10 to 15 years, extensive research into the pharmacokinetics and bioavailability of cocoa flavanols in humans have been crucial to establish a link between cocoa flavanol intake and health outcomes.

4.1 a) Metabolism

After ingestion of cocoa, the monomer (-)-epicatechin reaches the small intestine and is exposed to enterocytes where they undergo passive transportation across the cell membrane wall of the small intestine (Cifuentes-Gomez et al., 2015). The (-)-epicatechin then undergoes phase II metabolism whereby actions of sulfotranferases, uridine-5'-diphosphate glucuronosyltransferases and catechol-*O*-methyltransferases, transform the (-)-epicatechin into sulfate, glucuronide and methylated sulfate/glucuronide metabolites (Cifuentes-Gomez et al., 2015). Within the liver un-metabolized (-)-epicatechin undergoes glucuronidation, sulfation and O-methylation. At the level of the large intestine the still intact procyanidins and flavanol monomers experience C-ring fission caused by the microbiome within the gut, forming smaller phenolic acids and 5(hydroxyphenyl)-γ-valerolactones, which are then absorbed within the liver (Cifuentes-Gomez et al., 2015).

Several studies have observed that ingestion of flavanol-rich cocoa leads to the appearance of (-)-epicatechin or derivatives of (-)-epicatechin within the blood (Engler et al., 2004; Monahan et al., 2011). Ottaviani and co-workers (2012) showed that (-)-epicatechin consumption causes three main metabolites to be detected within the blood; (-)-epicatechin-3'-β-D-glucuronide, (-)-epicatechin-3'-sulfate, and a 3'-O-methyl-(-)-epicatechin-5/7-sulfate (Ottaviani et al., 2012). They also found that (-)-epicatechin-3'-β-D-glucuronide is the principal O-glucuronidated metabolic derivative that is found within the blood after (-)-epicatechin intake (Ottaviani et al., 2012). Baba and colleagues (2000) showed that after consumption of 35g of cocoa powder (2.73 g total polyphenols, 760 μ mol (-)-epicatechin, 214 μ mol (+)-catechin, 159 μ mol procyanidin B2), 24.1 ± 8.1% of total (-)-epicatechin and ~80% of total metabolites

were excreted within 8-hours post-ingestion. Elimination half-life of catechins have been shown to be between 3-5 hours, implying that catechins are rapidly absorbed and excreted (Baba et al., 2000).

4.1 b) Bioavailability

In addition to demonstrating that cocoa flavanols are absorbed, the aforementioned have also shown it has relatively low bioavailability due to short half-life, fast excretion and low Cmax (peak concentration within the blood) within the plasma (Manach et al., 2005). Many aspects affect the bioavailability of flavonoids, especially the complexity of the compound structure and the molecular weight, with more complex and larger compounds having lower bioavailability (Hollman, 2004).

Individual variability is another important factor to consider when assessing flavanol bioavailability within humans. Individual variation is partly due to genetic polymorphism in metabolic enzymes that are involved within flavanol metabolism; especially within phase II enzymes, namely sulfotransferase, uridine-5′-diphosphate glucuronosyltransferases and catechol-O- methyltransferases (Cifuentes-Gomez et al., 2015). Cifuentes-Gomez and colleagues (2015) showed that men possessing a low-activity genotype, COMT-AA, excreted lower levels of tea flavonoid metabolites in comparison to other genotypes, namely COMT-GG and COMT-AG. However, there is little information available currently on genotype-effect of other enzymes within phase II metabolism of flavanols, for example for sulfotransferase, uridine-5′-diphosphate glucuronosyltransferases.

Rodriquez-Mateos *et al.* (2015) conducted a study assessing variability of absorption, metabolism and excretion of cocoa flavanols within younger and older healthy adults

to evaluate the differences that occur with bioavailability with ageing. They found significant differences between the two cohorts in plasma levels of sulphated and glucuronidated epicatechin metabolites with the ingestion of a high flavanol cocoa drink containing 800 mg total flavanols (106 mg (-)-epicatechin). Within the older participants the area under the curve$_{(0-6h)}$ (AUC) of (−)-epicatechin-3'-β-D -glucuronide (E3'G) was significantly higher, while AUC$_{(0-6h)}$ of methyl-sulfated metabolites were lower compared to the younger cohort. No difference was seen within the urine for amounts of sulphated and glucuronidated metabolites for (-)-epicatechin and acetaminophen. Rodriquez-Mateos and colleagues suggested that these findings may be explained by differences in elimination. Supporting this theory, the older participants' half-life, apparent clearance and renal clearance of E3'G was lower than that observed within the younger participants. Therefore, the known decline of renal function with age (Weinstein & Anderson, 2010) may influence the plasma levels of sulphated and glucronidated metabolites. Alongside this Rodriquez-Mateos and colleagues found that even though there were no differences in structurally related (-)-epicatechin metabolites (SREM), there was a decrease in γ-valerolactone metabolites excreted within the urine, which are degradation products that are seen within the plasma and the urine with high flavanol intake. Therefore, there may be changes within the gut microflora with age as capacity to yield γ-VL metabolites are lessened.

Other aspects can affect bioavailability, such as the matrix that the compound is delivered in. In this respect the possible influence that other compounds being ingested alongside cocoa flavanols exert on metabolism must be considered. For example, cocoa is most commonly ingested in the form of chocolate, which contains sugars and

fats (such as dairy), therefore we need to assess the ability of these added elements to impact, either positively or negatively on flavanol absorption and metabolism. Njike and co-workers (2011) demonstrated that cocoa with the addition of sugar lessened the observed increase in flow mediated dilation (FMD), where sugar-free cocoa increased FMD by 2.4% compared to sugared cocoa increasing FMD by 1.5%. Although the sugared cocoa still improved FMD, this study demonstrates the augmented beneficial effect of consuming cocoa without the addition of sugar. Serafini and Crozier (2003) examined the impact of ingesting high flavanol dark chocolate with or without milk, or milk chocolate with the equivalent total flavanols, on the plasma (-)-epicatechin concentration over a 4-hour period. They found that the addition of milk, either during the manufacturing process (milk chocolate) or during ingestion, inhibited the uptake of (-)-epicatechin into the blood stream. Serafini and Crozier proposed that milk proteins may form bonds with the cocoa flavanols, inhibiting absorption and therefore reducing cocoa flavanols ability to impart the positive physiological effects. Mullen and co-workers (2009) reported that ingestion of cocoa powder with milk (10g cocoa powder; 22.3 ± 0.3 µ mol catechin and 23.0 ± 0.4 µ mol epicatechin) did not have any significant effect on Tmax (time at which Cmax is reached) or Cmax values and had no effect on catechin detection within the plasma. However, milk did cause significantly lower flavan-3-ol metabolite concentrations to be excreted within the urine, particularly within the first few hours after intake (0-2 hours and 2-5 hours post); specifically, 10.5 ± 1.1% vs 18.3 ± 1.9% of intake metabolites were excreted within the first 24-hours for milk and water cocoa drinks respectively (Mullen et al., 2009). Thus, Mullen and colleagues (2009) demonstrated that milk has a clear impact on absorption

of cocoa flav-3-ols, as indicated by content within the urine, although had no effect within the plasma concentrations.

Alternatively, studies have shown that intake of flavanol rich cocoa alongside other compounds can enhance (-)-epicatechin absorption and therefore make cocoa flavanols more bioavailable. Sansone and colleagues (2017) showed that ingestion of methylxanthines simultaneously with high flavanol cocoa increases the amount of (-)-epicatechin detected within plasma above and beyond ingestion of cocoa flavanols alone (5 ± 4-fold versus 12 ± 4-fold increase). Moreover, this elevated (-)-epicatechin content was associated with enhanced positive physiological effects, indicating a synergistic mode of action. Although not in humans, theobromine, the most abundant methylxanthine within cocoa, has been observed to enhance absorption of cocoa flavanols within Wister rats (Yamamoto et al., 2014). Yamamoto and colleagues reported a dose response relationship to intake of cocoa flavanols with increasing amounts of theobromine, showing that with the same amounts of flavanols the higher quantities of theobromine caused more (-)-epicatechin and 3'-*O*-Methyl-epicatechin to be detected within the plasma (Yamaoto et al., 2014).

4.1 c) Pharmacokinetics

Manach and colleagues (2005) reviewed data from human flavonoid bioavailability studies and found that cocoa intake interventions produced a wide range of plasma concentrations of catechins that were possibly due to variation in dose, but also likely due to methodological differences in catechin-derived metabolite detection within the plasma. However, these studies reported extremely consistent Tmax of 2-hours (Richelle et al., 1999; Baba et al., 2000; Rein et al., 2000; Wang et al., 2000; Holt et

al., 2002; Schramm et al., 2003), urinary excretion at 25.3% and 26.8%, and elimination half-life ranging from 1.7-3 hours (although urinary excretion and elimination half-life data were only reported for 2 studies; Manach et al., 2005). This pharmacokinetic data gives us the information to be able to study the acute impact of cocoa flavanols, specifically (-)-epicatechin on human health.

Collectively, these aforementioned studies show that there is an extensive list of factors that can affect the bioavailability and pharmacokinetics of cocoa flavanols, including age, genetics and the matrix in which cocoa is taken. Of these, the matrix is the main factor researchers can manipulate, therefore optimizing the delivery of these compounds via addition of methylxanthines and avoidance of milk and sugar, is key for conducting cocoa flavanol research. Controlling for individual differences via good standardisation of participants recruited for studies may also allow for more reliable data on the influence cocoa flavanols have within specific groups.

4.2. Impact of cocoa flavanols on human peripheral vascular function

Flavanols have been shown to modify important markers of endothelial function, particularly FMD of the brachial artery, both with acute and longer-term intake in healthy populations, as well as in populations at risk (e.g. Fisher et al., 2012; Heiss et al., 2015) and with disease (e.g. Heiss et al., 2005; Hermann et al., 2006; Farouque et al., 2006; Balzer et al., 2008; Berry et al., 2010) (summarized in **Table 2**).

FMD of the brachial artery is a key biomarker of endothelial function and has been shown to have clinical significance, as it is closely linked with cardiovascular health and disease risk (Vita, 2005). A Framingham Heart Study showed strong associations between decreased FMD and cardiovascular disease risk factors, especially

increasing age, high BMI, elevated systolic blood pressure and smoking cigarettes (Benjamin et al., 2004). Other studies have identified that utilising FMD to measure endothelial dysfunction predicts the occurrence of cardiovascular events such as; myocardial infarction, unstable angina, ischemic stroke and cardiac death. Even once known risk factors are controlled for (Widlansky et al., 2003). Witte and colleagues (2005) showed that within low risk populations for a percentage increase in Framingham risk, FMD decreased by 1.42%, however, this association was not seen within medium and high-risk populations. A meta-analysis conducted by Inaba *et al.* (2010), including data from 5,547 participants revealed that for a 1% decrease in FMD an associated 8% increase in risk of future cardiovascular events was observed. Therefore, this research technique is of great importance when analysis of vascular function and cardiovascular disease risk is warranted. As such, the fact that cocoa flavanols have been shown to increase FMD supports the notion that the intake of flavanol-rich foods or beverages have the potential to modify cardiovascular disease risk.

The initial work in this area investigated the time course of the acute influence of flavanol-rich cocoa (FRC) ingestion on FMD. For example, Taubert and colleagues (2003) measured the FMD response at 0-, 2-, 4- and 6-hours post-ingestion and found that the maximal increase in FMD was at 2-hours (%FMD; 0 h ~2.5%, 2 h ~5.2%, 4 h ~2.9% & 6 h ~2.7%), a finding that has been replicated numerous times since (Hermann et al., 2006; Schroeter et al., 2006; Heiss et al., 2007; Vlachopoulos et al., 2005). Taubert and colleagues then used this acute time course finding to construct a double-blinded crossover study that compared a single dose flavanol-poor cocoa (FPC) drink to a FRC drink, and its effects on FMD, endothelium-independent dilation

and circulating nitrate and nitrite levels (nitric oxide metabolites, collectively known as RNO). The results of this study showed a correlation between increased RNO and a higher flavanol intake, as well as improvements in FMD response while there was no effect on endothelium-independent dilation. These results indicated that a single dose of FRC could elicit improvements in endothelial-dependent but not independent dilation, and that these responses might be driven by increased nitric oxide (NO) bioavailability. Cooper and colleagues (2008) stated that although this finding has been shown in several studies, such previous work also illustrates the variation in Cmax around this 2-hour time point, since it is likely there is individual variability in absorption of the compound that may confound potential correlations in bioavailability and bio-efficacy. Copper and colleagues therefore suggested that studies taking plasma samples around this 2-hour mark are merely only a check for compliance rather than a measure for bioavailability. Regardless of this issue, studies investigating a main outcome of FMD in response to FRC ingestion have repeatedly shown a peak around 2-hours (Hermann et al., 2006; Schroeter et al., 2006; Heiss et al., 2007; Vlachopoulos et al., 2005), although there will undoubtedly be individual variability in response.

To date, 14 studies have investigated the acute effects of cocoa ingestion on FMD in the form of a cocoa powder drink or bar at 2-hours post-ingestion. In order to summarise this body of work, the findings from these studies have been pooled, split and subdivided into age and health categories to calculate averages and ranges for each, allowing us to get a clearer picture of the influence of cocoa flavanols on brachial FMD within these populations (see **Fig. 2**).

FMD data were compiled from a total of 418 healthy young participants; resting FMDs were on average 6.52% (ranging from 4.30% to 10.70%), whilst 2-hours after ingestion

of high flavanol cocoa FMD increased to 9.07% (ranging from 5.90% to 11.70%). The total amounts of flavanols used in these interventions ranged from 450 mg to 1000+ mg, which likely explain in part the range of FMD changes observed. Studies with interventions including milk, sugar or fat added to the cocoa were not included due to known issues regarding the bioavailability of the flavanols as discussed above (**4.1**). The average increases in FMD from baseline to 2-hours post-ingestion was 2.55%, with the median being 2.00% and range from 0.90% to 5.70%.

Furthermore, three studies testing healthy older participants were compiled and analysed (total participants 62); as expected they showed a lower resting FMD in comparison to the young healthy cohorts, where the mean response was 4.25% (range: 3.60% to 4.90%). After ingestion of cocoa mean FMD increased to 6.00% (range: 5.90% to 6.10%). The improvement seen within the older cohorts were similar

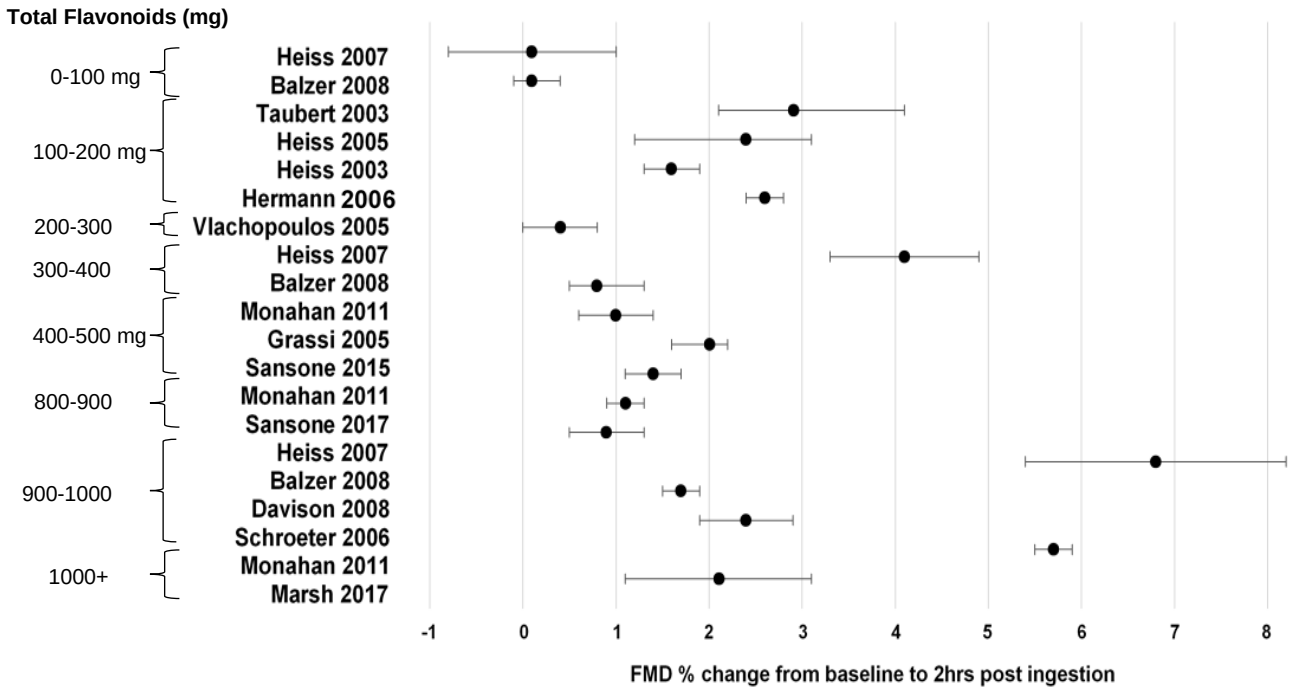


Figure 2. Summary of studies showing means and standard deviations of 14 acute (2hr) studies investigating the effect of cocoa on FMD (%)

as the improvements observed from the young, although the younger participants on average increased their FMD more than the older participants: 2.55% versus 1.75% increase, respectively. Studies have also investigated the impact of flavanol intake in unhealthy and diseased populations. For example, in overweight/obese participants (2 studies, N=65), an average baseline FMD of 4.9% (range: 3.40% to 6.40%) increased to 7.5% (range: 6.20% to 8.80%) post-acute cocoa ingestion. In smokers (3 studies, N=47) a mean baseline FMD of 4.2% (range: 3.70% to 4.50%) increased to 6.67% (range: 6.10% to 7.00%) post-cocoa ingestion. While in patients with hypertension, diabetes or carotid artery disease (5 studies, N=150), baseline FMD of 4.62% (range: 5.50% to 8.90%) post-ingestion.

Interestingly, no matter what participant pool cocoa is given to and therefore what the baseline FMD is, the average increase in FMD post-ingestion is around ~2%. However, there was a larger range within the younger healthy cohort regarding percentage increase in FMD from baseline to post-ingestion as opposed to any other group; range of 4.80% versus 0.75% (older healthy), 1.90% (disease states), 1.30% (overweight) and 0.20% (smokers). It is noted that there were more studies compiled using younger healthy participants and this may be a part of the reason for this large range. Nevertheless, this may also be due to physiological differences between the cohorts, with populations with disease states having more similar pathologies than the physiological variation in younger groups. Alongside this, the age range within the 'younger' studies spans 23 to 57 years old. It is known that vascular physiology alters throughout life, with stiffening of arteries and increases in plaque deposits (Seals et al., 2009; Xu et al., 2017), showing changes in vascular structure and function right through ones' life (Mitchell et al., 2004). Another factor that may influence the FMD

outcome is fitness, which was not reported in the aforementioned studies. Given the likely variation in fitness of individuals within these studies, and the well-known cause of vascular modification (Wray et al., 2006; Seals et al., 2009; Thijssen et al., 2010), this could also explain the wider range in FMD at baseline and in response to flavanols for this studied young cohort.

It is also possible that responsiveness to the cocoa treatment may also be varied, as participants normal dietary intake of flavanols may differ and impact on this acute response to high cocoa flavanols. Alongside this, studies vary in the amount of total flavanols within their interventions, which may have affected the results as flavanols impact on vascular function has been shown to be dose-dependent in nature (Heiss et al., 2007; Balzer et al., 2008; Davison et al., 2010; Monahan et al., 2011; Rodriquez-Mateos et al., 2015; Vlachojannis et al., 2016). To explore this further, **Figure 2** includes the dose-response from the 14 studies complied here (Taubert et al., 2003; Heiss et al., 2003; Grassi et al., 2005a; Heiss et al., 2005; Vlachopoulos et al., 2005; Hermann et al., 2006; Schroeter et al., 2006; Heiss et al., 2007; Balzer et al., 2008; Davison et al., 2008; Monahan et al., 2011; Sansone et al., 2015; Sansone et al., 2017; Marsh et al., 2017), with total flavanols versus %FMD response to flavanols at 2 h post-intake. However, it should be taken into account that the participants included within this graph vary from young healthy to older unhealthy, which likely explain some of the variability within the studies (represented by the wide standard error bars). Alongside this there are disparities in number of studies included in each point of the graph due to lack of interventions with certain flavanol amounts, this is broken down into the following; baseline (N=14), 0-100 mg (N=2), 100-200 mg (N=4), 200-300 mg

(N=1), 300-400 mg (N=2), 400-500 mg (N=3), no studies reported for 500-800 mg, 800-900 mg (N=3), 900-1000 mg (N=4) and 1000+ mg (N=2).

In summary, although there is variation in FMD outcomes within the studies analysed above, the current evidence we have to date indicates that cocoa ingestion can improve vascular function across healthy and diseased groups. Furthermore, this improvement seems to be around a 2% increase in the FMD response, no matter the cohort studied. Altogether this highlights that flavanol intake within the diet might be an important factor to maintain vascular health throughout the lifespan and reduce the risk of CVD.

4.3 Cocoa derived flavanols and cerebrovascular function: acute interventions

From the FMD studies reviewed above there is sufficient evidence to suggest that flavanol-rich cocoa improves vascular responsiveness, enhancing the ability for vessels to dilate and decreasing cardiovascular disease risk. However, how this translates to the cerebrovasculature is much less known. Nevertheless, given the wealth of data supporting peripheral vascular effects, flavanol-rich cocoa certainly has the potential to exert similar effects within the brain vasculature. As such, these compounds have the potential to prevent cerebrovascular disease and decrease stroke risk, as well as other neurological diseases such as Alzheimer's.

Cerebrovascular reactivity is an important experimental measure of cerebrovascular function and is defined as the degree of vasodilation or vasoconstriction in response to a vasoactive task or agent (Halani et al., 2015). Utilising a vasodilating (i.e. breathing elevated levels of CO_2) or a vasoconstricting (i.e. hyperventilation to lower levels of CO_2) stimulus and recording the responsiveness of the cerebral arteries via imaging

techniques such as transcranial Doppler (TCD) or functional magnetic resonance imaging (fMRI), allows us to determine the reactivity of the vessels (Ito et al., 2003; Halani et al., 2015). Cerebrovascular reactivity is an important metric of cerebrovascular health and has been shown to be an indicator of vascular reserve and auto-regulatory efficiency (Ito et al., 2003). Indeed, an impairment of cerebrovascular reactivity has been associated with age-related cognitive decline, dementia and stroke (Halani et al., 2015).

Presently, there are a limited number of studies that have examined the impact of cocoa flavanols on cerebral blood flow reactivity within humans, irrespective of the measure of the cerebrovascular function. Sorond and colleagues (2008) investigated the effect of ingesting a high flavanol cocoa drink (451.1 mg total flavanols) versus a low flavanol cocoa drink (18.2 mg total flavanols; all other micro and macronutrients matched) on cerebral blood flow velocity via transcranial doppler (TCD) at 2-, 4-, 6- and 8-hours post-ingestion, within older participants (72 ± 6 years). They found that 2- and 4-hours post-ingestion of the flavanol rich drink, middle cerebral artery velocity (MCAv) was reduced non-significantly and increased back to baseline levels at 6- and 8-hours, there was no effect on cerebrovascular resistance (CVR) in this acute setting (Sorond et al., 2008). Based on the effects shown with FMD-based studies, the dosage used within this study was high enough to elicit an increase in brachial vascular function. Therefore, the decrease in MCAv seen at 2-hours post likely timed with the peak for systemic response to cocoa. Sorond and colleagues attributed the decrease to caffeine, however, they do not report the acute results for the flavanol poor drink and given the matched micronutrients in both drinks the caffeine-effect should be expected with both drinks.

More recently, Marsh and colleagues (2017) conducted a study analysing both systemic and cerebral arterial function to chocolate intake, within the same cohort. Specifically, they investigated the influence of dark (DC; 80% cocoa, total flavonoids 3600 mg/kg, (-)-epicatechin 587.1 µg/g), milk (MC; 35% cocoa, total flavonoids 980 mg/kg, (-)-epicatechin 288.4 µg/g), and white chocolate bars (WC; 0% cocoa, total flavonoids 370 mg/kg, (-)-epicatechin undetected) on brachial and cerebrovascular function in postmenopausal women (57.3 ± 5.3 years). They reported that 1 hour 20 minutes post-ingestion FMD increased for DC as expected, while no change was seen for MC or WC. MCAv and cerebrovascular conductance decreased for both MC and DC in response to cognition tasks, but no change was reported for WC. As with the Sorond *et al.*, study, the flavonoid and (-)-epicatechin concentrations were high enough in both DC and MC that an increase in %FMD would be expected, but an increase was only seen within DC, which may be due to the reported inhibitory effects of milk on flavonoid absorption (Serafini & Crozier, 2003; Mullen et al., 2009). In disagreement with Sorond and colleagues (2008), Marsh saw a significant decrease in MCAv and cerebrovascular conductance within DC and MC in response to a cognition test. The authors concluded that their findings showed an increased cerebral efficiency as their data interfered continual performance in the presence of lower blood flow (velocity) as the outcomes of the cognition tests did not change pre to post.

Other studies have looked at cerebral haemodynamics, blood flow and perfusion utilizing functional near-infrared spectroscopy (NIRS) and fMRI to assess blood oxygenation level-dependent (BOLD) signals and arterial spin labelling (ASL) (Francis et al., 2006; Lamport et al., 2015; Decroix et al., 2018). For example, Francis *et al.* (2006) reported that a flavanol rich cocoa (450mg total flavanols) increased ASL-fMRI

measures of cerebral blood flow to the grey matter at 2-hours post-ingestion and returned to baseline by 6-hours. Another study by Lamport and colleagues investigated the effect of matched flavanol rich and low cocoa drinks (total flavanols; 494 mg and 23 mg respectively) on cerebral perfusion in healthy older adults using ASL-fMRI. The authors found that high flavanol cocoa intake resulted in an increase in regional cerebral blood flow, specifically within the anterior cingulate cortex and regions of the parietal lobe (Lamport et al., 2015). More recently, Decroix and colleagues (2018) explored the influence of flavanol rich cocoa (530 mg total flavanols & 100 mg (-)-epicatechin) on cerebral haemodynamics using NIRS, within a young healthy cohort. Results from this study showed that cocoa had no effect on neural activity, measured by electroencephalogram, but cocoa did enhance the haemodynamic response (oxygenated haemoglobin was greater) in the right prefrontal cortex during cognitive effort (risk decision making, visual tracking, complex scanning and spatial orientation) in both hypoxia and normoxia conditions. These results showed for the first time that even under physiological stress cocoa flavanols can improve cerebral blood flow.

4.4. Impact of acute vascular changes on long-term adaptations to flavanol intake

It is of vital importance that we can assess whether these positive acute changes in vascular function translate to chronic situations as protective effects against diseases will only occur when these changes are seen long-term. As mentioned earlier, epidemiological studies indicate that long-term protective effects of high flavonoid containing diets seem to have positive effects of various health outcomes. Studies investigating both acute and chronic effects of cocoa ingestion on peripheral vascular

function are summarized within **Table 2** and cognitive and cerebrovascular function within **Table 3**.

Davison and colleagues (2008) conducted a study spanning 12-weeks with 2-hour acute FMD measurements done at baseline, week 6 and week 12 after daily ingestion of a flavanol rich cocoa (902 mg total flavanols) or a low flavanol cocoa (36 mg total flavanols).They found that acute ingestion increased FMD from baseline to 2-hours, and chronic daily intake increased baseline FMD significantly at week 6 and week 12, with no significant difference between week 6 and 12. The authors also found that diastolic and mean arterial blood pressure were both significantly reduced at week 6 and week 12, with no changes in body weight, systolic blood pressure or heart rate. This study demonstrates that sustained intake of high flavanol cocoa powder over the course of weeks improves baseline FMD and therefore decreases cardiovascular disease risk. The authors further demonstrated that the positive effects of chronic cocoa ingestion may plateau past 6-weeks of daily intake.

Another study looked at cocoa ingestion (total flavanols; 963 mg per day vs 75 mg per day) within type 2 diabetics over 30 days with an acute 2-hour single dose measurement done on day 0, 8 and 30 (Blazer et al., 2008). Blazer and colleagues (2008) saw that baseline FMD increased from day 0 to day 8 and again on day 30. This acute increase in FMD at 2-hours post-ingestion was seen during every visit showing an ‘acute-on-chronic’ effect (Blazer et al., 2008), consistent with Davison and colleagues’ findings (Davison et al., 2008). Thus, even with improvements in baseline FMD with chronic cocoa consumption, acutely ingesting cocoa can cause a further increase in FMD.

More recently, Heiss and colleagues (2015) investigated the effect of cocoa within both an acute (1-hour) and chronic (14 days) setting, in both young and older healthy males. They found that both cohorts' %FMD improved acutely and chronically to a similar absolute extent, with flavanol bioavailability being comparable between the two cohorts. This study therefore shows that these chronic adaptations are manifested within older populations to a similar extent as they are within younger (data shown in **Table 2**). The authors further observed a decrease in blood pressure within the older participants and a decrease in PWV within both cohorts between day 1 and 14. Such observations indicate an attenuation in age-related increases in blood pressure and arterial stiffness with daily high flavanol cocoa intake, findings which are close to what is seen within drug studies such as ACE inhibitors (O'Rourke & Hashimoto, 2007; Heiss et al., 2015). Further, this study by Heiss and colleagues demonstrated that the chronic FMD improvements seen within the two previous studies discussed (Davison et al., 2008; Blazer et al., 2008) can start to be observed at 14 days of daily cocoa consumption within both young and older populations.

In regard to cerebrovascular function, Sorond and co-workers (2008) evaluated the changes in cerebral blood flow both acutely (2, 4, 6- and 8-hours post) and chronically (7 and 14 days) to an intake of a single dose of 450 mg versus 18.2 mg total cocoa flavanols, or daily dose of 900 mg versus 36.4 mg total cocoa flavanols. After 14-days of daily high cocoa flavanol consumption, peak cerebral blood flow velocity was significantly elevated in response to 5% CO_2 (a stimulus to assess cerebrovascular reactivity), however this was only observed acutely after 8-hours post-ingestion of the flavanol drink with no changes seen at 2-, 4- and 6-hours, and there was no difference in baseline cerebral blood flow velocity on day 7 or 14. They also found that after 7 or

14-days of daily high flavanol consumption there was no effect on blood pressure, cerebrovascular reactivity or cerebrovascular resistance. These results are interesting as chronic intervention studies conducted with flavanol rich drinks of similar duration (7 to 14 days) do report significant decreases in blood pressure and significant increases in FMD (Grassi et al., 2005a; Grassi et al., 2008; Heiss et al., 2015). Therefore, the cerebrovascular results within this Sorond *et al.* study may allude to possible differences in both the acute and chronic time frames needed to see changes in cerebrovascular function in comparison with the time frames it takes to see changes in peripheral vascular function.

A study conducted by Brickman *et al.* (2014) examined the impact of cocoa flavanols (total flavanols 900 mg & 138 mg (-)-epicatechin vs total flavanols 10 mg & <2 mg (-)-epicatechin per day) on cerebral blood volume (CBV) within the dentate gyrus (DG) in older adults over a course of 12-weeks. They found that consumption of the high flavanol cocoa for 12-weeks significantly increased CBV within the DG and the subiculum downstream of the hippocampus. This study illustrated that chronic consumption of high flavanol cocoa can improve blood perfusion within specific regions of the brain within older populations.

The mechanistic reasons behind these improvements in endothelial function both acutely and chronically may be different, as alluded to by the acute-on-chronic outcomes seen within Blazer *et al.* (2008) and Heiss *et al.* (2015). As discussed above (Section **4.1**) it's been observed that increased FMD correlated with (-)-epicatechin plasma levels in acute settings (Monahan et al., 2011). This acute improvement in brachial vascular function has been explained by reduced superoxide anion-mediated loss of nitic oxide (NO) and oxidative stress via inhibition of nicotinamide adenine

dinucleotide phosphate (NADPH) oxidase activity, causing an increased circulating bioactive NO pool (Schewe et al., 2008). This increased NO bioavailability allows improvements in endothelium-dependent vasodilation, as the ability of the endothelium to vasodilate is dependent on NO availability. However, this cannot be the same mechanistic pathway that allows sustained improvements in endothelial function chronically as once (-)-epicatechin has been cleared from the blood the increased circulating NO decreases expediently back to baseline levels. A review by Aprotosoaie and colleagues (2016) described the mechanistic changes with repeated cocoa flavanol ingestion, and they suggested that the effect may be mediated via multiple pathways including changes in gene expression and protein synthesis of endothelial nitric oxide synthase (eNOS), which allows for improvements in NO bioactivity and bioavailability. Sustained intake of flavanol rich cocoa has also been linked with other mechanisms; such as: suppression of proinflammatory cytokines IL-1β, IL-2 and IL-8 production; inhibition of ET-1 release; lessened activity of xanthine oxidase and myeloperoxidase; modulation of leukotrienes and PGI2; reduction in vascular damage biomarkers such as monocyte CD62L expression; formation of elevated endothelial microparticles, and mobilization of circulating endothelial progenitor cells (EPCs; Heiss et al., 2010; McFarlin et al., 2015; Kerimi et al., 2015; Aprotosoaie et al., 2016). These mechanisms allow for increased NO bioavailability and inhibition of endothelial vasoconstriction, allowing for reduction in blood pressure and improvements in FMD.

4.5 Impact of cocoa flavanols on human cognitive function

Randomized controlled human trials investigating cocoa flavanols influence on cognition, both acutely and chronically, have generally supported the epidemiological evidence, although there is still very limited data available (**Table 2).** Scholey and

colleagues (2010) conducted a study looking at an ingestion of either low (46 mg total flavanols), medium (520 mg total flavanols) or high (994 mg total flavanols) flavanol cocoa drinks 1-hour post-ingestion on state-trait anxiety inventory (STAI) scale and a cognitive demand battery, which included: two subtraction tasks (Serial Threes and Serial Sevens), rapid visual information processing (RVIP) tasks and a 'mental fatigue' scale. The authors found that this acute consumption of cocoa flavanols improved cognitive performance within all tasks and reduce 'mental fatigue' during high effort cognitive processing.

Brickman and colleagues (2014) utilised a task called the Modified-Benton (ModBent) that is specifically sensitive to DG function and localised to the hippocampal circuit, areas of the brain that are involved in learning, memory and is severely affected by ageing (Small et al., 1999; Yassa et al., 2011). The authors demonstrated an improved reaction time by an average of 630 milliseconds within the ModBent tasks for participants taking the flavanol rich drink, with this change in reaction time being equivalent to around 30 years of ageing. Further, this reduced reaction time was accompanied by an inverse relationship to changes in CBV within the DG and improved DG function; i.e., faster reaction time was associated with increased CBV (Brickman et al., 2014).

Mastroiacovo and colleagues (2015) examined the effect of high, medium and low cocoa flavanol drinks (total flavanols daily; 993 mg vs 520 mg vs 48 mg respectively) on cognitive function across 8-weeks using MMSE, the Verbal Fluency Test (VFT) and the Trail Making Test (TMT) A and B. They found no change in MMSE score in any of the interventions ($p = 0.52$), but they did find a significant reduction in reaction time within the medium and high flavanol groups for both TMT A and B. Improvements in

the VFT scores were seen within all groups, and these improvements in performance were was seen in a dose-dependent manner. Specifically, a measure of overall cognitive function, a composite overall ʒ score, showed improvements within the two higher flavanol drinks but not the low flavanol drink. Interestingly, improvements were seen in all the cognitive tests except the MMSE score, which is a finding consistent with others (Desideri et al., 2012; Sorond et al., 2013). Recently, Haskell-Ramsay (2018) has proposed that the MMSE may not be sensitive enough to be able to measure small alterations in cognitive ability within a short intervention time frame, as compared to the improvements that are noted within epidemiological studies (Commenges et al., 2000; Ng et al., 2008).

Overall, the current literature highlights that cocoa flavanols improve cognitive ability and protects against age-related cognitive decline, however these effects are more likely to be seen with chronic interventions rather than following an acute dose. Although the mechanisms by which this enhanced cognitive function occurs is not well understood, improvements in cerebrovascular function (e.g. resting flow and/or reactivity) might be one of the mechanisms underlying these improvements.

5. Conclusions and Future Directions

Current work evaluating flavanols' influence over vascular and cognitive function in humans, seems promising, but additional acute and long-term intervention studies, specifically focussing on cerebrovascular function are needed. The impact of cocoa flavanols on peripheral vascular function and cardiovascular mortality has been well researched thus far, however how these peripheral effects translate to the cerebrovasculature is unclear. Research is needed to better establish how flavanol

rich cocoa effects brain health, especially how cerebrovascular changes might relate to the well-established improvements in peripheral vascular function.

Table 2. Human acute peripheral vascular function intervention trails with cocoa

Intervention	Flavonoids' Daily Dose	Duration of the Intervention	Subjects	Vascular Measures	Blood Pressure	Other Measures	Reference
Cocoa Drink	176mg flavan-3-ols vs <10mg flavan-3-ols	Acute 2hrs (crossover - 2 days)	20 adults (41 ± 14 yrs.) with at least 1 cardiovascular risk factor	↑FMD (3.4% - 6.3%)	-	↑plasma RNO levels (22-36)	Taubert *et al.* (2003)
Cocoa Drink	821mg flavanols vs <41mg flavanols	5 days + Acute 90min	27 healthy adults (44 ± 3 yrs.)	↑ PWA after 4 days & after 90 mins single dose day 5; ↔ PWA with L-NAME on day 0; ↓ PWA with L-NAME on day 4	↔ BP to cocoa; ↑ SBP sig. & ↓ DBP non-sig. with L-NAME day 0; ↑ BP with L-NAME after 4 days	-	Fisher *et al.* (2003)
Dark chocolate bars	High-flavanol dark chocolate bars (213 mg total procyanidins & 46 mg epicatechin) vs Low-flavanol dark chocolate bar (trace total procyanidins & trace epicatechin)	2-week intervention	21 healthy adults (33 ± 3 yrs.)	↑FMD after 2-weeks with HF bar (10.7% - 12%) but not LF bar (10.2% - 9.8%)	-	↑ Plasma epicatechin concentration (HF bar); ↔ LDL oxidation, 8-isoprostanes or ORAC	Engler *et al.* (2004)
Cocoa Drink	176mg flavanols vs <11.5mg flavanols	Acute 2hrs (crossover - 2 days)	11 healthy smokers (31 ± 1 yrs.)	↑FMD 2 hrs (4.5% - 6.9%)	-	↑RXNO	Heiss *et al.* (2005)
Chocolate bars	100g dark chocolate (~500mg polyphenols) vs 90g white chocolate (~0mg polyphenols)	15-day intervention (crossover, 7-day cocoa free run in + wash out periods)	20 hypertensives (44 ± 8 yrs.)	↑ FMD with DC (7.4% - 8.9%)	↓ SBP, DBP & ABPM after 15 days with DC not WC	↓HOMA-IR; ↑QUICKI & ISI; ↓Insulin levels (fasted)	Grassi *et al.* (2005a)

Chocolate bars	100g dark chocolate (~500mg polyphenols) vs 90g white chocolate (~0mg polyphenols)	15-day intervention (crossover, 7-day cocoa free run in + wash out periods)	15 healthy adults (34 ± 8 yrs.)	-	↓ SBP for DC not WC after 15 days	↓HOMA-IR; ↑QUICKI	Grassi *et al.* (2005b)
Dark chocolate bars	100 g procyanidin-rich dark chocolate (74% cocoa, 2.62 g procyanidins) & 250 ml of water vs sham-eating & 250 ml of water	Acute 180 mins (30, 60, 90, 120, 150- & 180-mins post; crossover design)	17 healthy adults (29 yrs.)	↑ Brachial artery diameter 90 (↑ 0.15 mm) increased up to 180 mins post; ↑ Hyperaemic brachial artery diameter 60 (↑ 0.15 mm) - 180 (↑ 0.18 mm) mins post; ↑ FMD 60 mins (4.5% - 5%), 120 mins (4.7%), 180 mins (4.9%); ↓ FMD at 90 & 150 mins post; ↓ Endothelium-independent vasodilation; ↑ Brachial artery BF (90 mins); ↔ Hyperaemic BF & % relative reactive hyperaemia; ↑ HR (max 180 mins); ↔ BP; ↓Augmented pressure & AIx (max 180 mins); Stepwise ↓ PWV (90 min-180 mins, NON-SIG)	-	↔ Plasma TAC & MDA	Vlachopou-los *et al.* (2005)
Chocolate bars	40g dark chocolate (74% cocoa) vs 40g white chocolate (4% cocoa)	Acute 2, 4, 8 & 24hrs post ingestion (parallel study)	25 male smokers	↑FMD 2hrs (4.4% - 7%), remained elevated until 8hrs; ↔Endothelium-independent dilation	-	↓Shear stress dependent platelet function (2hrs); ↑Δ-antioxidant status (2hrs)	Hermann *et al.* (2006)
Cocoa Drink	High flavanol-cocoa drinks (917mg total polyphenols) vs low flavanol-cocoa drink	Acute 1-4hrs post ingestion	Healthy male adults	↑FMD 1-4hrs, peak increase at 2 hrs (4.8% - 10.5%) & correlated with ↑epicatechin; ↑PAT	-	↑RXNO (1-3hrs); ↑Plasma flavanols/metabolites	Schroeter *et al.* (2006)

Cocoa Drink	446mg total flavanols vs 43mg total flavanols	11wks (0-2wks run in, 2-8wks treatment, 8-11wks washout), parallel study	32 hypercholesterolemic postmenopausal women (58 ± 2 yrs.)	↑FMD at week 2-8 in HF (12% - 14.1%); ↓ FMD week 2-8 in LF (12.5% - 11%); ↑Hyperaemia BF	↔SBP or DBP with high CF; ↓ SBP & DBP with low CF	↓s-VCAM-1; ↑HDL-C; ↔TC, LDL, TC:HDL ratio & TG; ↑ADP/collagen-induced PFA-100 closure time	Wang-Polagruto *et al.* (2006)
Chocolate bars & cocoa drink	444mg flavanols (~107mg epicatechin) daily vs 19.6mg flavanols (~4.7mg epicatechin) daily	6wks	40 participants with CAD (61 ± 9 yrs.)	↔Brachial diameter; ↔BF; ↔ Conduit vessel FMD or GTN-induced vasodilation (90min); ↔SAC	-	-	Farouque *et al.* (2006)
Cocoa Drink	**Acute Study:** 36mg vs 330mg vs 918mg **OR** 28mg vs 179mg vs 483mg flavanol content. **Short Term Study:** 36mg vs 928mg total flavanols per day	**Acute Study:** 0, 1, 2, 3, 4 & 6hrs. **Short Term Study:** 7-day intervention (measure 1, 3, 5 & 8 days, 7day washout, baseline measure day 15)	11 healthy males (27 ± 1yrs) with smoking related endothelial dysfunction	**Acute Study:** Dose dependent ↑FMD (peaked at 2hrs, returned to baseline at 6hrs), **Peak 2 hr: day 1** ↑FMD (3.7% - 6.1%), **day 3** ↑FMD (5.2% - 7.6%), **day 5** ↑FMD (6.1% - 8.8%), **day 8** ↑FMD (6.6% - 9%), **day 15** ↔FMD; ↔BP. **Short Term Study:** ↑FMD (day 1-5) ↔FMD (day 5-8) ↓FMD (day 15- to baseline); ↔BP	-	**Acute Study:** Dose-dependent ↑Nitrate (time course correlating with FMD). **Short Term Study:** ↔Nitrate levels	Heiss *et al.* (2007)
Chocolate bars & cocoa drink	**Chocolate bars:** 74g dark chocolate (22g cocoa powder) vs 74g placebo (0g cocoa powder). Cocoa drinks: Sugar-free cocoa (2 cups containing 22g cocoa powder,	Acute 2hrs (randomised crossover trail), 7-day wash out period between each intervention	45 healthy adults (10 mean, 35 women; ~53 yrs.)	**Dark Chocolate:** ↑FMD (7.4% - 11.7%), ↑BP. **Sugar-free Cocoa:** ↑FMD 5.6% - 11.3% (vs placebo & sugared-cocoa); ↑BP. **Sugared Cocoa:** ↑FMD 5.8% - 7.8% (vs placebo); ↔BP	-	-	Faridi *et al.* (2008)

	sweetened) vs sugared cocoa (2 cups containing 22g cocoa powder & 45.3g sugar) vs placebo (2 cups containing 0g cocoa powder)						
Cocoa Drink	**Feasibility Study:** 75mg vs 371mg vs 963mg total flavanols (single dose). **Efficacy Study:** 321mg vs 25mg total flavanols (3x per day)	**Feasibility Study:** Acute 6hrs (crossover design) **Efficacy Study:** 30 days (parallel design)	**Feasibility Study:** 10 diabetics (65 ± 10yrs) **Efficacy Study:** 41 diabetics (64 ± 9 yrs.)	**Feasibility Study:** ↑FMD dose-dependently; peak ↑FMD at 2 hrs (963 mg flavanols; 3.7% - 5.5%), returned to baseline by 6hrs; ↔ endothelium-independent vasodilation. **Efficacy Study:** ↑FMD at baseline across the 30 days (**Day 0;** 3.3% **Day 8;** 4.1% **Day 30;** 4.3%), largest ↑FMD acutely (2hrs) was on day 30 (**Day 0;** 4.8% **Day 8;** 5.7% **Day 30;** 5.8%); ↔ endothelium-independent vasodilation	-	**Feasibility Study:** ↑ Plasma flavanol metabolites correlated with ↑FMD (dose-dependently). **Efficacy Study:** ↑ Plasma flavanol metabolites with high-flavanol containing cocoa drink across 30 days	Balzer *et al.* (2008)
Cocoa Drink and exercise	902mg (HF) vs 36mg (LF) total flavanol per day (each split into exercise or sedentary groups)	**Acute:** 1 dose at 2hrs (day 0); **12-week:** exercise/sedentary intervention with high or low flavanol cocoa drink (split into 4 groups;	49 sedentary adults (45 ± 4 yrs.)	**Acute:** ↑FMD HF not LF (4.3% - 6.7%). **12wk Intervention:** ↑FMD week 6 (4.3% - 5.8%) & 12 (4.3% - 5.9%) HF sig. diff vs baseline/LF; ↓ diastolic BP & MAP (HF vs LF)	-	↔ Metabolic parameters (cocoa or exercise); ↓ Abdominal body fat (exercise); ↔ BMI (cocoa or exercise); Improvement in all HOMA2 parameters (HF vs LF)	Davison *et al.* (2008)

		HF+Ex/LF+Ex/HF-Ex/LF-Ex)					
Chocolate bars	100g flavanol-rich dark chocolate vs 100g flavanol-free white chocolate	7-day cocoa free washout phase - 15-day intervention (crossover trial)	19 patients with grade I essential hypertension (45 ± 8 yrs.)	**Dark Chocolate:** ↑FMD (5.1% - 6.5%); ↓BP & ABMP; ↔ Endothelium-independent Vasodilation	-	**Dark Chocolate:** ↓HOMA-IR; ↑QUICKI; ↑ISI; ↑β-cell; ↓ Insulin resistance; ↑ Insulin sensitivity; ↓Serum total cholesterol & LDL cholesterol; ↔HDL cholesterol or triglycerides	Grassi *et al.* (2008)
Cocoa drink & cycling exercise	High flavanol-cocoa drink (701 mg total cocoa flavanols) vs low flavanol-cocoa drink (22 mg total cocoa flavanols)	Acute 2 h (crossover design, each participant did 2x both interventions)	21 overweight/obese (>25 kg/m²) otherwise healthy males/post-menopausal women (54 ± 9 yrs.)	↑FMD HF (6.2%) vs LF (3.4%); ↔ BP pre-exercise (HF vs LF); ↔ HR during exercise (HF vs LF); ↓ BP response to exercise (HF vs LF)	-	-	Berry *et al.* (2010)
Cocoa Drink	33mg, 372mg, 712mg or 1052mg total flavanols per day	6-week double blind, parallel comparison	52 male & post-menopausal females (53 ± 7 yrs.)	↓BP (only with 1052mg flavanols per day); ↔BP (33mg, 372mg, 712mg flavanols per day)	-	-	Davison *et al.* (2010)
Cocoa drink	Sugar-free cocoa (805 mg total flavanols per day) vs sugared cocoa (805 mg total flavanols per day) vs placebo cocoa (9	6-week intervention & 4-week wash out period (crossover design)	44 healthy overweight adults (52 ± 11 yrs.)	↑FMD sugar-free (6.4% - 8.8%) and sugared cocoa (6.5% - 8%); Greatest ↑FMD (sugar-free vs sugared cocoa- not significant); ↔BP	-	↔ BMI; ↔ HDL, LDL & triglyceride; ↔ CRP, LDL oxidation, lipid hydroperoxide & endothelin	Njike *et al.* (2011)

	mg total flavanols per day)						
Cocoa powder added to a liquid fatty meal	14.68mg total flavanols vs 918 mg total flavanols (included in the fatty meals)	Acute 6 h study (crossover design – 1-week wash out period), inducing acute lipemia-induced endothelial dysfunction with a fatty meal	18 healthy young adults (25 ± 3 yrs.)	↓ FMD 1 - 4 hrs; ↓ FMD was less pronounced in the high-flavanol cocoa containing meal, 2, 3 & 4 hrs (**HF= Baseline;** 9%, **1hr;** 7.4%, **2hr;** 7.7%, **3hr;** 7.6%, **4hr;** 8.3%, **6hr;** 8.5%. **VS. LF= Baseline;** 8.5**%, 1hr;** 6.6**%, 2hr;** 6.5**%, 3hr;** 6.6**%, 4hr;** 7.5**%, 6hr;** 8.3**%**)	↔ BP	↔ Cholesterol HR; ↑ Triglycerides & FFA (2, 3 & 4 hrs)	Westphal *et al.* (2011)
Cocoa drink	0 g (330 mg total polyphenols, 0 mg epicatechin), 2 g (420 mg total polyphenols, 6.3 mg epicatechin), 5 g (420 mg total polyphenols, 17.7 mg epicatechin), 13 g (840 mg total polyphenols, 45 mg epicatechin) or 26 g (1470 mg total polyphenols, 96 mg epicatechin) cocoa powder	Acute 2.5 hrs, 5 experimental interventions (crossover design)	23 healthy sedentary-recreationally active adults (63 ± 2 yrs.)	↑FMD 5 g (Baseline 4.0%, 1 h 4.9%, 2 h 5.0%) 13 g (Baseline 3.6%, 1 h 4.6%, 2 h 5.1%) and 26 g cocoa (Baseline 3.6%, 1 h 5.2%, 2 h 6.1%); Dose-dependent ↑FMD (largest ↑ seen at 26 g cocoa); ↔ Endothelium-independent vasodilation	-	Dose-dependent ↑ epicatechin (1 and 2 h post, correlated with ↑ FMD); ↔ Insulin, norepinephrine and oxidized LDL	Monahan *et al.* (2011)
Cocoa drink	451 mg total flavanols	Acute 8hrs	19 healthy older adults (71 ± 7 yrs.)	**Habitual Flavonoid Intake:** Sig. correlation with endothelial function (RH-PAT response); Sig. correlated with basal PW PAT response. **Acute Cocoa**	-	-	Fisher *et al.* (2012)

				Intake: PAT response sig. correlated with ↑ flavanol concentration in the blood			
Cocoa drink	High-flavanol cocoa (450 mg total flavanols, 2x daily) vs Placebo coca (0 mg total flavanols)	2-week intervention & acute 1 h post intake	22 young healthy males (26 ± 1 yrs.) & 20 older healthy males (60 ± 2)	**Acute:** ↑FMD at 1 h young (6.1% - 7.4%) & older (4.9% - 5.9%); ↔ FMD at 1h (day 14); ↓ PWV (young & older); ↔ PWV at 1h (day 14). **2 weeks:** ↑ FMD day 14 vs day 0, young (6.1% - 7.8%) & older (4.9%-6.3%); ↓ PWV (day 14 vs day 0; young & older); ↑ FBF (young & older); ↑ RBC deformability & maximal perfusion (young & older)	-	↓ SBP & AIX at all time points compared with baseline (older participants); ↓ DBP & TPR (day 14); ↔CO, HR & SV (all time points); ↑ Total plasma flavanols	Heiss *et al.* (2015)
Cocoa drink	**Intake-Response:** Nitrate (0.1, 0.3, 1, 3, 8.5 & 10 mg/kg bw) vs Cocoa Flavanols (1.4, 2.7, 5.5 & 10.9 mg/kg bw). **Interaction:** *Low intake*- control vs nitrate (3 mg/kg bw) vs cocoa flavanols (2.7 mg/kg bw) vs nitrate (3 mg/kg bw) & cocoa flavanols (2.7 mg/kg bw) VS *High intake*- control vs nitrate (8.5 mg/kg bw) vs	Acute 4 hrs (crossover design within 4 study arms)	15 healthy adults (25 ± 1 yrs.) split into **Intake Response:** nitrate (N=5) & cocoa flavanols (N=5). **Interaction:** low intake (N=10) & high intake (N=10)	**Intake-Response:** dose-dependent ↑ FMD (similar in nitrate and flavanols), peaked at 1 h (1.4 mg/kg bw = 7.5% - 7.9%, 2.7 mg/kg bw = 7.5% - 8.2%, 5.5 mg/kg bw = 7.5% - 8.7%, 10.9 mg/kg bw = 7.5% - 10.1%) , decreased to baseline by 4 hrs; ↔ BP. **Interaction:** ↑ FMD (low dose nitrate & flavanols vs just nitrate or cocoa); ↔ FMD (high dose nitrate & flavanols vs just nitrate or cocoa)	-	**Interaction:** ↓ Plasma nitrite (flavanols & nitrate ingested vs nitrate alone); ↔ Plasma & urinary nitrate & salivary nitrite/nitrate (flavanols & nitrate ingested vs nitrate alone); ↑ Expelled NO (flavanols or nitrate ingested); ↔ Plasma/urinary levels of flavanols (flavanols vs flavanols & nitrate)	Rodriguez-Mateos *et al.* (2015)

	cocoa flavanols (10.9 mg/kg bw) vs nitrate (8.5 mg/kg bw) & cocoa flavanols (10.9 mg/kg bw)						
Cocoa drink	**Pilot Study:** 450 mg of cocoa flavanols (2x day). **Main Study:** 450 mg of cocoa flavanols per drink (2x day) vs cocoa flavanol-free drink (2x day)	**Pilot Study:** Chronic 1-month trail. **Main Study:** Chronic 1-month trail (parallel design) **- Acute within each visit (0 h, 1 h, 2 h post)**	**Pilot Study:** 5 healthy males (44 ± 9 yrs.) **Main Study:** 100 female and male healthy adults (**Flavanol group;** 45 ± 8 yrs., **Control group;** 44 ± 8 yrs.)	**Main Study:** ↑ FMD acutely 1 h & 2 hs, greatest ↑ at 2 hs (day 1 = 6% - 7.4%, & day 7 = 6.6% - 7.9%); ↔ FMD acutely (days 14, 21 & 28); ↑ fasting FMD chronically (from day 1 to day 7 = **6% - 6.6%**; day 7 to day 14 = **6.6% - 7.8%**); ↔ fasting FMD chronically (days 21 and 28); ↓ office SBP/DBP & central SBP/DBP (over month); ↓ PWV & AIX (over month)	-	**Main Study:** ↓ total & LDL-cholesterol (1 month); ↑ HDL (1 month); ↔ TAG, fasting plasma glucose & HbA1c (1 month); ↑ plasma flavanol metabolites (acutely at 2 hrs); ↔ plasma flavanol metabolites (fasting levels); ↓ Framingham Risk Score (1 month)	Sansone *et al.* (2015)
Chocolate bars	Dark chocolate (80% cocoa, 3600 mg total flavonoids) vs milk chocolate (35% cocoa, 980 mg total flavonoids) vs white chocolate (cocoa fats, 370 mg total flavonoids)	Acute 2 hrs (crossover design)	12 healthy post-menopausal women (57 ± 5 yrs.)	↔ MAP & HR (all conditions); ↓ CBFv in response to the cognitive tasks (all conditions vs no-chocolate session); ↓ CBFv in response to cognitive tasks, MC & DC vs WC; ↓ CVC during cognitive tasks, MC & DC. **WC:** ↔ CBFv & CVC; ↔ FMD. **MC:** ↓ CBFv (60 $cm \cdot s^{-1}$ - 54 $cm \cdot s^{-1}$) & CVC (0.65 - 0.55 $cm \cdot s^{-1} \cdot mmHg^{-1}$); ↔ FMD. **DC:** ↓ CBFv (65 $cm \cdot s^{-1}$ - 55 $cm \cdot s^{-1}$)	-	-	Marsh *et al.* (2017)

				& CVC (0.68 - 0.55 $cm \cdot s^{-1} \cdot mmHg^{-1}$); ↑ FMD (6.9% - 9%)			
Drinks	**Study Protocols 1-3:** CF (820 mg/75kg bw total flavanols, 1.6 mg/kg bw theobromine, 1.2 mg/75kg bw caffeine) **vs** Methylxanthines (0mg/75kg bw total flavanols, 112.8 mg/kg bw theobromine, 10.2 mg/75kg bw caffeine) **vs** CF & Methylxanthines (820 mg/75kg bw total flavanols, 111.0 mg/kg bw theobromine, 11.4 mg/75kg bw caffeine). **Study Protocol 4:** Epicatechin (75 mg) **vs** Epicatechin & theobromine (400 mg) & caffeine (26 mg)	**Study 1:** Acute 2 hrs (crossover design) **Study 2:** Acute 2 hrs (parallel design, 4 arms) **Study 3:** Acute 5 hrs (crossover design) **Study 4:** Acute 4 hrs (crossover design)	47 healthy males (25 ± 2 yrs.). **Study 1:** N=12 **Study 2:** N=24 (N=6/group) **Study 3:** N=5 **Study 4:** N=6	**Study 1:** ↑ FMD at 2 hrs with CF (6.5% - 7.4%) and CF + Mx (6.6% - 8.2%); Greater ↑ FMD (Mx & flavanols drink vs flavanols only drink); ↔ FMD (Mx drink); ↔ Endothelium-independent vasodilation (all drinks); ↔ DBP/SBP & HR. **Study 2:** Dose-dependent ↑ FMD at 2 hrs (after intake of >105 mg flavanol drink); Dose-dependent ↑ FMD at 2 hrs (flavanol with 122 mg Mx; this ↑ FMD was greater with the combined drink than with flavanols alone); ↔ FMD (Mx alone); Dose-dependent ↑ FMD (Increasing amounts of MX with 820 mg flavanols). **Study 3:** ↑ FMD (sig. greater with combined Mx & flavanol drink vs flavanol drink alone). **Study 4:** N/A	-	**Study 1:** ↑ CD34+/KDR+ CACs & ↓ bPWV (both drinks containing flavanols, these changes were bigger with consumption of the flavanol and Mx drink). **Study 2:** ↔ ED50 (all drinks) **Study 3:** ↑ Cmax of SREMs & AUC0-5 h of plasma conc. (sig. higher in combined drink than flavanols alone) **Study 4:** ↑ SREM Cmax at 2 hs (epicatechin & combination drinks; higher in combined drink vs epicatechin alone); AUC0-4 h (higher in combined than in epicatechin only)	Sansone *et al.* (2017)

Table 3. Human acute cerebral blood flow and cognition intervention trails with cocoa

Intervention	Flavonoids' Daily Dose	Duration of the Intervention	Subjects	Cognitive Measures	Other Measures	Reference
Cocoa drink	HF (172 mg flavanols per day) vs LF (13 mg flavanols per day)	Short term 5-day intervention (crossover design)	16 young females (18-30 yrs.)	↔ Switch cost (HF vs LF); ↑ BOLD signal change for switch & nonswitch (HF)	↑ HR (switch vs nonswitch, both drinks); ↑ CBF at 2 hrs, returned to baseline at 6 hrs (HF)	Francis *et al.* (2006)
Cocoa drink	Flavanol-rich cocoa (451.1 mg total flavanols per drink, 2x per day) vs flavanol-poor cocoa (18.2 mg total flavanols per drink, 2x per day)	**Time Course Study:** Acute (0, 2, 4, 6 & 8 hrs post) & short term (2-weeks). **FRC vs FPC:** Short-term (1-week)	**Time Course Study:** 13 healthy adults (73 ± 4 yrs.). **FRC VS FPC:** 21 healthy adults (72 ± 6 yrs.)	-	**Time Course Study:** ↑ Peak CBF response (2-weeks of FRC intake); ↓ Resting MCA MFV to single dose day 0 (2 & 4 hrs, returned to baseline at 6 & 8 hrs); ↓ MCA MFV to single dose day 7 (2 & 4 hrs); ↑ MCA MFV to single dose day 7 (6 & 8 hrs); ↑ Resting CBF day 14 (smaller ↓ MCA MFV at 2 & 4 hrs, small ↑ at 6 hrs, sig. ↑ at 8 hrs); ↔ MAP & CVR (2-weeks). **FRC vs FPC:** ↔ BP, CVR or cerebral vasoreactivity; FRC larger % ↑ MFV (large variability so didn't reach sig.)	Sorond *et al.* (2008)
Cocoa drink	HF (994 mg total flavanols, 184 mg epicatechin) vs LF (520 mg total flavanols, 94 mg epicatechin) vs Control (46 mg total flavanols, 4 mg epicatechin)	Acute 90 min (crossover design)	30 healthy participants (~22 yrs.)	↔ State Anxiety; Cognitive Demand Battery = ↑ No. correct Serial 3 Subtractions (HF & IF), ↔ No. correct Serial 7 Subtractions, ↔ RVIP accuracy, ↓ RVIP speed at 30 & 40 min (HF), ↑	-	Scholey *et al.* (2010)

				Improved Mental Fatigue VAS all time points other than 30 mins (IF)		
Chocolate bars	35 g Dark chocolate (773 mg total flavanols) vs 35 g white chocolate (trace amounts of flavanols) - Not matched with caffeine, energy, theobromine, etc	Acute 2 hrs (crossover design)	30 healthy young participants (18-25 yrs.)	↑ Contrast sensitivity 0.96% & 0.58% (DC); ↓ non-sig. motion coherence threshold (DC); ↓ Motion integration time threshold (DC); ↑ Visual spatial working memory (DC); ↓ Choice reaction time (DC)	-	Field *et al.* (2011)
Dairy based cocoa drink	HF (990 mg flavanols per day) vs IF (520 mg flavanols per day) vs LF (45 mg of flavanols per day)	8-weeks (parallel design)	90 elderly adults	↔Mini Mental State Score (HF, IF or LF); ↓ Time to complete TMT A & B (HF & IF, not LF); ↑ TMT A & B scores (HF & IF, not LF); ↑ Verbal Fluency test scores (HF & IF, not LF); ↑ Cognitive z score (HF & IF, not LF)	↓ SBP & DBP (HF & IF, not LF) ↓ Plasma glucose levels (HF & IF, not LF); ↔ Plasma insulin levels; ↓ HOMA-IR (HF & IF, not LF); ↔ Total cholesterol, LDL, HDL & triglycerides concentrations; ↓ Plasma total 8-iso-PGF2a (HF & IF, not LF)	Desideri *et al.* (2012)
Cocoa drinks	20g dark chocolate drinks; HF (500 mg flavanols) vs LF (250 mg flavanols) vs placebo (0 mg flavanols)	**Acute:** 4 hrs **Chronic:** 30-day intervention (parallel design)	71 healthy middle-aged participants split into groups; placebo N=22, LF N=25 & HF N=24	**Acute:** ↔ Cognition or mood (HF or LF). **Chronic:** ↔ Cognition (HF or LF); ↑ Calmness (HF); ↑ Contentedness (HF)	-	Pase *et al.* (2013)

Diet and exercise intervention	High flavanol intake group (900 mg cocoa flavanols & 138 mg epicatechin per day) with or without exercise VS Low flavanol group (10 mg cocoa flavanols & <2 mg epicatechin per day) with or without exercise; Exercise = 1 h per day 4x per week	Chronic 3 month (parallel design, 4-armed intervention)	37 healthy sedentary participants (~58 yrs); HF & Ex (N=8) vs HF & no-Ex (N=11) vs LF & Ex (N=9) vs LF & no-Ex (N=9)	↓ ModBent reaction time (HF, independent of exercise); ↔ Delayed retention (HF & LF); ↑ Cognitive performance (HF vs LF); ↔Exercise effect on any outcome measure including VO2max	↑ Cerebral blood volume in DG (HF); Changes in ModBent were associated with CBV changes	Brickman *et al.* (2014)
Cocoa drink	LF drink (29 mg total flavanols, 3 mg epicatechins) vs HF drink (494 mg total flavanols, 89 mg epicatechins)	Acute 2 hrs (crossover design)	18 healthy older adults (~61 yrs.)	-	↑ Regional perfusion at 2 hrs (2 main clusters; anterior cingulate cortex & cluster extending from the central opercular cortex of the left parietal lobe to a sub-cluster in the temporal lobe)	Lamport *et al.* (2015)
Dairy based cocoa drinks	HF drink (993 mg flavanols per serving) vs IF drink (520 mg flavanols per serving) vs LF (48 mg per serving)	Short term 8-week intervention after 1-week run in (parallel design)	90 elderly adults (70 ± 12 yrs.); split into N=30 per cohort (HF, IF & LF)	↔ MMSE score (HF, IF or LF); ↓ TMT A & B scores (HF & IF); ↑ VFT scores, dose-dependent improvements (HF, IF & LF, largest ↑ with HF); ↑ z scores (HF & IF)	↓ SBP (HF & IF); ↓ DBP (during study in HF & IF but not at 8-weeks) ↓ Plasma glucose, insulin & HOMA-IR (HF & IF); ↓ Circulation concentrations of total cholesterol, LDL cholesterol & triglycerides (HF & IF); ↑ HDL cholesterol (HF & IF); Improved HDL-LDL ratio (LF & sig. greater improvement in HF & IF); ↓ Plasma 8-iso-prostaglandin F2a concentrations (HF & IF)	Mastroiacovo *et al.* (2015)

THE IMPACT OF COCOA FLAVANOLS ON PERIPHERAL VASCULAR, CEREBROVASCULAR AND COGNITIVE FUNCTION IN YOUNGER AND OLDER HEALTHY ADULTS

Introduction to Study

In recent years the prevalence of age-related cardiovascular and cognitive diseases has increased, in part, due to the rise in life expectancy across the world. As a significant portion of these diseases can be terminal and can greatly reduce quality of life (Stroke Association. 2018; British Heart Foundation. 2019), the need to delay the onset of these diseases or decrease their prevalence becomes an important health concern to overcome.

Multiple large epidemiological studies have demonstrated that populations with high intakes of certain foods and beverages experience vastly decreased incidences of vascular diseases (Hertog et al., 1993; Knekt et al., 1996; Kalmijn et al., 1997; Yochum et al., 1999; Commenges et al., 2000; Arts et al., 2001; Geleijnse et al., 2002; Letenneur et al., 2007). For example, in particular food stuffs that include polyphenols such as; red wine, tea, apples, blueberries and cocoa, have been shown to lower blood pressure (Hooper et al., 2008; Galleano et al., 2009), improve cognition (Scholey et al., 2010), reduce age related cognitive decline (Letenneur et al., 2007) and promote scarcer rates of dementia (Commenges et al., 2000) and cardiovascular diseases (Hertog et al., 1993; Knekt et al., 1996; Arts et al., 2001; Geleijnse et al., 2002).

Cocoa in particular, included within a sub-group of polyphenols known as flavonoids, has demonstrated an innate ability to offset risk factors associated with cardiovascular disease (Shrime et al., 2011). Therefore, within this thesis the effects of high flavanol

cocoa will be investigated in relation to its influence on peripheral and cerebral vascular function as well as cognitive function, across both younger and older healthy adults. This study aims to identify whether cerebrovascular vessels respond differently to cocoa ingestion compared to peripheral vessels. Further, the secondary aim of this study is to explore whether age alters how vessels respond to acute cocoa ingestion. It is hypothesised that cerebral vessels will respond in a similar way to cocoa ingestion as peripheral vessels.

Methods

Study Participants

All participants included within this study were informed of the procedures and requirements of the study and provided written informed consent at least 48-hours prior to inclusion within the study. Participants were screened at least 48-hours prior to their first data collection visit for; cardiovascular, respiratory or neurovascular diseases, high blood pressure, raised blood cholesterol, medication usage, smoking or allergies to cocoa. Included within the study were twenty younger healthy male (age 21 ± 4yrs) and fourteen older (age 71 ± 5yrs) healthy male or female participants (mean ± SD). Younger menstruating females were excluded from this study due to known alterations in hormonal profile throughout the month, which have been shown to influence the vasculature. Self-reported levels of physical activity varied from sedentary to high levels of physical activity within our cohort.

Experimental Conditions

The protocols of this experiment as discussed below were conducted within a temperature-controlled laboratory (~22°C) at the University of Birmingham, School of

Sport, Exercise and Rehabilitation Sciences. An acute randomized, placebo-controlled, double-blinded, cross-over human study design was used. All participants visited the laboratory on three occasions; the first for a familiarization visit, done at any time of day, and the following two for data collection done at the same time of day starting at between 7:30-9:00am with a minimum of 4 days in-between these visits. Participants were instructed to avoid certain foods and beverages high in polyphenols (see **Appendix A.1**; E-Flavonoid rich foods to avoid for 24-hours prior to each study day), beverages high in caffeine, alcohol or exercise 24-hours prior to visiting the laboratory on data collection sessions, they were also asked to complete a 12-hour overnight fast. To try and measure adherence to this avoidance protocol a dietary recall questionnaire was utilised at the beginning of each visit (see **Appendix A.2**; 24h Hour Recall Questionnaire), if participants had not adhered they were requested to visit the laboratory on another day to collect data after adhering to the avoidance list. Participants were provided Buxton® water throughout the laboratory visits, due to low levels of nitrite within this bottled water. The study was approved by the University of Birmingham Ethics Board (ERN_17-1591) and conformed to the standards set by the Declaration of Helsinki.

Study Protocol

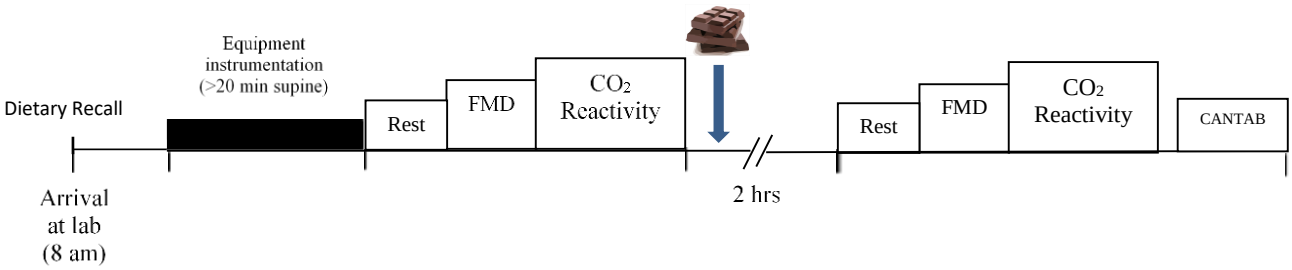


Figure 3. Schematic of experimental protocol for set up, resting and functional measures prior and post cocoa ingestion ( )

Familiarisation Visit

To acquaint the participants with the study they were required to partake in a familiarisation laboratory visit. Participants were sent the information sheet at least 24-hours before agreeing to enter the laboratory for this visit. On arrival at the laboratory participants signed the consent form before reading through and completing the health questionnaire (see **Appendix A.3**; General Health and lifestyle Questionnaire) as well as the cocoa allergy questionnaire (see **Appendix A.4**; Cocoa Questionnaire). The researcher then checked these filled in forms for eligibility within the study, before moving on to the familiarisation protocol.

The protocol and the measurements of the study (both for the familiarisation and the data collection visits) were explained to the participant, highlighting what the participant should expect to feel throughout. Participants then lay supine on the laboratory bed and their brachial artery image was assessed alongside their middle cerebral artery (MCA) and posterior cerebral artery (PCA) signals before undergoing both the flow mediated dilation (FMD) and the cerebrovascular reactivity (CVR) protocols (full details below). Following at least 48-hours, participants returned to the laboratory to complete the full experimental trial, with the knowledge that they were able to exclude themselves from the study at any time up to and including two weeks post completion of the study.

Data Collection Visits

Prior to the experimental sessions' participants were asked to refrain from vigorous exercise and the consumption of alcohol or caffeine for 24-hours prior to testing. Participants were fasted (12-hours) prior to their arrival to the laboratory for each

experimental session; instructed not to eat or drink anything after 8 pm. In addition, participants were asked to avoid certain foods and drinks containing high levels of polyphenols for 24-hours before each visit (see **Appendix A.1**; E-Flavonoid rich foods to avoid for 24-hours prior to each study day).

Upon arrival to the laboratory participants dictated answers to dietary recall form filled in by the researcher. A 3-lead electrocardiogram (ECG) was attached (see **Fig. 7**) before lying supine on the laboratory bed. Participants were then fitted with a portapres (see **Fig. 2**) and transcranial Doppler Ultrasound (TCD) headset (see **Fig. 4**). Following 20 minutes of supine rest, baseline measures of resting heart rate (HR), end-tidal CO_2 ($P_{ET}CO_2$), blood pressure (BP), and both middle cerebral and posterior cerebral blood flow velocities (MCAv and PCAv, respectively) were recorded. Following resting measures, the functional vascular tests were completed (FMD and CVR tests, as detailed below). Participants then ingested 8.3 g of a low flavonoid or high flavonoid cocoa (for composition of powders see **Table 4**) power mixed in 300 mL of cold water, given in an opaque cup. Participants then rested in the laboratory for 2-hours, after which they repeated the battery of vascular measures before completing cognitive testing utilising the Cambridge Neuropsychological Test Automated Battery (CANTAB), following at least 5 min recovery after CO_2 reactivity test.

Participants then returned to the laboratory following at least a four-day gap and repeated the same protocol on the alternative flavonoid drink intervention. All drinks were coded, and the order randomized, therefore both the participant and the researcher were blinded to the intervention including the order they were administered.

Table 4. Composition of cocoa interventions

		High-flavanol cocoa (8.3 g)	Low-flavanol cocoa (8.3 g)
(-)-Epicatechin	mg	150.0	> 4
(+)-Catechin	mg	35.5	> 4
Total polyphenols	***mg***	***1052.5***	***143.4***
Theobromine	mg	179.8	179.8
Caffeine	mg	19.5	19.3
Fat	g	1.1	0.9
Carbohydrates	g	1.8	0.8
Protein	g	1.8	1.8
Energy	kcal	28	25

Experimental Protocols

Flow Mediated Dilation (FMD)

FMD non-invasively measures a vessels vasodilatory capacity, therefore providing a tool to assess arterial endothelial function. FMD measures the vasodilation of an artery in response to an acute increase in blood flow, this elevation in blood flow increases laminar shear forces parallel to the walls of the artery, this shear stress is then detected by mechanoreceptors which signals to the endothelial cells to increase endothelial nitric oxide production, ultimately relaxing smooth muscle cells and causing vasodilation (Harris et al., 2010; Markos et al., 2013). This increase in arterial diameter is then compared to baseline diameter and is expressed as a percentage of this baseline (Harris et al., 2010).

In this study participants lay with their right arm outstretched resting comfortably on a table, an occlusion cuff strapped to their lower arm approximately two centimetres

distal to the elbow (see **Fig. 4**), before lying for 20 minutes to allow the participants vital signs to stabilise and whilst the researcher found the optimal probe placement, prior to fixing into position using a mechanical arm. Once the Cardio-Vascular Suite software was set up, the test began with 1 minute of baseline recording, followed by the pressure in the occlusion cuff being raised to 220-240 mmHg for 5 minutes, after this occlusion period the pressure within the cuff was released and data recorded for 5 minutes. Throughout this time continuous recordings of arterial diameter, blood flow velocity and shear stress were taken by the software, before automatically calculating the FMD immediately post completion of the test (see **Fig. 4**).

Figure 4. Full Flow Mediated Dilation (FMD) setup. Equipment shown as follows; 1) Terason Duplex Doppler Ultrasound, 2) PC laptop running CardioVascular Suite software, 3) Occlusion cuff, 4) Supports for raised arm, 5) 15-4Mhz transducer attached to Terason, and 6) Three-axes movement probe holder.

Cerebrovascular Reactivity (CVR)

CVR is the regulation of CBF in response to a vasoactive stimulus given by a researcher whether via injection of vasoactive chemicals, manipulation of mean arterial pressure (MAP) or alterations in the partial pressure of arterial CO_2 (P_aCO_2). Within the current protocol the latter was used, initially increasing P_aCO_2 (hypercapnia), to induce vasodilation, by feeding air gas mixtures with elevated levels of CO_2 (2% CO_2 or 5% CO_2 with synthetic air balance) and decreasing P_aCO_2 (hypocapnia), to induce vasoconstriction, by instructing participants to mildly hyperventilate. Participants whilst lying supine were instrumented with a mouthpiece and MCAv, PCAv, BP, $P_{ET}CO_2$ and HR data was continuously collected throughout the protocol. The mouthpiece was

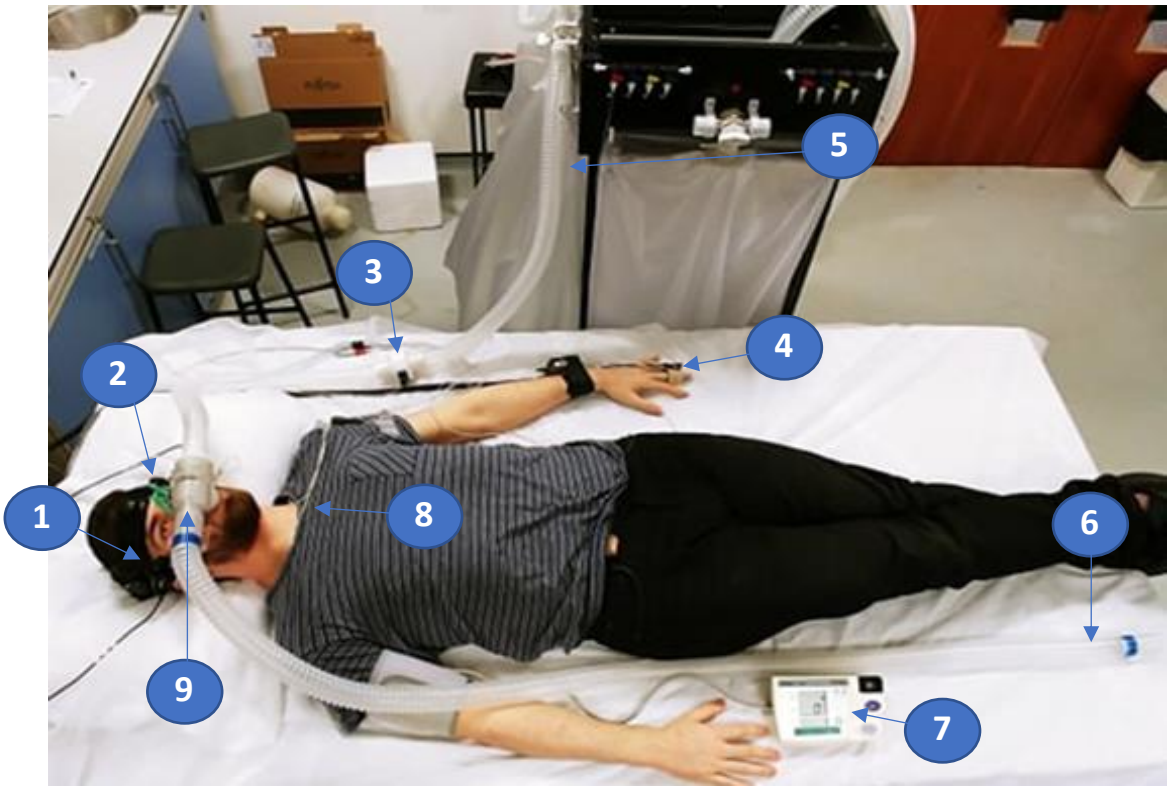


Figure 5. Full Transcranial Doppler Ultrasound setup. Equipment shown as follows; 1) Transcranial Doppler Ultrasounds probes attached to a head band, 2) Nose clip, 3) Spirometer, 4) Portapres set up, 5) Douglas bag gas administration set up, 6) Exhalation tube, 7) Brachial blood pressure cuff set up, 8) Three-lead electrocardiogram, and 9) 2-way T-shaped breathing value with gas sampler and intake and exhalation tubes attached.

attached to a Douglas bag setup, one bag containing a 2% CO_2 mixture and the other contained a 5% CO_2 gas mixture (see **Fig. 5** & **Fig. 6**). A CVR test was then carried out, participants underwent a steady state hypercapnic stimulus-response test (run consecutively) which required them to breathe a 2% and a 5% CO_2 gas mixture via a mouthpiece attached to a Douglas bag, for 4 minutes each. Participants were then allowed to rest until $P_{ET}CO_2$ and vital signs returned to baseline state before completing the hypocapnic stages in which they were verbally guided to mildly hyperventilate until they reached a target $P_{ET}CO_2$ of equal and opposite to the increase seen for 2% and 5% CO_2, this level was then held for 2 minutes for both stages.

Cambridge Neuropsychological Test Automated Battery (CANTAB)

All cognition tests were done in the same laboratory as the physiology tests but were done in silence sat on a chair comfortably whilst not instrumented. Auditory instructions for the tasks were given by the CANTAB software, however if the participants were unclear on the rules of a task the researcher explained the task until the participant understood. The participants were instructed to complete every movement as quickly as possible and with only the index finger of their dominant hand for all tasks.

Information Input: Reaction Time (RTI)

This cognitive test assessed participants' reaction times, movement times and response accuracy. Within this test participants respond to a five-choice reaction time task (see **Fig. 11**) in which the participant holds down the button at the bottom of the touchscreen and were instructed to only let go of this button when they saw a yellow light flash up in one of the circles at the top of the touchscreen. They then must touch the circle they saw the yellow light in before returning to hold down the button. This

task was repeated over 30 trials post several practice trials to become familiar with the task.

Using Information to Guide Behaviour: Attention Switching Task (AST)

This cognitive test assesses executive function, providing a measure of cued attentional shifting. Within this test participants saw an arrow flash up on the screen pointing either left or right, they were instructed to press either the right or left button at the bottom of the screen indicating the side the arrow flashed up on or the direction the arrow was pointing (see **Fig. 11**). The first set of trials involved pressing the buttons indicating the direction the arrow was pointing, and the second set involved pressing the button indicating the side the arrow appeared on. After these first two trials a word, either 'direction' or 'side' flashed up at the top of the screen instructing the participants on which rule to follow. At the start of the trials for each new rule participants had several practice trails to become familiar with the new rule prior to assessment.

Using Information to Guide Behaviour: Spatial Working Memory (SWM)

This cognitive test requires retention of visuospatial information and manipulation of this information, which assesses executive function and provides a measure of strategy and response accuracy. Within this test participants saw boxes appear on the screen (see **Fig. 11**), starting with 3 and getting progressively harder by increasing by 3 in each round (3, 6, 9 & 12). Participants had to touch the boxes to reveal if they were either empty or contained a 'token', once a token was found they clicked on this token to move it to the column on the right-hand side of the screen. The aim was to find all the tokens to fill the empty column, the 'token' never reappeared in a box, so the participant should not have returned to this box in the same round. This task

recorded ‘errors’, i.e. touching empty boxes and returning to boxes that have already been found to contain a token in that round. Prior to starting the assessment participants were given several practice trials to become familiarised with the rules of the task.

Information Storage: Paired Associates Learning (PAL)

This cognitive test assesses visual memory and learning, providing a sensitive measure episodic memory. Within this test participants see boxes on the screen, each box opened individually showing patterns hidden inside, a pattern was then displayed in the centre of the screen and participants touched the box where this pattern was originally seen (see **Fig. 11**). If the participant touched the incorrect box, the boxes re-opened to allow the participant a reminder of where the patterns were located. The task became gradually harder starting with finding 2 patterns and increasing each round from 2 to 4, 8 and 12. Participants had 3 attempts per round, with failure in all 3 attempts resulting in the task being terminated. Prior to starting the assessment, participants were given several practice trials to become familiarised with the rules of the task.

Measurements and Instrumentation

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

The amount of CO_2 exhaled at the end of each breath is known as the partial pressure of end-tidal CO_2 ($P_{ET}CO_2$), although not the exact same as the P_aCO_2, $P_{ET}CO_2$ closely indicates the amount of CO_2 within the blood and was used as a non-invasive surrogate measure of P_aCO_2 within this study (see **Fig. 6**). Throughout data collection, breath-by-breath $P_{ET}CO_2$ was sampled using a fast-responding gas analyser (ML206,

ADInstruments, Dunedin, New Zealand), in addition to ventilation rate and volume being measured using a spirometer attached to the intake value of the mouthpiece. Prior to testing, the spirometer was calibrated using a 3-L syringe (5530 series, Hans Rudolph, USA) and the gas analyser with known concentrations of CO_2 and O_2. Daily barometric pressure was recorded and used to account for changes in barometric pressure between days using **Eq. 1**.

$$\Delta P_b = \frac{\%CO_2 * (P_b - 47)}{100} \quad (1)$$

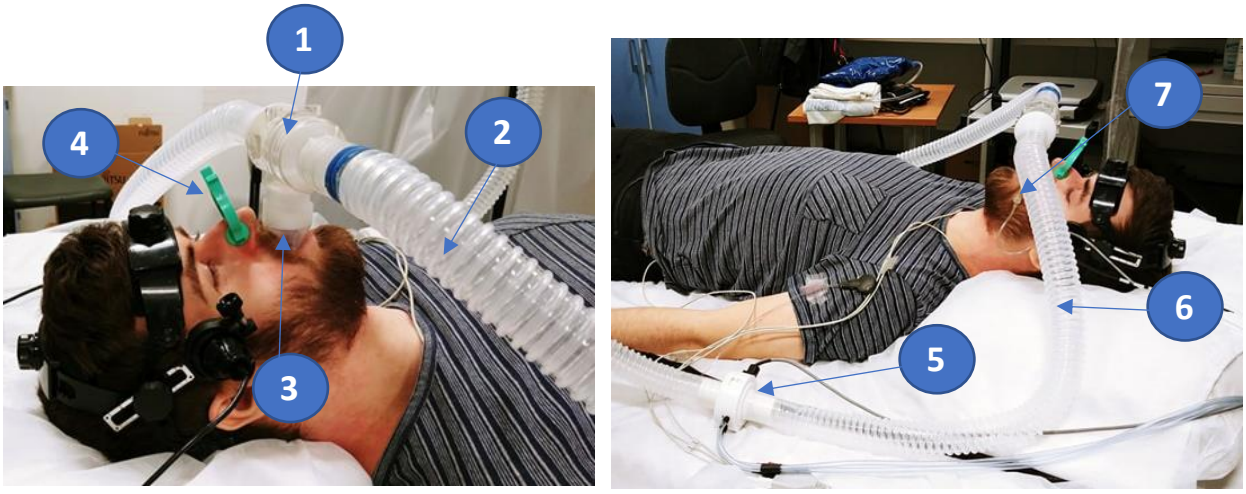


Figure 6. Full Gas setup attached to douglas bags (not shown in figure, see **Fig 2.**). Equipment shown as follows; 1) 2-way T-shaped breathing value, 2) Exhalation tube, 3) Mouthpiece, 4) Nose clip, 5) Spirometer attached to tube connected to douglas bag setup to left of the picture, 6) Intake tube, 7) Gas sampler.

Continuous Assessment of Blood Pressure: Portapres

Utilising a portapres (Finapres Medical Systems, The Netherlands), which includes a cuff placed around the middle finger on the left hand of the participants, allowed for continual beat-to-beat assessment of blood pressure non-invasively (see **Fig. 7**). Using this system, measurements of systolic (SBP), diastolic (DBP) and MAP were recorded continuously.

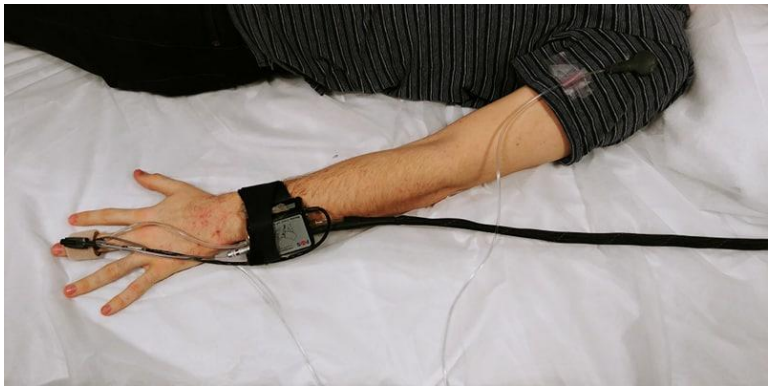


Figure 7. Portapres set up.

Intermittent Assessment of Blood Pressure: Brachial Cuff

The beat-by-beat blood pressure taken by the portapres was verified by using an automated brachial blood pressure cuff attached on the right arm (see **Fig. 8**) of our

Figure 8. Brachial blood pressure cuff set up.

participants (Omron 705IT, OMRON, Japan). The brachial cuff measured both SBP and DBP, MAP was then calculated from **Eq. 2.**

$$MAP = \frac{(SBP+2)(DBP)}{3} \quad (2)$$

Transcranial Doppler Ultrasound (TCD)

TCD is a useful measure utilised within cerebral vascular research that allows for non-invasive assessment of cerebral haemodynamics and works via the Doppler shift effect. Within this study cerebral blood flow velocity was recorded at the level of the MCA and the PCA, insonated via the temporal window situated above the zygomatic arch using a 2MHz Doppler-box™X system (DWL, Compumedics LTD, Germany).

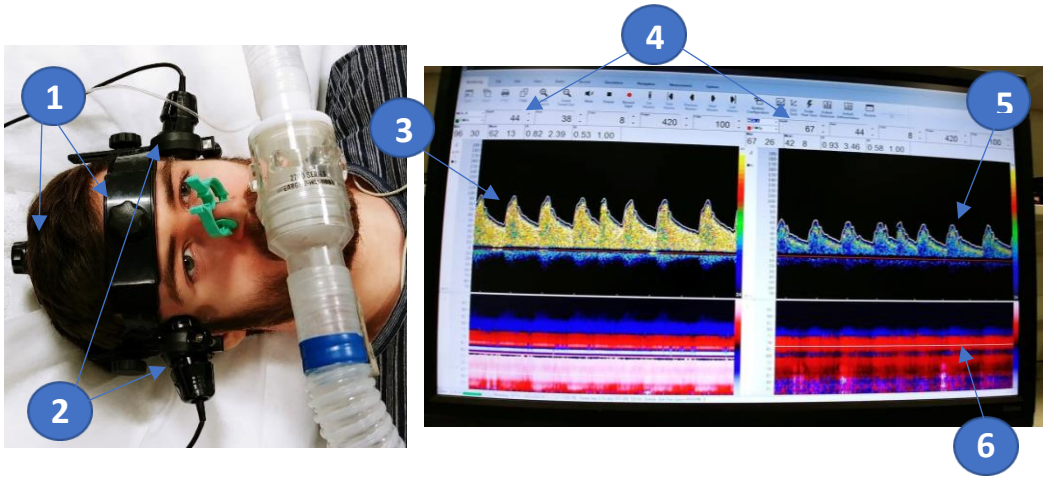


Figure 9. Transcranial Doppler Ultrasound set up and data collection. Equipment and data are displayed as follows; 1) Transcranial Doppler Ultrasound head set, 2) 2MHz Doppler probes with the top recording posterior cerebral artery (PCA) blood flow velocity and the lower recording middle cerebral artery (MCA) blood flow velocity , 3) MCA blood flow velocity, 4) Settings including depth and gain along with mean blood flow velocity data, 5) PCA blood flow velocity, 6) Depth of probes set in accordance with position of the red line indicating artery depth.

Ultrasound gel was used to match acoustic impedance to find the optimal signal. Probes were held in place using an adjustable head band (see **Fig. 9**).

To find the MCA, the probe was angled straight or slightly anteriorly, the depth of the probe was set between 30-60mm and was adjusted within this range once the vessel was found. The velocity of the vessel was assessed, expected to be between 50-80 $cm \cdot s^{-1}$ for younger participants and between 40-60 $cm \cdot s^{-1}$ for older participants.

To locate the PCA, the probe was angled posteriorly, the depth set to between 60-80 mm and was adjusted within this range once the vessel was found. The velocity of the vessel was assessed, expected to be around 40 $cm \cdot s^{-1}$ for younger adults and lower for older adults. Identifying insonation of the PCA was done utilising a visual stimulation test, where participants were asked to close their eyes before opening and following the researcher's finger with their eyes. A positive test resulted in cerebral velocity decreasing during eyes closed and increasing with eyes open and movement, due to the occipital lobe being supplied by the PCA. If this test was inconclusive, compression of the common carotid artery was done to confirm whether the signal was from the PCA. This technique results in blood being shunted through the circle of Willis as blood is redirected through the vertebral arteries in order to maintain perfusion to the anterior cerebral circulation, and is reflected by a disappearance in blood flow through the MCA and a large increase in blood flow through the PCA. To make sure the same area of the vessel was being insonated in each visit, photos of the probes were taken, and velocity, depth and gain values were recorded and replicated between visits.

Duplex Doppler Ultrasound

Using a 15-4Mhz (15L4 Smart MarK™) transducer attached to a Terason Duplex Doppler system (Usmart 3300 NexGen Ultrasound; Terason, United States) participants' right brachial artery was imaged for the FMD response test (protocol discussed above). Duplex Doppler allows for measurement of blood flow velocity within the artery insonated but also allows for the artery to be imaged. Therefore, providing a continuous measurement of diameter throughout testing, and allowing for accurate measurements of blood flow and measurements of shear rate. Once the image was optimised the transducer was held in place by a mechanical arm (three-axes movement probe holder, Quipu). The coefficient of variation of the researcher performing the FMD measures was 12% (established from repeat testing of 9 young male individuals prior to starting data collection for this study).

Within this measure the same section of the artery must be imaged each time, to do this within the current study the following measures were taken; photographs of the probe on the participants arm, photos of the artery during imaging, distance measurements of the probe placement on the arm, written recording of certain stand out landmarks on the image and written recordings of resting diameter.

This study utilised automatic edge-detection and wall-tracking software (CardioVascular Suite, Quipu). The image on the Terason Doppler system was connected to the software via a video converter (Epiphan AV.io HD Frame Grabber, Quipu). Within this software, a region of interest (ROI) box was drawn manually by the researcher within the optimum area on the artery, allowing the software to frame-by-frame detect the edges of the arterial walls within this ROI (see **Fig. 10**). A second ROI

is then selected around the pulse-wave velocity recording, allowing the software to detect the edges of the pulse wave form giving a measurement of velocity (see **Fig. 10**).

CANTAB

A battery of computer-based cognitive function tests were completed to assess four cognitive components: 1) a Reaction Time Test (RTI) to provide measures of motor and mental speeds as well as response accuracy; 2) an Attention Switching Task (AST) testing executive function, measuring cued attentional set-shifting; 3) Spatial Working Memory (SWM) which involves executive function demands via measures of errors and strategy; and 4) Paired Associates Learning (PAL) which assessed visual memory and new learning. All cognitive tests were conducted on an iPad (iPad model A1566; Apple, California United States) using CANTAB software (Cambridge cognition, University of Cambridge, England). The cognition tests were all conducted

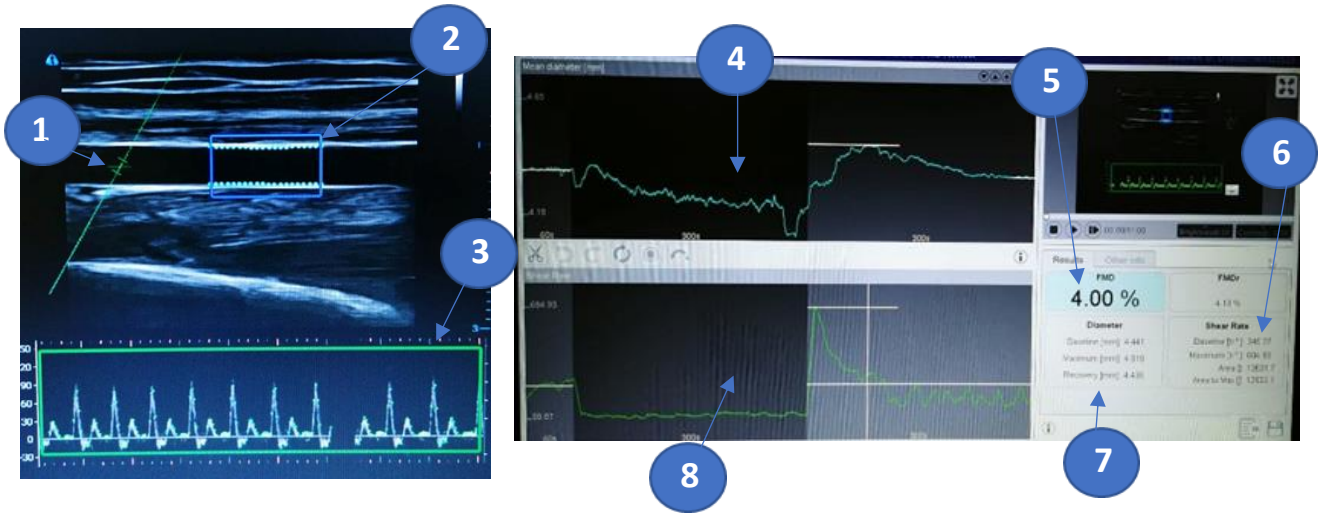


Figure 10. CardioVascular Suite software FMD studio data collection and analysis. Sections of the software are shown as follows; 1) Pulse-wave velocity sampling , 2) Arterial region of interest showing edge detections lines on arterial walls, 3) Pulse-wave velocity region of interest, 4) Continuous arterial diameter graph split into baseline, occlusion and recovery, 5) Flow mediated dilation automated calculation, 6) Shear rate calculated data, 7) Diameter calculated data, and 8) Continuous shear rate graph split into baseline, occlusion and recovery.

in the same room as the physiological tests in quiet conditions with only the researcher present. The CANTAB software included recorded voice commands that instructed the participants as to the rules of each test, as well as allowing them practice runs with and without audible feedback. The participants were also asked if they understood the instructions provided by the software and if not, they were explained by the researcher before the formal testing began. All participants were instructed to only use their index finger of their dominant hand to touch the iPad screen, and were made aware that speed and accuracy was highly important for all tests.

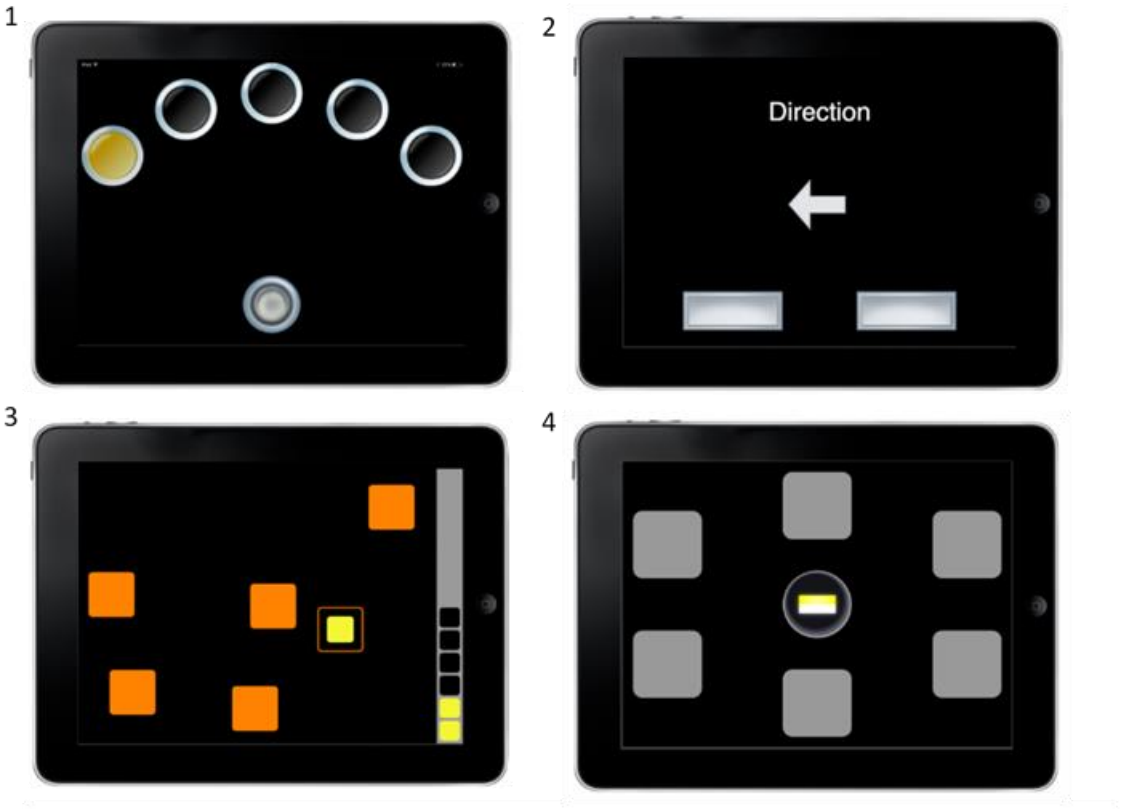


Figure 11. Cambridge Neuropsychological Test Automated Battery (CANTAB) tests. The four tests; 1) Reaction time (RTI), 2) Attention Switching Task (AST), 3) Spatial Working Memory (SWM), and 4) Paired Associates Learning (PAL).

Further Secondary Measures and Instrumentation

A three-lead ECG was attached to the participants, sensors were placed on each collar bone and one on the left lower rib, allowing continuous heart rate measurement (see **Fig. 12**).

Gas was administered (2% and 5% CO_2 gas mixture) using a 200 L Douglas bag via a T-Shaped 2-way non-rebreathing value (2700 series, Hans Rudolph, USA), and participants wore a nose clip to ensure mouth only breathing (see **Fig. 6**). End tidal CO_2 and O_2 gases were sampled using a fast-responding gas analyser (ML206, ADInstruments, Dunedin, New Zealand), and ventilation rate and volume measured used a spirometer attached to the intake value of the mouthpiece. Prior to testing, the spirometer was calibrated using a 3-L syringe (5530 series, Hans Rudolph, USA) and the gas analyser with known concentrations of CO_2 and O_2.

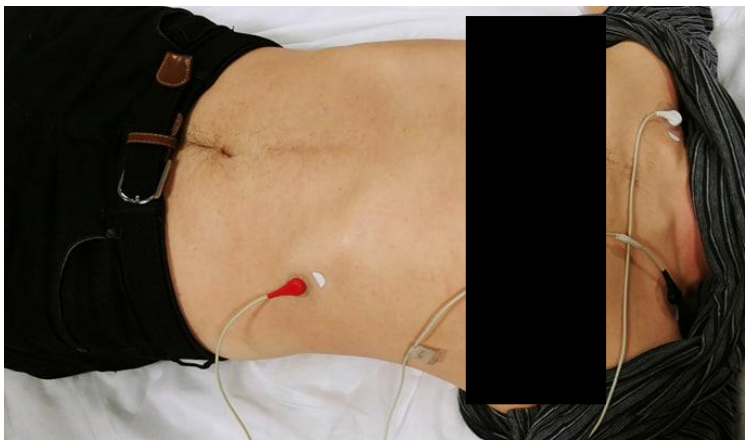


Figure 12. Three-lead electrocardiogram set

Data Acquisition

Physiological data were recorded via an analogue-to-digital converter (Powerlab PL3516, ADInstruments, Australia) and displayed in real time via LabChart software (ADInstruments, Australia) on a computer. During flow mediated dilation brachial artery

physiological data were recorded via a Terason ultrasound machine (Usmart 3300 NexGen Ultrasound; Terason, United States) onto a laptop (Fujitsu Lifebook A series, Japan), displayed and analysed in real time via QUIPU Cardiovascular Suite software (Smart Medical, United Kingdom).

Data Analysis

Flow Mediated Dilation

Assessment of each FMD video was done by running the analysis of each video a minimum of three times, moving the ROI along the artery, within optimum edge detection sections (high clarity and contrast of arterial walls) to evaluate whether the FMD percentage given was similar throughout. The reliability and accuracy of the arterial wall edge detection was done by the researcher scrutinizing the edge detection throughout the video, and utilising techniques such as using landmarking to evaluate any movement of the artery. Once at least three videos analyses were determined reliable, the FMD percentage, arterial diameter (at baseline, peak and recovery), shear rate, area to max dilation and velocity were recorded and averaged for each FMD done in the laboratory. The change in FMD percentage from baseline to 2-hours post-ingestion was then calculated for both interventions.

Cerebrovascular Reactivity

Data collected from the last 30 seconds of baseline, hypercapnia and hypocapnia was used to generate means and standard deviations. These values were then used to calculate data of interest, namely cerebrovascular conductance (CVC) and CVR. CVC for both MCA and PCA (CVC_{MCA} and CVC_{PCA}) vessels was calculated using **Eq. 3.**

$$CVC = \frac{cerebral\ velocity}{MAP} \quad (3)$$

CVR was calculated from both the change in blood flow velocity data (CVR) and change in CVC (CVC-CVR) in relation to the change in $P_{ET}CO_2$ induced from hypercapnia and hypocapnia, for both the MCA and the PCA (e.g. CVR_{MCA} and $CVC\text{-}CVR_{MCA}$). Of note, the calculated CVR measures presented within this thesis are for the relative changes in blood velocity from baseline to the inhaled 5% CO_2 (hypercapnic reactivity), and from baseline to the level of $P_{ET}CO_2$ induced from guided hyperventilation equal and opposite to that induced from the inhaled 5% CO_2 (hypocapnic reactivity).

Specifically, the two indices of CVR were calculated using **Eq. 4 and 5**.

$$CVR = \frac{\%\Delta cerebral\ velocity}{\Delta PETCO_2} \quad (4)$$

$$CVC - CVR = \frac{\Delta CVC}{\Delta PETCO_2} \quad (5)$$

CANTAB

Data for all cognition tests were analysed within the CANTAB software and outcome variables were exported from the software to Microsoft Excel. Means and standard deviations for each outcome measure were then calculated.

Statistical Analysis

Comparisons between the interventions for each age group were done via a 2-way repeated measures ANOVA (IBM SPSS Statistics Data Editor, version 23), for each outcome measure. If interaction or main effect were present, post hoc analysis was corrected using Bonferroni corrections. To compare between ages at baseline, unpaired student t-tests was used. For each CANTAB outcome an unpaired student t-test was run to analyse responses comparing both age and condition. Data are reported as means ± SD or as means alongside individual data points. Statistical significance was accepted if $P < 0.05$.

Results

Baseline Characteristics of Study Population

All participants included within this study met the inclusion criteria stated above. The study population consisted of 14 older participants (male: N=8, 57% of total older cohort; female: N=6, 43% of total older cohort) and 20 younger male participants. The average age of the cohorts was, older 71 ± 5 years and younger 21 ± 4 years. All participants were within normal diastolic and systolic blood pressure range (see **Table 5**).

Table 5. Resting physiological measures pre and 2 h post intake of either a low or high flavanol cocoa. All values are displayed as means ± SD with main and interaction significance values also displayed; * difference between pre-ingestion; † difference between conditions.

	High Flavanol Cocoa		**Low Flavanol Cocoa**		**Main Effect**		**Interaction Effect**
	Pre	*Post*	*Pre*	*Post*	*Time*	*Condition*	*Time*Condition*
Younger:							
MCAv	70 ± 11	66 ± 11*	69 ± 9	66 ± 9*	*.000*	*.898*	*.594*
PCAv	51 ± 8	47 ± 9*	51 ± 9	48 ± 10*	*.001*	*.817*	*.689*
CVC_{MCA}	1.8 ± 0.5	1.8 ± 0.5	1.7 ± 0.4	1.8 ± 0.3	*.423*	*.650*	*.164*
CVC_{PCA}	2.3 ± 0.5	2.4 ± 0.5	2.2 ± 0.4	2.4 ± 0.5	*.066*	*.856*	*.186*
Brachial cuff							
SBP	121 ± 10	123 ± 10	122 ± 10	123 ± 8	*.236*	*.753*	*.389*
DBP	65 ± 7	65 ± 7	66 ± 7	65 ± 6	*.713*	*.690*	*.938*
MAP	84 ± 7	84 ± 7	85 ± 7	85 ± 6	*.825*	*.591*	*.790*
Portapress							
MAP	78 ± 11	87 ± 17*	77 ± 13	79 ± 9*	*.035*	*.132*	*.205*
HR	57 ± 8	54 ± 7*	59 ± 6	55 ± 6*	*.004*	*.497*	*.350*
V_E	10 ± 7	10 ± 7	9 ± 5	8 ± 5	*.095*	*.408*	*.273*
$P_{ET}CO_2$	41 ± 4	41 ± 3	42 ± 2	42 ± 2	*.295*	*.377*	*.271*
Older:							
MCAv	64 ± 12	58 ± 12*	66 ± 17	59 ± 13*	*.000*	*.582*	*.740*
PCAv	-	-	-	-	-	-	-
CVC_{MCA}	1.9 ± 0.5	2.1 ± 0.5*	1.9 ± 0.6	2.1 ± 0.6*	*.041*	*.633*	*.634*
CVC_{PCA}	-	-	-	-	-	-	-
Brachial cuff							
SBP	138 ± 15	146 ± 18*	137 ± 15	144 ± 18*	*.001*	*.307*	*.770*
DBP	77 ± 8	79 ± 9	77 ± 8	78 ± 8	*.112*	*.751*	*.646*
MAP	97 ± 9	101 ± 9*	97 ± 10	100 ± 10*	*.005*	*.471*	*.619*
Portapress							

MAP	87 ± 11	93 ± 13*	77 ± 8	83 ± 16*	*.033*	*.093*	*.887*
HR	61 ± 11	59 ± 11	59 ± 14	59 ± 12	*.587*	*.712*	*.511*
V_E	11 ± 5	11 ± 6	13 ± 8	14 ± 10	*.907*	*.348*	*.587*
$P_{ET}CO_2$	39 ± 3	38 ± 3	39 ± 3	38 ± 4	*.363*	*.923*	*.971*

Abbreviations: Middle Cerebral Artery velocity (MCAv); Posterior Cerebral Artery velocity (PCAv); Middle Cerebral Artery Cerebrovascular Conductance (CVC_{MCA}); Posterior Cerebral Artery Cerebrovascular Conductance (CVC_{PCA}); Systolic blood pressure (SBP); Diastolic blood pressure (DBP); Mean Arterial Pressure (MAP); Heart rate (HR); Ventilation rate (V_E); End-tidal CO_2 ($P_{ET}CO_2$)

Resting blood pressure and ventilation measures following flavanol intake in young and older adults

In younger participants mean arterial pressure recorded from the portapres increased from pre- to post-ingestion in both interventions ($F(1,18)= 5.210$, $p = 0.035$), while heart rate decreased in both interventions ($F(1,19)= 10.976$, $p = 0.004$; see **Table 5**). There were no significant differences between the conditions for any of the resting data collected (all $p > 0.05$; **see Table 5**).

In older participants there was an increase in systolic ($F(1,13)= 16.463$, $p = 0.001$) and mean arterial blood pressure ($F(1,13)= 11.512$, $p = 0.005$) obtained from the brachial cuff, which was also seen in the MAP measure recorded via the portapress; ($F(1,8)= 6.648$, $p = 0.033$; see **Table 5**).

As expected, MAP, SBP and DBP was higher (all $p \leq 0.001$) in the older cohort compared to the younger group. End-tidal CO_2 ($P_{ET}CO_2$) was also significantly lower within the older cohort ($p \leq 0.05$).

Flavanol intake improves endothelial function (FMD) in young and older adults

Within the high flavanol condition younger participants FMD percentage increased from pre-ingestion to post-ingestion (+1.04 ± 0.77%; $F(1,14)= 27.264$, $p < 0.001$; see **Fig. 13**). In contrast, FMD percentage decreased (-0.61 ± 0.68%, $F(1,14)= 12.034$, $p = 0.004$; **Fig. 13)** after ingestion of the low flavanol drink in the younger participants. This differential effect between conditions was reflected by the significant interaction effect ($F(1,14)= 16.684$, $p = 0.001$), with posthoc comparisons showing a significant difference between conditions at 2 h only ($F(1,14)=43.190$, $p < 0.001$; **Fig. 13**).

Older participants also had an increase in FMD response post-ingestion of the high flavanol cocoa (+1.21 ± 0.40%, $F(1,11)= 102.270$, $p = 0.000$; see **Fig. 13**), but no changes were observed following the low-flavanol cocoa. Similar to the younger group, this different response between conditions was reflected by the significant interaction effect ($F(1,11)= 67.632$, $p < 0.001$), with posthoc comparisons showing a significant difference between conditions at 2 h only ($F(1,11)=51.446$, $p < 0.001$; see **Fig. 13**)

Baseline %FMD was lower for older participants compared to younger participants (2.59% vs 5.58%; $p < 0.001$; see **Fig. 13**). Similarly, at 2-hours post intervention, there were significant age differences in FMD for both interventions, with FMD being 2.70% ($p < 0.001$) and 2.55% ($p < 0.001$) lower within the older cohort in comparison to young, for the high and low flavanol cocoa conditions, respectively.

Resting brachial diameter, blood velocity and blood flow following flavanol intake in young and older adults

In young adults, there were no significant changes in brachial artery diameter after ingestion of either high flavanol (4.03 ± 0.48 mm to 4.07 ± 0.56 mm; $F(1,14)= 3.524$, $p > 0.05$) or low flavanol cocoa (4.01 ± 0.46 mm to 4.06 ± 0.48 mm; $F(1,14)= 3.524$, $p > 0.05$). There was also no difference in arterial diameter at 2 h post-ingestion between the high and low flavanol conditions ($p > 0.05$). There was a significant decrease in brachial artery velocity from pre to post-ingestion for both interventions in younger adults (high flavanol: 18.26 ± 5.68 $cm \cdot s^{-1}$ to 11.77 ± 6.48 $cm \cdot s^{-1}$; low flavanol: 19.16 ± 6.77 $cm \cdot s^{-1}$ to 11.32 ± 4.95 $cm \cdot s^{-1}$; $F(1,12)= 17.357$, $p = 0.001$), with no difference between interventions ($F(1,12)= 0.049$, $p > 0.05$). There was a decrease in brachial blood flow rate from pre to post-ingestion within both conditions (high flavanol: 138 ±

51 mL/min to 91 ± 56 mL/min; low flavanol: 145 ± 64 mL/min to 87 ± 43 mL/min; $F(1,12)= 13.486$, $p = 0.003$), however there was no difference between the conditions ($F(1,12)= 0.063$, $p > 0.05$).

There was no change in brachial artery diameter for the older cohort within the high flavanol condition (4.42 ± 0.53 mm to 4.46 ± 0.58 mm; $F(1,9)= 0.49$, $p > 0.05$) or low flavanol condition (4.35 ± 0.53 mm to 4.43 ± 0.47 mm; $F(1,9)= 0.49$, $p > 0.05$) and no difference between the conditions ($p > 0.05$). Brachial artery velocity was not significantly changed from pre- to post-ingestion in both the high flavanol condition (14.50 ± 6.62 $cm \cdot s^{-1}$ to 9.50 ± 4.71 $cm \cdot s^{-1}$; $F(1,11)= 1.625$, $p > 0.05$) and the low flavanol condition (14.48 ± 6.98 cm/s to 14.23 ± 11.31 $cm \cdot s^{-1}$; $F(1,11)= 1.625$, $p > 0.05$) within the older cohort, and there were no differences between the conditions ($p > 0.05$). There were no significant changes in blood flow rate from pre- to post-ingestion in either condition (high flavanol: 127 ± 63 mL/min to 91 ± 52 ml/min; low flavanol: 140 ± 68 mL/min to 137 ± 137 mL/min; $F(1,9)= 1.006$, $p > 0.05$), and no differences between the conditions.

There were no age differences observed for either brachial artery diameter, velocity or blood flow rate (all $p > 0.05$).

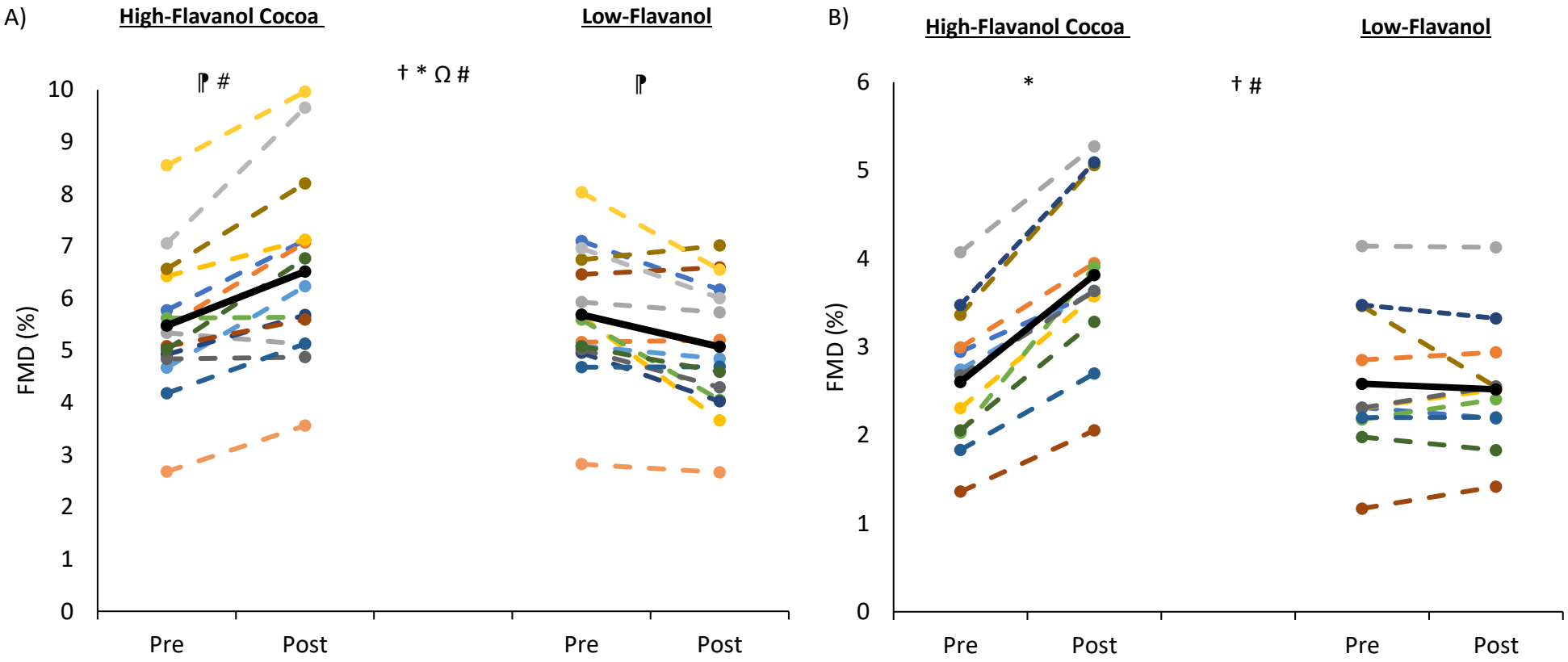


Figure 13. Shows the change in FMD (%) from pre-ingestion (pre) to post-ingestion (post) within both high flavanol cocoa and low flavanol cocoa, in younger (A) and older (B) participants. All individual data is displayed including the means for each cohort (as shown by the black line). Main effect significance; * P<0.05, comparison between time points (pre vs post), † P<0.05, comparison between conditions (high flavanol cocoa vs low flavanol cocoa), # P<0.05, age differences (younger vs older cohorts). Interaction effect significance; ⁋ P<0.05, condition (high flavanol cocoa vs low flavanol cocoa) and time interaction (pre vs post), ¥ P<0.05, time (pre) and condition interaction (high flavanol cocoa vs low flavanol cocoa), Ω P<0.05, time (post) and condition interaction (high flavanol cocoa vs low flavanol cocoa).

Resting Cerebrovascular Responses to Flavanol intake in young and older adults

Younger participants resting MCAv decreased by 5% and 4% from pre to post-ingestion of the high flavanol cocoa and low flavanol cocoa, respectively ($F(1,19)= 27.848$, $p< 0.001$; see **Table 5**). Resting PCAv also decreased by 7% and 8% from pre to post-ingestion of the high flavanol cocoa and low flavanol cocoa, respectively ($F(1,14)= 18.358$, $p = 0.001$; see **Table 5**). There were no changes in resting middle or posterior cerebral artery cerebrovascular conductance (CVC_{MCA}, CVC_{PCA}) from pre- to post-ingestion within either condition for younger adults ($p > 0.05$).

Older participants resting MCAv significantly decreased by 10% and 11% from pre to post-ingestion of the high flavanol cocoa and low flavanol cocoa, respectively ($F(1,13)= 26.367$, $p < 0.001$; see **Table 5**). There was also an increase in resting middle cerebral artery cerebrovascular conductance (CVC_{MCA}) from pre to post-ingestion within both conditions ($F(1,11)= 5.351$, $p = 0.041$; see **Table 5**). Due to age-related difficulties insonating the PCA, not enough data were obtained from the older cohort to warrant analysis, therefore no PCA data from the older cohort will be presented.

Expected age-related differences were only observed (significantly) for MCAv at the high-flavanol post-ingestion time point ($p = 0.049$). All other resting cerebrovascular measures were not significantly different between age groups.

Blood velocity Cerebrovascular Reactivity (CVR)

Within the younger cohort, hypercapnic CVR_{MCA} decreased from pre- to post-ingestion within both conditions ($F(1,19)= 12.556$, $p = 0.002$), and while the decrease in the high flavanol condition was on average greater, this difference was not significantly different between the conditions (interaction effect: $p = 0.083$; **Table 6** and **Fig. 14**). There was

no significant change in the younger cohort's hypercapnic CVR_{PCA}, hypocapnic CVR_{MCA} or hypocapnic CVR_{PCA} from pre- to post-ingestion in either condition, with no differences between the conditions ($p > 0.05$ for all; see **Fig. 15** and **Table 6**).

Within the older cohort, there was no significant change in hypercapnic CVR_{MCA} from pre- to post-ingestion in either condition, and no differences between the two conditions were seen ($p > 0.05$; see **Table 6** and **Fig. 14**). Hypocapnic CVR_{MCA} also did not change from pre- to post-ingestion in either condition within the older cohort, and no differences between the conditions were seen ($p > 0.05$; see **Table 6**).

There were no statistically significant differences between age groups for any measured hypercapnic or hypocapnic CVR time point.

Conductance Cerebrovascular Reactivity (CVC-CVR)

A significant interaction was observed for hypercapnic CVC-CVR_{MCA} in the younger cohort ($F(1,18)= 10.698$, $p = 0.004$; see **Table 6 and Fig. 16**). Posthoc analysis revealed that in the high flavanol condition hypercapnic CVC-CVR_{MCA} significantly decreased from pre to post (0.189 ± 2.43 to -2.089 ± 2.64, $F(1,18)= 34.199$, $p < 0.001$), while no significant change was seen in the low flavanol condition. In contrast, no changes for either condition were observed for CVC-CVR_{PCA} in the younger participants. In response to hypocapnia, younger participants' CVC-CVR_{MCA} response significantly increased from pre- to post-ingestion ($F(1,17)= 6.604$, $p = 0.020$; see **Table 6**), however there was no difference between the conditions ($p > 0.05$). Similarly, the CVC-CVR_{PCA} response to hypocapnia also increased from pre- to post-ingestion ($F(1,11)= 5.869$, $p = 0.034$) for the younger cohort, but there were no differences between the conditions ($F(1,11)= 0.327$, $p = 0.579$, see **Table 6**).

There was a significant decrease in hypercapnic CVC-CVR_{MCA} within the older cohort from pre to post-ingestion in both conditions ($F(1,11) = 40.949$, $p < 0.001$), however there was no difference between the two conditions ($p > 0.05$). No changes in hypocapnic CVC-CVR_{MCA} were seen within the older cohort ($p > 0.05$; see **Table 6**).

In the high flavanol condition during hypercapnia there was a significant age difference with younger participants having a higher CVC-CVR_{MCA} both pre-ingestion ($p = 0.002$; see **Table 6** and **Fig. 16**) as well as post-ingestion ($p = 0.002$; see **Table 6** and **Fig. 16**). Within the low flavanol condition, there was no age difference pre-ingestion for hypercapnic CVC-CVR_{MCA}, but there was post-ingestion ($p = 0.008$; see **Table 6** and **Fig. 16**). There were age differences throughout the hypocapnic challenges in both conditions during pre and post-ingestion, in which older participants had a higher hypocapnic CVC-CVR_{MCA} ($p < 0.05$) than the younger participants.

Table 6. Cerebrovascular velocity reactivity (CVR) and conductance reactivity (CVC-CVR) to 5% CO_2 (hypercapnia) and voluntary hyperventilation (hypocapnia) both pre- and post-ingestion of high and low flavanol conditions. All values are displayed as means ± SD with main and interaction significance values also displayed; * difference between pre-ingestion; † difference between conditions.

	High Flavanol Cocoa		**Low Flavanol Cocoa**		**Main Effect**		**Interaction Effect**
	Pre	*Post*	*Pre*	*Post*	*Time*	*Condition*	*Time* Condition*
Younger:							
Hypercapnia							
CVR_{MCA}	4.89 ± 1.34	3.57 ± 1.21*	4.27 ± 1.78	4.13 ± 1.29*	*.002*	*.923*	*.083*
CVR_{PCA}	4.41 ± 1.92	3.67 ± 1.20	4.09 ± 2.16	3.92 ± 1.39	*.204*	*.924*	*.491*
$CVC\text{-}CVR_{MCA}$	0.19 ± 2.43	-2.09 ± 2.64*†	-0.89 ± 2.45	-1.41 ± 3.29	*.000*	*.625*	*.004*
$CVC\text{-}CVR_{PCA}$	-6.44 ± 3.22	-7.84 ± 3.46	-6.57 ± 4.06	-7.21 ± 4.01	*.184*	*.647*	*.532*
Hypocapnia							
CVR_{MCA}	2.72 ± 1.04	2.70 ± 0.62	2.69 ± 0.69	2.78 ± 0.65	*.713*	*.890*	*.587*
CVR_{PCA}	2.89 ± 0.55	2.85 ± 0.87	3.07 ± 0.50	3.16 ± 0.77	*.833*	*.186*	*.610*
$CVC\text{-}CVR_{MCA}$	4.02 ± 1.82	4.24 ± 1.24*	4.05 ± 1.30	4.47 ± 1.26*	*.020*	*.689*	*.455*
$CVC\text{-}CVR_{PCA}$	5.85 ± 1.53	6.09 ± 1.24*	5.79 ± 1.09	6.67 ± 1.79*	*.034*	*.579*	*.134*
Older:							
Hypercapnia							
CVR_{MCA}	4.21 ± 1.63	3.85 ± 1.00	4.02 ± 1.32	3.65 ± 1.14	*.180*	*.335*	*.994*
$CVC\text{-}CVR_{MCA}$	-2.57 ± 2.32	-4.88 ± 2.16*	-2.06 ± 2.86	-4.33 ± 2.55*	*.000*	*.357*	*.964*
Hypocapnia							
CVR_{MCA}	3.14 ± 1.05	2.98 ± 0.67	3.21 ± 0.76	3.13 ± 1.09	*.450*	*.496*	*.885*
$CVC\text{-}CVR_{MCA}$	6.34 ± 2.83	6.63 ± 1.96	5.99 ± 2.26	7.63 ± 4.64	*.114*	*.712*	*.320*

Abbreviations: Middle Cerebral Artery Cerebrovascular Reactivity (CVR_{MCA}); Posterior Cerebral Artery Cerebrovascular Reactivity (CVR_{PCA}); Middle Cerebral Artery Cerebrovascular Conductance Reactivity ($CVC\text{-}CVR_{MCA}$); Posterior Cerebral Artery Cerebrovascular Conductance Reactivity ($CVC\text{-}CVR_{PCA}$)

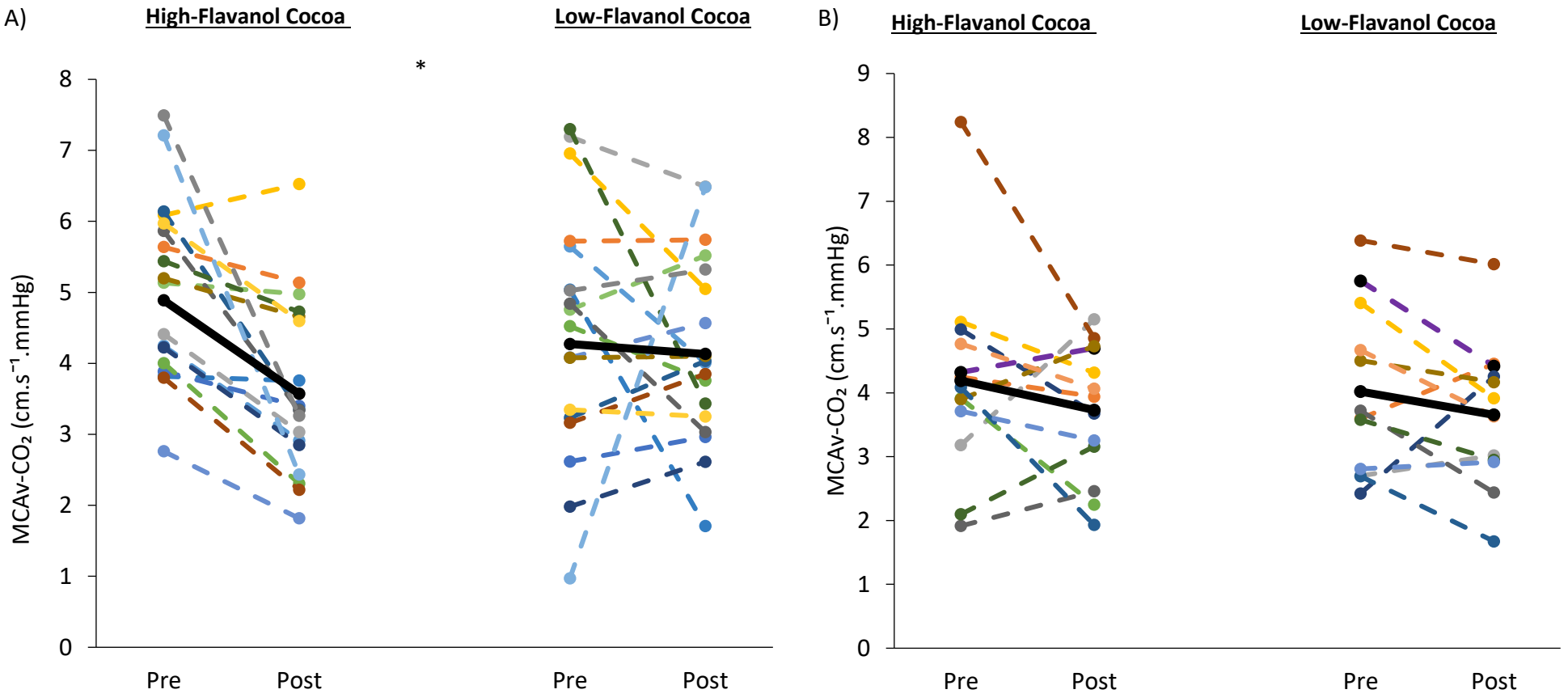


Figure 14. Shows Middle Cerebral Artery velocity in relation to $P_{ET}CO^2$ ($MCAv\text{-}CO_2$) in hypercapnia pre-ingestion and post-ingestion of both high flavanol cocoa and low flavanol cocoa, in younger (A) and older (B) participants. All individual data is displayed including the means for each cohort (as shown by the black line). Main effect significance; * P<0.05, comparison between time points (pre vs post), † P<0.05, comparison between conditions (high flavanol cocoa vs low flavanol cocoa), # P<0.05, age differences (younger vs older cohorts). Interaction effect significance; ⁋ P<0.05, condition (high flavanol cocoa vs low flavanol cocoa) and time interaction (pre vs post), ¥ P<0.05, time (pre) and condition interaction (high flavanol cocoa vs low flavanol cocoa), Ω P<0.05, time (post) and condition interaction (high flavanol cocoa vs low flavanol cocoa).

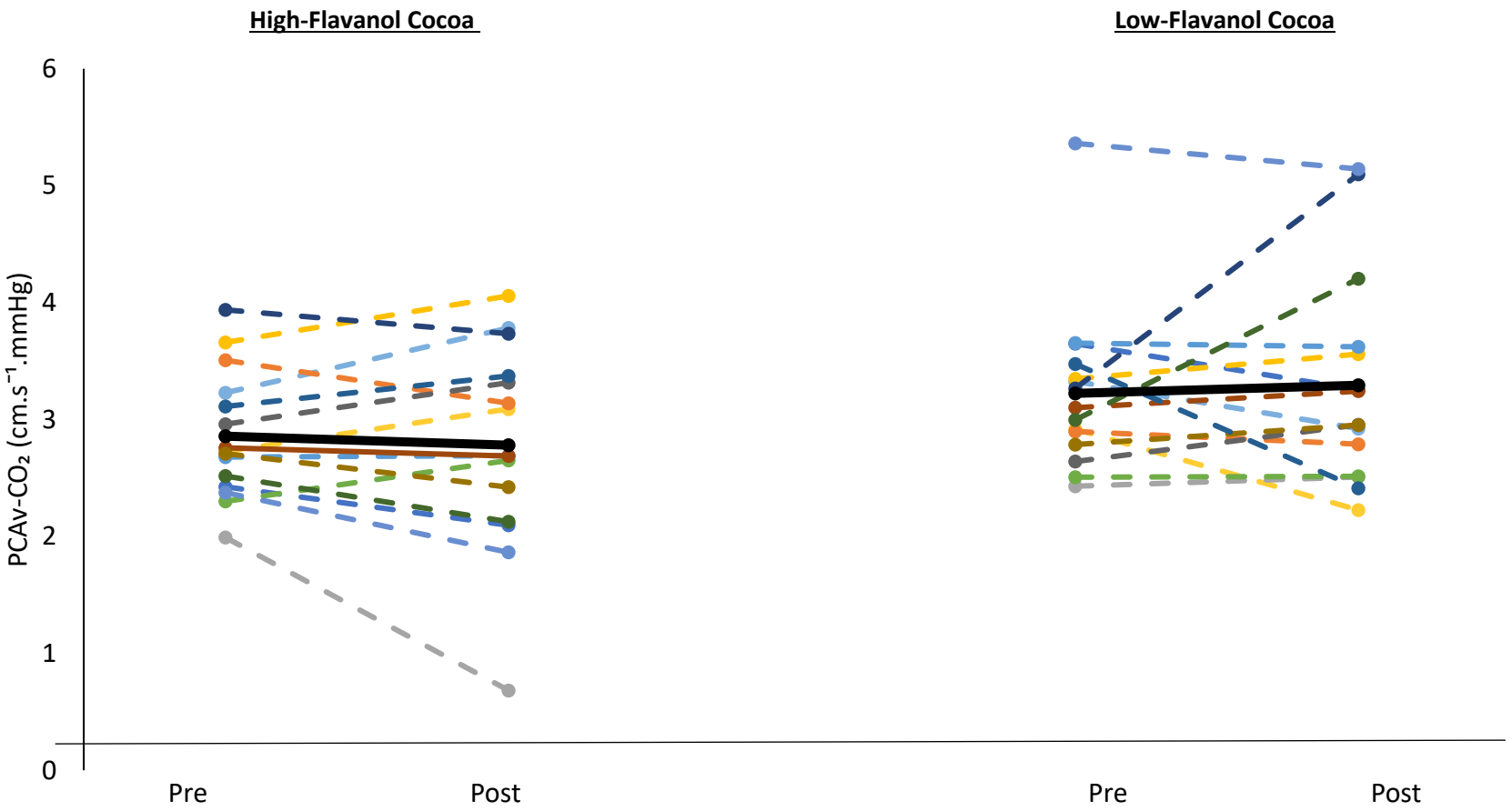


Figure 15. Shows Posterior Cerebral Artery velocity in relation to $P_{ET}CO^2$ ($PCAv\text{-}CO_2$) in hypercapnia pre-ingestion and post-ingestion of both high flavanol cocoa and low flavanol cocoa, in younger participants. All individual data is displayed including the means (as shown by the black line). Main effect significance; * P<0.05, comparison between time points (pre vs post), † P<0.05, comparison between conditions (high flavanol cocoa vs low flavanol cocoa), # P<0.05, age differences (younger vs older cohorts). Interaction effect significance; ⁋ P<0.05, condition (high flavanol cocoa vs low flavanol cocoa) and time interaction (pre vs post), ¥ P<0.05, time (pre) and condition interaction (high flavanol cocoa vs low flavanol cocoa), Ω P<0.05, time (post) and condition interaction (high flavanol cocoa vs low flavanol cocoa).

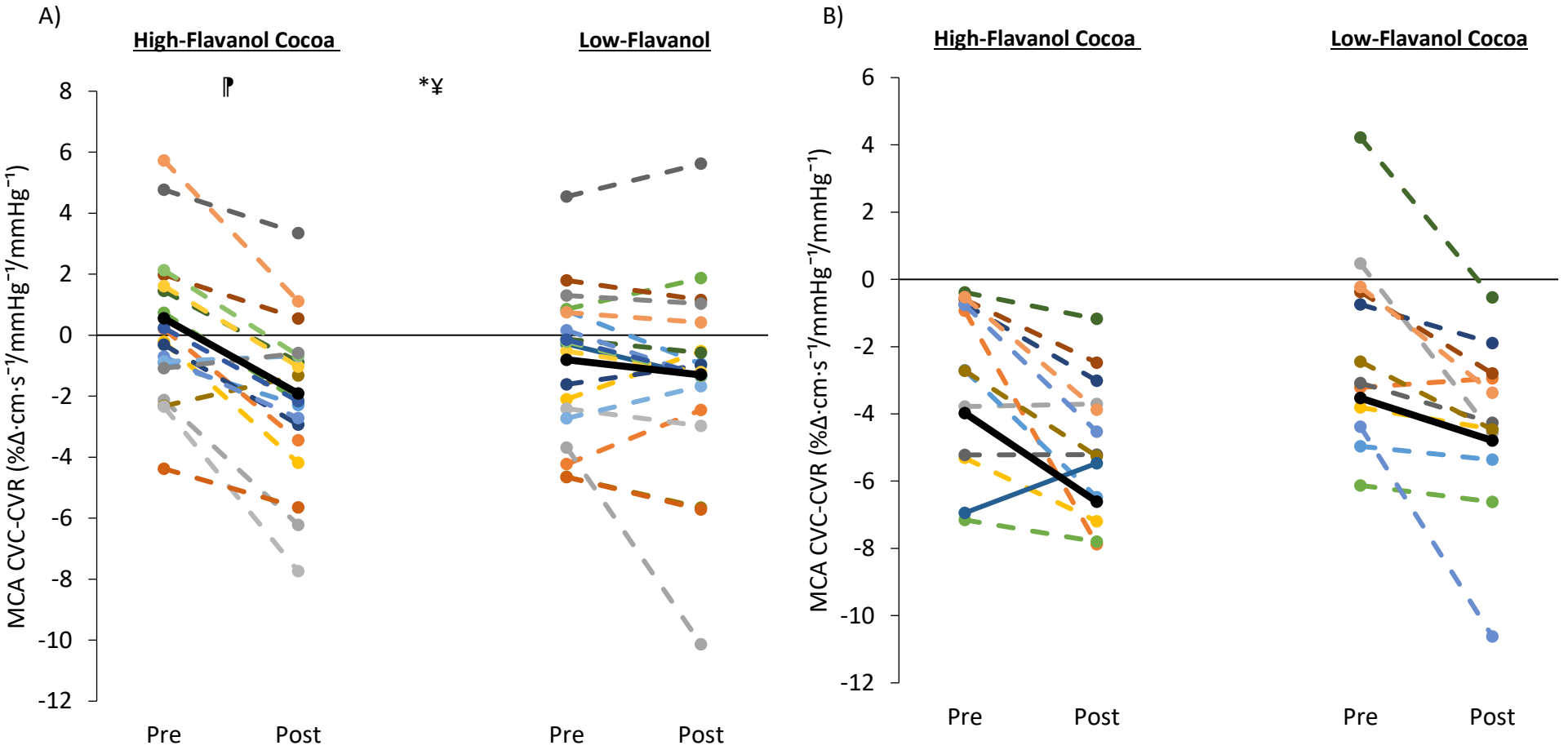


Figure 16. Shows Middle Cerebral Artery velocity accounting for blood pressure in relation to $P_{ET}CO^2$ (MCA CVC-CVR) in hypercapnia (5% CO_2) pre-ingestion and post-ingestion of both condition high flavanol cocoa and low flavanol cocoa, in younger (A) and older (B) participants. All individual data is displayed including the means for each cohort (as shown by the black line). Main effect significance; * P<0.05, comparison between time points (pre vs post), † P<0.05, comparison between conditions (high flavanol cocoa vs low flavanol cocoa), # P<0.05, age differences (younger vs older cohorts). Interaction effect significance; ⁋ P<0.05, condition (high flavanol cocoa vs low flavanol cocoa) and time interaction (pre vs post), ¥ P<0.05, time (pre) and condition interaction (high flavanol cocoa vs low flavanol cocoa), Ω P<0.05, time (post) and condition interaction (high flavanol cocoa vs low flavanol cocoa).

Cognitive performance in response to flavanol intake in young and older adults

Paired Associates Learning (PAL)

Within PAL, the younger participants on average made less errors within the low flavanol cocoa condition in comparison to the high flavanol cocoa condition ($F(1,19) = 4.772$, $p = 0.042$; see **Table 7**).

The older participants showed no change in errors made between the two conditions (see **Table 7**).

As expected, younger participants made significantly less errors than the older participants, regardless of condition (both $p < 0.001$).

Spatial Working Memory (SWM)

Within the SWM task, the average number of errors participants made prior to completing the task (SWMS) was not different between the conditions for the younger cohort (see **Table 7**). The number of errors a participant made for the final level with 12 tokens (SWMBE12) and as an average for levels with 4, 6 and 8 tokens (SWMBE468) was similar in both conditions (see **Table 7**).

Within the older cohort there was no difference between the two conditions for any of the spatial working memory outcome measures (SWMS, SWMBE12 or SWMBE468; see **Table 7**).

Similar to the PAL findings, younger participants performed better than the older participants for the SWMS, SWMBE12 or SWMBE468 outcomes of the SWM task. Specifically, compared to the older cohort, younger participants made less errors prior to completing the task across all difficulty levels (all t-tests: $p<0.01$, see **Table 7**).

Attention Switching Task (AST)

Within the AST, the median latency response from the stimulus appearance to the participant pressing the button on congruent trails (ASTLCMD) was not different between the conditions for the younger participants (see **Table 7**). The median latency response from the stimulus appearance to the button press during rule switching (ASTLSWMD) was similar between conditions the younger participants (see **Table 7**).

The older cohort also had no significant differences between the two conditions for either ASTLCMD or ASTLSWMD (see **Table 7**).

As with the other CANTAB tests, younger participants' performance was better than the older cohort. Specifically, both AST latency response outcome measures (ASTLCMD, ASTLSWMD) were significantly longer for the older cohort, regardless of high or low flavanol conditions (all $p < 0.001$; see **Table 7** and **Fig. 19**).

Reaction Time (RTI)

Both reaction time and movement time for the younger participants was not different between the two conditions (see **Table 7**). Similarly, both reaction and movement time for the older cohort did not differ between the two conditions (see **Table 7**).

As expected, reaction and movement time was faster in the younger cohort, regardless of flavanol condition (all $p < 0.001$; see **Table 7**, **Fig. 17** and **Fig. 18**).

Table 7. Number of incorrect choices within cognition test outcomes; Paired Associates Learning, Spatial Working Memory (12), Spatial Working Memory (4, 6 & 8) and Spatial Working Memory no. of incorrect before correct choice, and response time (ms) within cognition test outcomes; Attention Switching Task (switching task), Attention Switching Task (congruent), Reaction Time Task (reaction time) and Reaction Time Task (movement time), for both the high flavanol cocoa and low flavanol cocoa conditions, in younger and older participants. All values are displayed as means ± SD with main and interaction significance values also displayed.

	High Flavanol Cocoa		**Low Flavanol Cocoa**		**p value** (high vs. low flavanol post-ingestion).	
	Young	*Old*	*Young*	*Old*	*Young*	*Old*
Paired Associates Learning *(# of incorrect choices)*	5 ± 5	29 ± 14	3 ± 3	22 ± 9	*.042*	*.088*
Spatial Working Memory *(# of incorrect boxes revisited for 12 tokens)*	12 ± 9	36 ± 13	13 ± 9	37 ± 12	*.734*	*.617*
Spatial Working Memory *(# of incorrect boxes revisited for 4, 6 & 8 tokens)*	5 ± 5	20 ± 12	3 ± 3	14 ± 6	*.091*	*.067*
Spatial Working Memory *(# of incorrect choices before correct choice)*	4 ± 3	8 ± 3	4 ± 2	8 ± 3	*.674*	*.748*
Attention Switching Task *(response time in switching task; ms)*	533 ± 94	949 ± 156	518 ± 77	984 ± 171	*.419*	*.359*
Attention Switching Task *(congruent task response time; ms)*	479 ± 70	729 ± 79	471 ± 59	729 ± 74	*.474*	*.986*
Reaction Time Task *(reaction time; ms)*	374 ± 41	439 ± 35	369 ± 41	434 ± 42	*.362*	*.598*
Reaction Time Task *(movement time; ms)*	203 ± 47	329 ± 97	202 ± 44	317 ± 79	*.854*	*.444*

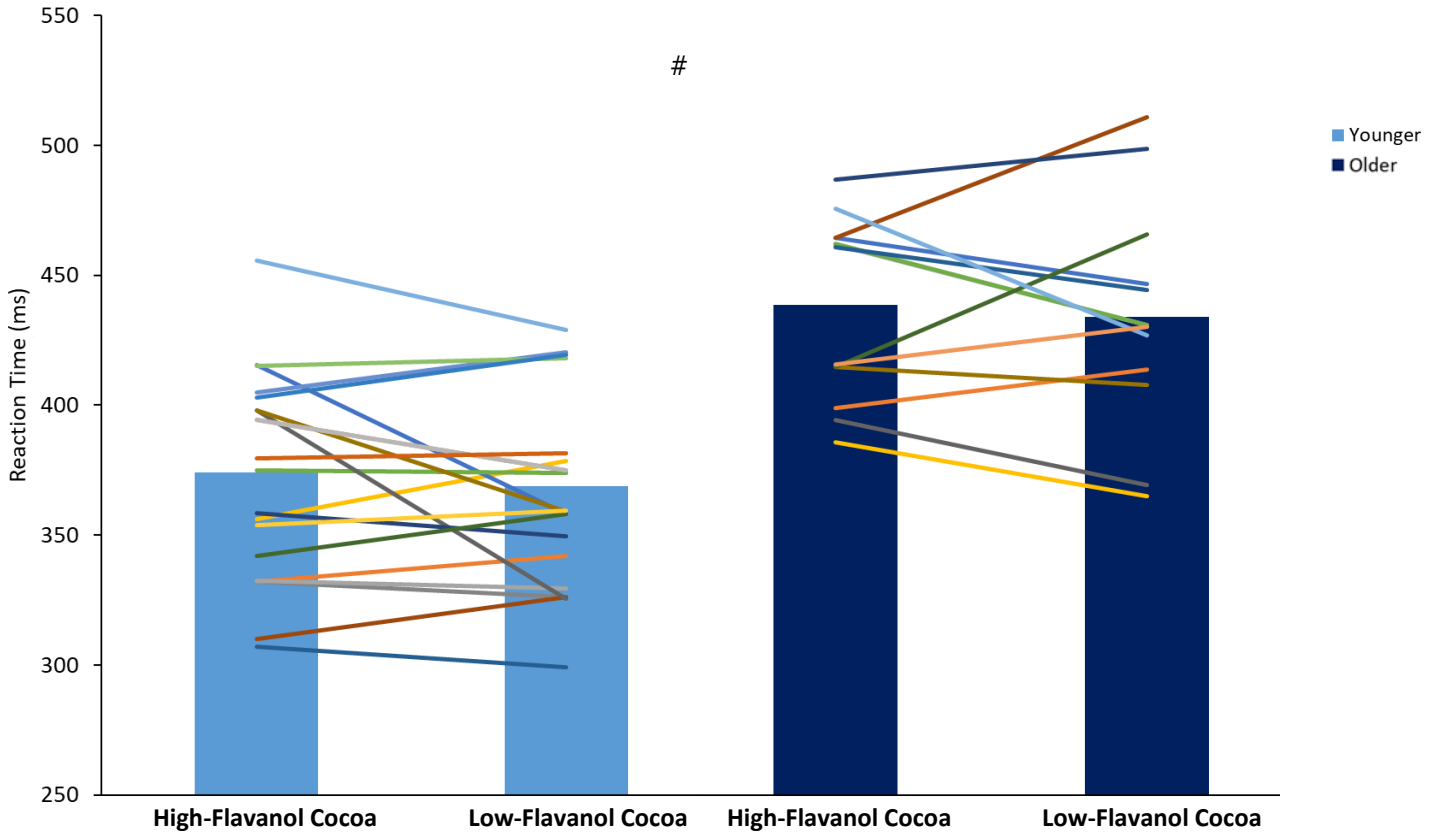


Figure 17. Shows reaction time data within the Reaction Time task. All individual data is displayed including the means (as shown by bar charts). Main effect significance; * P<0.05, comparison between time points (pre vs post), † P<0.05, comparison between conditions (high flavanol cocoa vs low flavanol cocoa), # P<0.05, age differences (younger vs older cohorts). Interaction effect significance; ⁋ P<0.05, condition (high flavanol cocoa vs low flavanol cocoa) and time interaction (pre vs post), ¥ P<0.05, time (pre) and condition interaction (high flavanol cocoa vs low flavanol cocoa), Ω P<0.05, time (post) and condition interaction (high flavanol cocoa vs low flavanol cocoa).

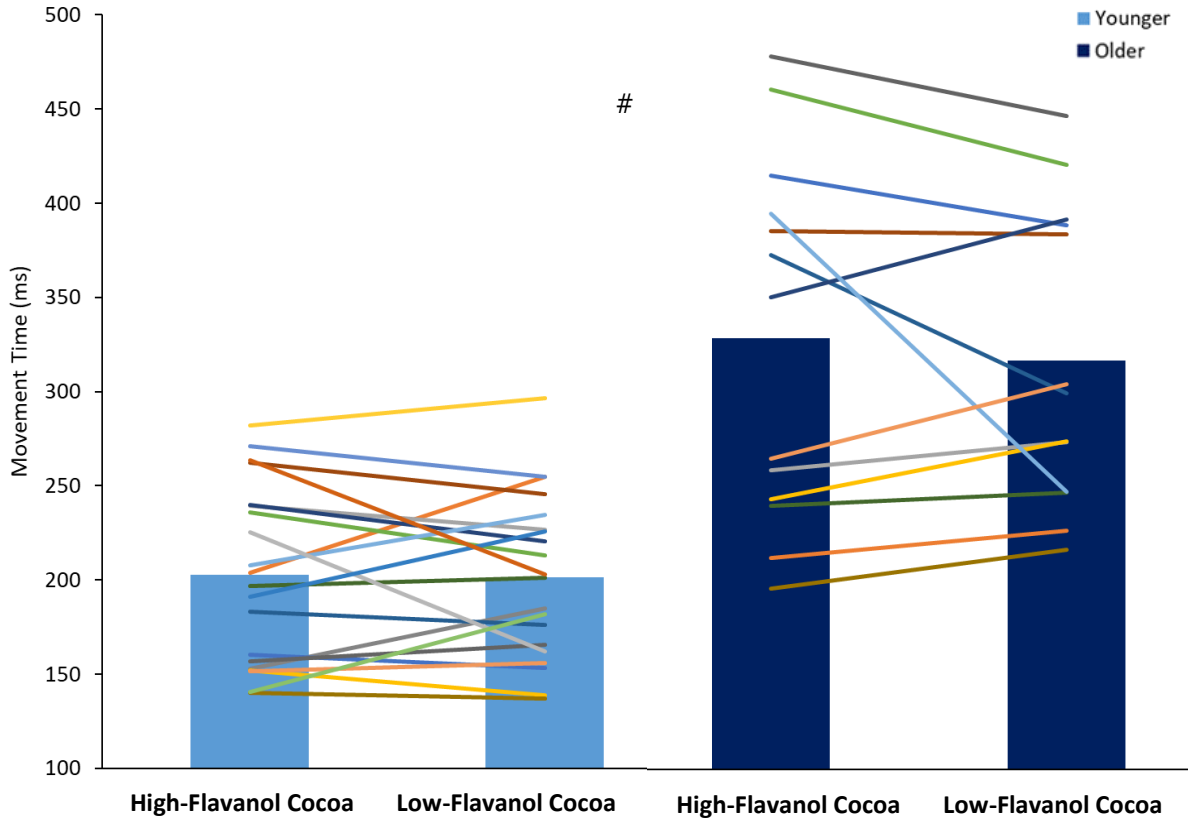


Figure 18. Shows movement time data within the Reaction Time task. All individual data is displayed including the means (as shown by bar charts). Main effect significance; * P<0.05, comparison between time points (pre vs post), † P<0.05, comparison between conditions (high flavanol cocoa vs low flavanol cocoa), # P<0.05, age differences (younger vs older cohorts). Interaction effect significance; ⁋ P<0.05, condition (high flavanol cocoa vs low flavanol cocoa) and time interaction (pre vs post), ¥ P<0.05, time (pre) and condition interaction (high flavanol cocoa vs low flavanol cocoa), Ω P<0.05, time (post) and condition interaction (high flavanol cocoa vs low flavanol cocoa).

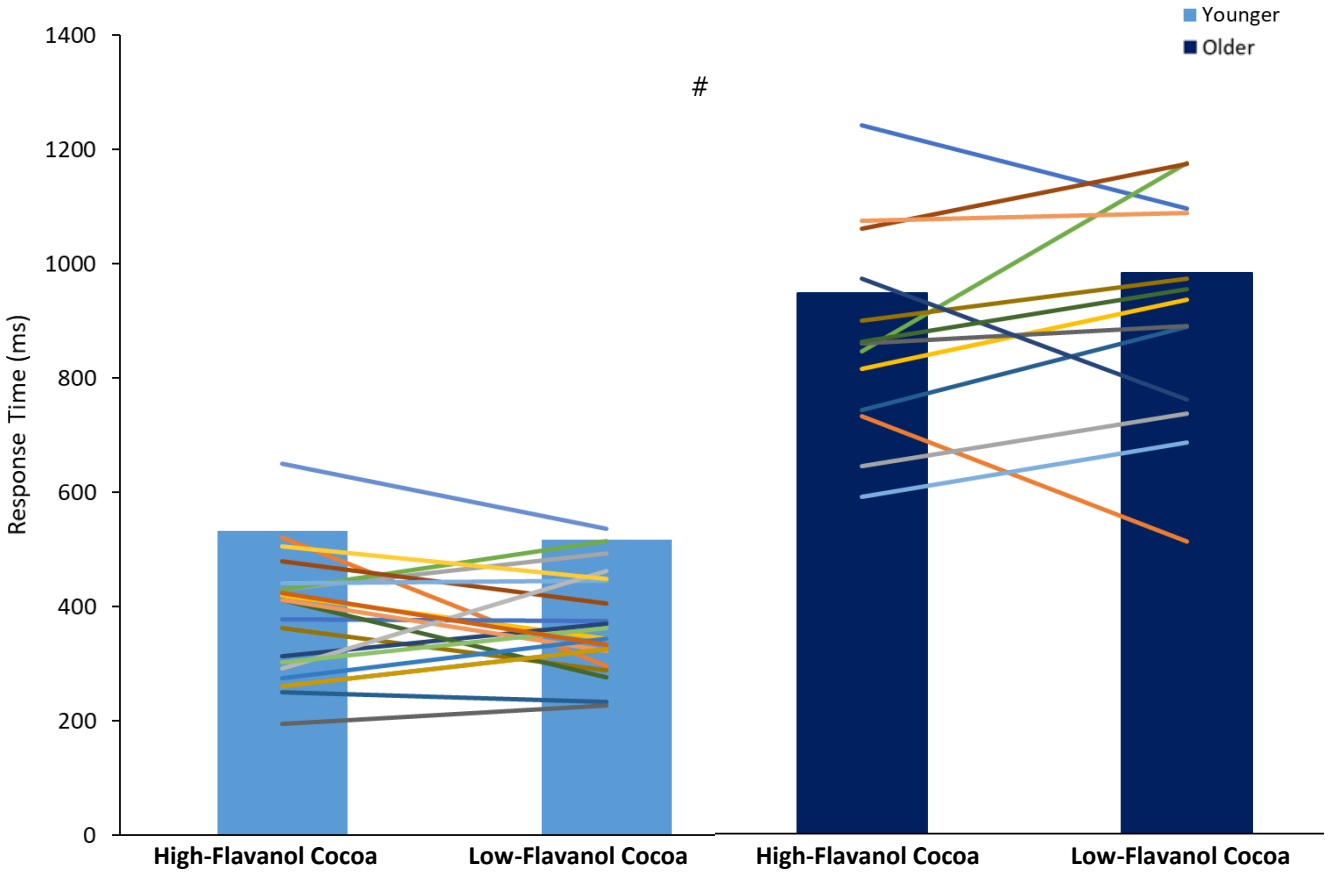


Figure 19. Shows the median latency response time data within the Attention Switching Task during rule switching. All individual data is displayed including the means (as shown by bar charts). Main effect significance; * P<0.05, comparison between time points (pre vs post), † P<0.05, comparison between conditions (high flavanol cocoa vs low flavanol cocoa), # P<0.05, age differences (younger vs older cohorts). Interaction effect significance; ⁋ P<0.05, condition (high flavanol cocoa vs low flavanol cocoa) and time interaction (pre vs post), ¥ P<0.05, time (pre) and condition interaction (high flavanol cocoa vs low flavanol cocoa), Ω P<0.05, time (post) and condition interaction (high flavanol cocoa vs low flavanol cocoa).

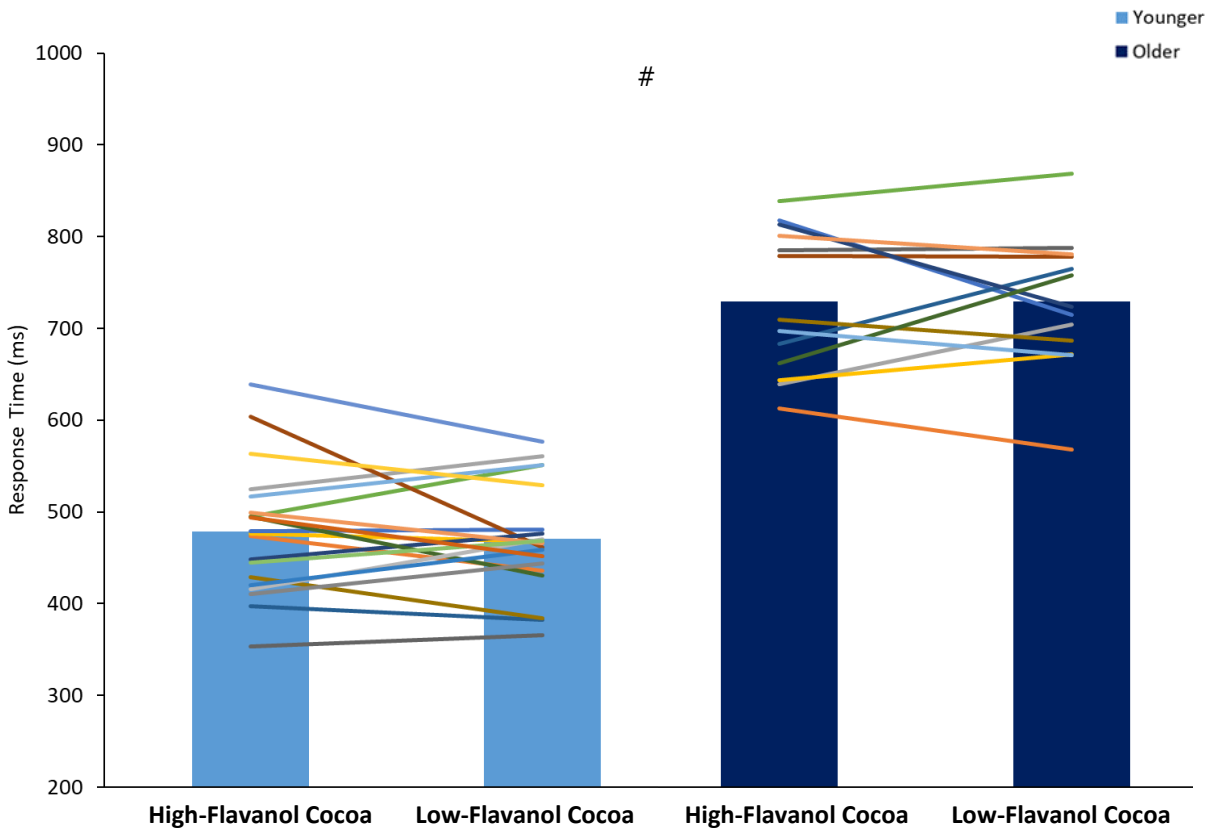


Figure 20. Shows the median latency response time data within the Attention Switching Task during congruent tasks. All individual data is displayed including the means (as shown by bar charts). Main effect significance; * P<0.05, comparison between time points (pre vs post), † P<0.05, comparison between conditions (high flavanol cocoa vs low flavanol cocoa), # P<0.05, age differences (younger vs older cohorts). Interaction effect significance; ⁋ P<0.05, condition (high flavanol cocoa vs low flavanol cocoa) and time interaction (pre vs post), ¥ P<0.05, time (pre) and condition interaction (high flavanol cocoa vs low flavanol cocoa), Ω P<0.05, time (post) and condition interaction (high flavanol cocoa vs low flavanol cocoa).

Discussion

The aim of the current study was to investigate the hypothesis that peripheral vascular function, cerebrovascular function and cognition is improved within healthy adults after acute ingestion of flavanol rich cocoa. Although the acute impact of flavanols in peripheral vascular function is well established in healthy adults, the extent to which benefits in the periphery translate into the cerebrovasculature is less understood. Furthermore, this study explored the difference in these physiological measures between younger and older participants, to examine whether cocoa ingestion can reduce the disparity in vascular and cognitive function between the age groups. As previously shown, endothelial function, as measured by brachial FMD, improved after intake of the high-flavanol cocoa in both young and older adults. Brachial blood flow decreased within the younger cohort for both interventions, but brachial artery diameter remained unchanged, no change was noted within the older adults. Middle cerebral artery reactivity decreased after both interventions' conditions for the older cohort, but within the younger cohort there was only a significant decrease seen within the high flavanol condition. No changes in cognition were noted in response to flavanol intake. These findings indicate that acute high flavanol cocoa ingestion can acutely enhance peripheral endothelial function in young and older adults, as previously demonstrated. Moreover, in young healthy adults high flavanol cocoa intake reduces cerebrovascular reactivity only in the middle cerebral artery, but not in the posterior cerebral artery, suggesting an acute and region-specific modulation of cerebrovascular function. Collectively, these findings illustrate potential opposing effects between the peripheral and cerebral vasculature for the functional tests used within this thesis. The following discussion will consider these findings in the context of the current literature as well as

the methodological considerations that may explain the unexpected reduction in cerebrovascular reactivity following cocoa ingestion.

High-flavanol cocoa improves endothelial function in healthy young and older adults

In the present study, acute ingestion of flavanol rich cocoa increased FMD significantly by 1.04% in younger participants and 1.21% in older participants. These findings agree with previous studies, showing that on average younger participants experience improvements in FMD of 2.55%, whilst older adults observe an improvement of 1.75% on average within 2-hours of cocoa ingestion (Taubert et al., 2003; Heiss et al., 2003; Grassi et al., 2005; Heiss et al., 2005; Vlachopoulos et al., 2005; Hermann et al., 2006; Schroeter et al., 2006; Heiss et al., 2007; Balzer et al., 2008; Davison et al., 2008; Monahan et al., 2011; Sansone et al., 2015; Sansone et al., 2017; Marsh et al., 2017). An increase in FMD percentage of this degree has clinical significance to reduce cardiovascular disease risk, in accordance with a meta-analysis composed by Inaba and colleagues (2010). If maintained over the long term, this FMD change would decrease risk of a future cardiovascular event within the younger cohort by 8.3% and within the older cohort by 9.7% (Inaba et al., 2010).

High-flavanol cocoa reduces cerebrovascular conductance reactivity in young but not in older adults

High flavanol cocoa decreased cerebrovascular reactivity (both CVR_{MCA} and CVC-CVR_{MCA}) within younger participants. This was in contrast to our hypothesis and opposite to what we observed for the peripheral vascular response test (i.e. FMD). We hypothesised that the enhanced cerebrovascular reactivity would be mediated by increased levels of circulating nitric oxide (NO), which has been shown to occur after

flavanol intake (Fisher et al., 2003; Rimbach et al., 2009). One explanation for our blunted CVR observation is that higher NO from the cocoa resulted in increased vasodilation at baseline within the MCA, therefore, the vessel would have a smaller vasodilatory reserve capacity prior to the CO_2 challenge. However, given the nature of the imaging system utilized (transcranial Doppler ultrasound), we were unable to directly measure the diameter of MCA to confirm the levels of dilation at rest post flavanol intake. Therefore, to explore this hypothesis a small separate study (see **Appendix A.5**) was conducted within a different group of young healthy participants (N = 8); where we used ultrasonography to image both the carotid (CA) and internal carotid (ICA) arteries (which are distal to and supply the MCA) following intake of both the low and high flavanol cocoa interventions used for this study. However, there was no difference in vessel diameter or blood flow in the CA (**Fig A1.**) or ICA (**Fig A2.**) following both interventions. Nevertheless, given the low number of volunteers in this second study (N=8), we cannot confidently rule out, at this time, that no differences in vessel dilation exist between dietary interventions.

However, assuming that at rest, vasodilation is similar between the two interventions (2 h post intake), we would predict that flavanols would increase reactivity to CO_2, rather than induce a blunted response like we observed. Reactivity to CO_2 has been shown to be at least partially dependent on the vasodilatory properties of NO (Wang et al., 1992; Schmetterer et al., 1997; Lavi et al., 2003), and that flavanols have been shown to modulate eNOS activity and result in increased levels of circulating NO (Schroeter et al., 2006). In agreement with this, there is evidence showing that exogenous administration of NO donors increases CVR to hypercapnia (Lindauer et al., 2001; Lavi et al., 2003). However, other studies have also shown that utilising L-

NMMA to block NO had no effect on MCA reactivity to hypercapnia (White et al., 1998; Ide et al., 2007). A possible reasoning for this disparity within the literature is due to the redundancy within cerebral regulatory mechanisms, once NO is blocked there is a compensation from other vasodilatory pathways (Coverdale et al., 2015). This redundancy in cerebral regulation is incredibly important for maintaining healthy cerebral blood flow (Coverdale et al., 2015), but might make interpretation of the effects of flavanols on the cerebrovascular vasculature more challenging. Flavanol-induced improvements in vasodilatory capacity in the brachial artery (as measured by FMD) have been attributed largely to NO-dependent mechanisms (Schroeter et al., 2006; Heiss et al., 2007; Balzer et al., 2008; Davison et al., 2008; Monahan et al., 2011; Marsh et al., 2017; Sansone et al., 2017), but our findings indicate that flavanols may not act on the cerebrovascular arteries in the same way they effect the peripheral arteries.

Interestingly, Marsh and colleagues (2017) observed a decrease in MCAv and CVC_{MCA} 1-hour post-ingestion of dark chocolate (DC; total flavanols 3600 mg/kg; Epicatechin 587.1 $\mu g/g^{1}$) and milk chocolate (MC; total flavanols 980 mg/kg; Epicatechin 288.4 $\mu g/g^{1}$) but not white chocolate (WC; total flavanols 370 mg/kg; Epicatechin not detected). While they detected no changes in cognitive performance. Interestingly, the decline in the functional reactivity to this cognition task is in line with what we observed in response to our CO_2 challenge post-ingestion. Marsh and colleagues suggest that cocoa flavanols may increase neurovascular coupling efficiency, meaning that less blood is needed to deal with the requirements in oxygenation induced by neuronal activity. In support of this conclusion from Marsh and colleagues, Francis *et al.* (2006) saw an increase in blood oxygenation during a cognitive task with no change in

cognitive performance after a 5-day intervention with a flavanol-rich cocoa drink. As such, it is possible that cocoa flavanols improve cerebral metabolism and therefore result in decreased oxygen demand. To identify whether CO_2 reactivity can be affected by changes in cerebral metabolism future studies that employ BOLD-fMRI or fNIRS techniques should be utilised during CO_2 challenges.

Contrary to our study, Sorond *et al.* (2008) observed that a high flavanol cocoa drink elicited a non-significant fall in MCAv 2- and 4-hours post-ingestion, but this was not noted post-ingestion of the low flavanol drink, and did not result in changes in CVR_{MCA} after 2 and 4 h of intake. To explain their findings, they suggested that the caffeine within the cocoa drinks caused this reduction in velocity, however as both drinks were matched for micro and macronutrients then this fall should have been seen within both conditions. Unfortunately, the authors did not present the acute MCAv data for the low flavanol cocoa drink, if they had it may have been the case that both conditions imparted a non-significant drop in MCAv that may have strengthened their conclusions. Further, the content of caffeine within the drinks was only ~20 mg, and while caffeine has been shown to dose-dependently reduce cerebral blood flow (Chen & Parrish 2009), the quantities used in that study are likely too low to explain any cerebral vasoconstriction-induced reduction in flow. Specifically, caffeine acts within the vascular smooth muscle as a competitive agonist of adenosine A2A and A2B receptors, interrupting adenosines ability to bind and ultimately cause vasodilation by opening ATP-dependent K+ channels, reducing conduction of Ca_2+ (Addicott et al., 2009). This action of caffeine has been shown to reduce blood flow within the cerebrovasculature, with a dose of 250 mg shown to reduce blood flow between 22-30% (Addicott et al., 2009). Others have observed a smaller effect, with 200 mg

decreasing cerebral blood flow by around 5-8%, which returned to baseline levels by 2-hours (Hasse et al., 2005). Moreover, this time course described by Hasse *et al.* (2005) is another reason it is unlikely caffeine (especially at such a low dose) caused the reduction in cerebral blood flow observed by Sorond *et al.* (2008). Nevertheless, Sorond and colleagues (2008) noted that after 1- and 2-weeks of daily (total flavanols 900 mg) high flavanol cocoa intake MCAv was significantly increased at 8-hours post-ingestion of a single cocoa drink, but no change in CVR_{MCA} was observed. These findings indicate that flavanols impact on the cerebrovasculature may be more likely to occur after chronic ingestion as opposed to acute intake, as tested within the current study.

Discrepancies in response to flavanols in peripheral and cerebral vasculatures

Contradictory to the hypothesis of the current study, the response of the peripheral and cerebral arteries was different, where the brachial artery increased in responsiveness and the MCA decreased in reactivity to the flavanol intervention. This finding was unexpected as both FMD (Thijssen et al., 2011) and CO_2 induced cerebrovascular vasodilation involve NO mechanisms (Schmetterer et al., 1997; Lavi et al., 2003). However, FMD relies virtually wholly on NO to induce a vasodilatory response to elevated shear stress caused by hyperaemia (Thijessen et al., 2011), as inhibiting NO synthase abolishes the vasodilatory response (Robinson et al., 1995; Doshi et al., 2001). In contrast, due to the redundancy of the regulatory mechanisms within the cerebrovasculature (Willie et al., 2014), NO is not the only pathway that elicits vasodilation in response to elevated P_aCO_2 (Coverdale, 2015). Although exogenous administration of NO has been shown to increase CVR response to hypercapnia (Lavi et al., 2003), previous studies have also observed that blocking NO

does not influence CVR in response to hypercapnia (Ide et al., 2007). Alongside this no correlation in nitrate and nitrite levels when assessed alongside CVR has also been reported (Meadows et al., 2005). Multiple K^+ channels within the cerebral vessels have been discovered and will affect cerebrovascular vasodilation; with ATP-sensitive K^+ channels and calcium-sensitive K^+ channels possibly playing key roles, as animal studies have shown blockage of both channels abolish hypercapnic vasodilation (Lindauer et al., 2003). Therefore, hypercapnia may utilise other channels to elicit a vasodilatory response within the cerebrovasculature. This difference in vascular regulation may partly be the reason there was a different response noted between the vessels assessed within this study. In accordance with the current study's findings, Marsh and colleagues (2017) observed a decrease in cerebrovascular conductance (at rest) and an increase in FMD in response to high flavanol dark chocolate consumption. Given this is the only other study that has investigated both systemic and cerebral vascular function in response to high flavanol cocoa consumption within the same cohort, further studies need to investigate this observed discrepancy in response to cocoa flavanols between the peripheral and cerebral vasculature.

Although there is a lack of information as to how cocoa flavanols influence different vasculatures, other studies have noted disparities between vessels in response to nutritional interventions. For example, Patik and colleagues investigated the influence of high-fat meals on vascular function, showing that FMD decreased in response to the intervention whereas cerebral reactivity remained unchanged (Patik et al., 2018). A single high-fat meal has been shown to impair nitric oxide bioavailability (Tsai et al., 2004; Patik et al., 2018), and therefore would be expected to attenuate both FMD (Thijssen et al., 2011) and cerebrovascular reactivity (Schmetterer et al., 1997; Lavi et

al., 2003) as both are influenced by NO. As such, the observations of Patik and colleagues' further sheds light on the possibility that nitric oxide may play a different more complex role within the cerebral circulation as opposed to the peripheral circulation. Further a study by Pretnar-Oblak *et al.* (2007) aimed to compare systemic endothelial function (via FMD) and cerebral endothelial function (via cerebrovascular reactivity to L-arginine). Since L-arginine has been shown to elicit vasodilation within the cerebral arteries via increased NO production (Rosenblum et al., 1990), this study allowed the investigation of how these assessments of systemic and cerebral arterial endothelial function compare. Pretnar-Oblak and colleagues observed that there was no correlation between FMD and cerebral reactivity, therefore demonstrating that these two techniques may be measuring different aspects of arterial function in the brain and in the periphery, meaning direct comparisons may not be valid. This lack of correlation between these techniques often employed to assess endothelial function, implies that studies utilising both methods may see differing outcomes if they expected their intervention to elicit similar responses between the vessels investigated. Indeed, this study may further explain why the current study observed opposing outcomes between FMD and CVR in response to flavanol intake.

In summary, it is important for future researchers to investigate influences on both systemic and cerebral circulations to fully understand the effect that interventions can have on the whole-body vasculature, as it seems unlikely that the effect imparted on the periphery will be mirrored within the cerebral arteries. To increase our understanding of how cocoa flavanols influence the cerebral vasculature differently to the peripheral vessels, studies utilising techniques that can allow for imaging of the cerebral vessels and assessment of both global and regional cerebral blood flow will

be important. The current study's findings may be a consequence of the CO_2 challenge itself, as the stimuli utilised for the two techniques differ it may mean that the two challenges may not capture the exact same aspect of vessel health, therefore not allowing for the techniques to be directly comparable. It therefore may be important to use a combination of imaging techniques (e.g. ultrasound, fMRI and fNIRS), which take advantage of the different strengths of each technique (e.g. temporal vs. spatial resolution; conduit vs. capillary function), as well as a range of stimuli (e.g. metabolic/neural or mechanical), to be able to fully assess cocoa-flavanols influence on the whole-body vasculature.

High-flavanol cocoa does not improve cognitive function in young and older adults

Contrary to our hypothesis there was no difference in any cognitive outcome with high flavanol intake compared to low flavanol intake. Previous acute studies that have investigated the impact of cocoa flavanols on cognitive ability have shown improved performance in cognition tasks peaking at around 2-hours post-ingestion (Scholey et al., 2010; Field et al., 2011). Scholey *et al.* (2010) demonstrated an improved performance in the Serial Threes task and a quickened response time within Rapid Visual Information Processing. Alongside this the authors observed that the most significant cognitive improvements were noted during the fourth Cognitive Demand Battery cycle, this cycle occurred approximately 2-hours post-ingestion, coinciding with peak improvements with FMD (Taubert et al., 2003), plasma epicatechin levels (Monahan et al., 2011) and peak cerebral blood flow reported by Francis *et al.* (2006). This suggests that the cocoa flavanols exert their peak effect throughout the body at around 2-hours post-ingestion, although more cerebral blood flow studies and acute cognition studies are needed to determine this. Within the current study participants

underwent cognitive testing at approximately 3-hours post-ingestion. Although it may be unlikely that this hour difference in testing time from the possible peak effects caused the results in both conditions to be similar, it is worth noting as a timeline for acute cognitive impact of flavanols has not been established.

Within the current study participants only underwent cognitive testing twice, at the end of each intervention visit, this therefore meant that there was high probability of a learning effect occurring. To improve this set up the cognitive tasks should have been first undertaken during the familiarisation visit and done pre-ingestion as well as post-ingestion. To try and reduce the impact of a learning effect our participants were randomised in condition order and practice trails, which were built into the CANTAB software, allowing participants to become acquainted with each test within the cognitive battery prior to testing. This was done to try and counteract the possibility of a learning effect occurring; however, this may not have completely mitigated the effect, although it should be noted that there were no differences between the two conditions. In the study by Scholey and colleagues (2010) the study design allowed participants to practice the cognition tasks within the familiarisation visit and they also took pre-ingestion cognition tests rather than purely post-ingestion. This allowed for a reduction in learning effect and permitted the researchers to have a baseline cognition measure on each day of the visits. This set-up may have allowed for more accurate cognitive data collection, any future studies evaluating acute cognition changes with cocoa flavanol ingestion should follow this paradigm.

It is possible that although no changes in cognition were present within the current study there may have been improvements in cerebral efficiency in accordance with findings observed by Francis *et al.* (2006). The authors noted no changes in cognitive

performance but did see an increase in blood oxygenation levels (BOLD), suggesting that cocoa flavanols may improve cerebral metabolism. Therefore, future studies examining flavanols effect on cognition may want to include cerebral blood flow measures during the completion of the cognitive tasks, specifically regional measures that correspond to the cognitive task undertaken to allow for analysis of cognitive performance and cerebral efficiency that may be occurring as a consequence of an alterations in neurovascular coupling.

Within a study conducted by Field and colleagues (2011), 2-hours post-ingestion imparted an improvement in motion and visual contrast sensitivity, greater spatial working memory accuracy and a faster choice reaction time. However, the researchers utilised dark and white chocolate that meant participants were not blinded to the condition, and any outcome may not be directly attributed to the cocoa flavanols as influence from other chocolate components cannot be ruled out (i.e. white vs. dark chocolate). These two studies (Scholey et al., 2010; Field et al., 2011) demonstrated positive improvements across a range of cognitive tasks at 2-hours post-ingestion, which conflicts directly with the current study's findings. Both studies (Scholey et al., 2010; Field et al., 2011) included 30 young healthy participants, whereas within the current study only 20 young and 14 older participants were included. While these numbers were adequate to see differences with our main outcome measures of vascular function, a power analysis for one of the main outcome measures of the cognitive tasks (reaction time) revealed that a 7% change in reaction time for the younger cohort should have included 39 participants and 31 for the older cohort. Therefore, it is likely that our study was underpowered not allowing us to see any change in cognitive outcome.

Decline in blood flow in peripheral and cerebral vasculature: A consequence of prolonged sitting?

The current study observed significant decreases in vascular reactivity and blood flow parameters (blood flow, blood flow velocity and shear rate) within both the brachial and cerebral arteries, independent of the dietary intervention. Within this study FMD was decreased within the younger cohort by 0.61% with ingestion of low flavanol cocoa, this was a surprising outcome, as to date, the majority of studies have not seen any decrease in FMD with low flavanol cocoa intake (Grassi et al., 2005; Heiss et al., 2005; Schroeter et al., 2006; Heiss et al., 2007; Blazer et al., 2008; Davison et al., 2008; Monahan et al., 2011; Marsh et al., 2017). One explanation for this is that the declines in blood flow and vascular function might be associated with the inactivity during the 2 h period between pre and post measurements. Although, we did not specifically control for sitting time, volunteers spent most of their time sitting during that period.

In support of this explanation, there is an increasing body of evidence indicating that prolonged sitting (+2 h) can affect the peripheral vasculature. For example, studies assessing acute sedentary time (2-6 h) have found that within the lower body vasculature (usually the femoral or the popliteal arteries) there is a decrease in %FMD, ranging 2-5% (Thosar et al., 2015; Restaino et al., 2015; Climie et al., 2018), but not within the upper body vasculature, e.g. brachial artery FMD (Thosar et al., 2014; Restaino et al., 2015; Carter et al., 2017). Furthermore, Padilla and colleagues (2009) showed that arm inactivity and increase hydrostatic load caused blunting in the brachial FMD response and a decrease in shear rate within 2.5-hours, which is consistent with the observations in the current study. Furthermore, Restaino and colleagues (2015) observed a reduced brachial blood flow in response to 6-hours of

sitting, with blunting of the upper limb microvasculature of ~30%. Interestingly, the decrease in lower limb vascular function was reversed by a 10-minute walk, but this was not reflected within the upper limbs. Similarly, Thosar and colleagues (2014) observed a decrease in brachial shear rate after 3-hours of sitting, although the authors did not calculate brachial blood flow, this finding indicates that 3-hours of sedentary time may be enough to see a reduction in blood flow and blood flow parameters within the brachial artery. Overall, the aforementioned studies agree with the current study's findings that sedentary time may have been a factor effecting the decrease in brachial shear rate and blood flow.

Similarly, Carter and colleagues (2018) showed that 4-hours of sitting resulted in a reduction in resting MCAv in young healthy adults, as observed in the current study, but no change in CVR_{MCA}, in contrast to our CVR indices (i.e. CVR_{MCA} and CVC-CVR_{MCA}). However, Carter *et al.* presented their CVR data as the linear regression between MCAv and $P_{ET}CO_2$, rather than the slope of this relation. As such, comparison of the CVR findings between their study and the current study is problematic. Of note, the reduction in cerebral blood *velocity* observed at rest (MCAv and PCAv) is further supported by the reduction in blood *flow* and increases in arterial diameter within the Carotid and Internal Carotid arteries that were recorded with Duplex Doppler from a follow-up study (see **Appendix A.5**). Collectively, findings from Carter *et al.* and the current study provide evidence that an acute about of sitting can have consequences to the cerebral vasculature, similarly to what has been observed in the peripheral vasculature.

Summary

This study aimed to assess how both peripheral and cerebral vessels react in response to high flavanol cocoa ingestion. Secondly, the study was intended to observe whether cocoa flavanols would reduce the disparity in vascular and cognitive function between younger and older adults. Similar effects in peripheral vascular function in response to flavanol intake for both age groups were also noted within this study, but distinct effects for cerebrovascular function were seen, with no differences in cognitive function. Due to a vast array of literature demonstrating that high flavanol cocoa causes increases in brachial FMD response, we hypothesised that cerebrovascular reactivity would also increase in response to high flavanol intake. Contrary to our hypothesis cerebrovascular reactivity was reduced in response to high flavanol cocoa. Initially we proposed that this may have been caused due to an increase in resting vasodilation of the cerebral vessels. However, after running a side study assessing the diameter of cerebral feeding vessels pre and post flavanol intake, this hypothesis was deemed unlikely. The possible explanation for this opposite response within the cerebrovasculature may be due to an increase in cerebral efficiency. Although studies utilising fMRI and fNIRS techniques during CO_2 challenges are needed to attest to this hypothesis. An alternative explanation may be that NO does not pay such a key role within the cerebrovasculature due to the redundancy in regulatory mechanisms within the cerebral vessels. Further, it is possible that the use of differing stimuli could have affected the disparity in vascular outcomes. Therefore, utilising multiple stimuli to assess different aspects of vascular health throughout the body may give us a clearer picture for the role of cocoa flavanols on vascular health.

Within the current study physical activity was accounted for to reduce any effect of exercise on the vasculature, however although physical activity was kept to a minimum during the study we did not control for sedentary time. This study also demonstrated that it may be important to control for sitting time within studies that assess vascular function, as previous work demonstrates that acute sedentary time alone can have impacts on vascular function.

Future Directions

To add to the current knowledge, it is important for researchers to explore the influence of cocoa flavanols within the cerebral circulation. Alongside these studies to look at the influence of cocoa flavanols on the cerebral vessels, more work needs to be done on how acute sedentary time effects cerebrovascular reactivity. As this will have important implications for all acute studies assessing cerebral vascular function where participants must be sedentary for any length of time. This would allow researchers to rule out influences of sitting time on their outcome measures.

Future studies need to utilise other Doppler or fMRI techniques to assess the diameter of MCA and PCA vessels at rest and during CO_2 challenges in response to high flavanol cocoa to have a clear understanding of how flavanols influence the vessels and brain function. Further, imaging techniques such as BOLD-fMRI or fNIRS would also allow us to assess the question of whether flavanols can alter cerebral efficiency and thus the neurovascular coupling relation. Further, studies using tests that directly target this neurovascular coupling response would be useful to identify whether the supraphysiological CO_2 stimuli could have been the cause of this decrease in CVR_{MCA}.

Moreover, NVC testing techniques may also more physiologically relevant and therefore might give more insight into how cocoa flavanols effect the cerebral vessels.

References

1. Addicott, M. A., Yang, L. L., Peiffer, A. M., Burnett, L. R., Burdette, J. H., Chen, M. Y., Hayasaka, S., Kraft, R. A., Maldjian, J. A. & Laurienti, P. J. (2009), 'The Effect of Daily Caffeine Use on Cerebral Blood Flow: How Much Caffeine can we Tolerate?', *Human Brain Mapping,* 30, pp. 3102-3114
2. AlGhatrif, M., Strait, J. B., Morrell, C. H., Canepa, M., Wright, J., Elango, P., Scuteri, A., Najjar, S. S., Ferrucci, L. & Lakatta, E. G. (2013), 'Longitudinal Trajectories of Arterial Stiffness and the Role of Blood Pressure: The Baltimore Longitudinal Study of Aging', *Hypertension,* 62, pp. 934-941
3. Alzheimer's Disease International (2015) **World Alzheimer Report, The global impact of dementia** [online]. Available from: https://www.alz.co.uk/research/WorldAlzheimerReport2015-sheet.pdf [Accessed 4th August 2018]
4. Andres-Lacueva, C., Monagas, M., Khan, N., Izquierdo-Pulido, M., Urpi-Sarda, M., Permanyer, J. & Lamuela-Raventós, R. M. (2008), 'Flavanol and Flavonol Contents of Cocoa Powder Products: Influence of the Manufacturing Process', *Journal of Agricultural and Food Chemistry,* 56 (9), pp. 3111-3117
5. Aprotosoaie, A., Miron, A., Trifan, A., Luca, V. S. & Costache, I-I. (2016), 'The Cardiovascular Effects of Cocoa Polyphenols – An Overview', *Diseases,* 4 (39), pp. 1-25
6. Arts, I. C. W., Jacobs, D. R., Harnack, L. J., Gross, M. & Folsom, A. R. (2001), 'Dietary catechins in relation to coronary heart disease death among postmenopausal women', *Epidemiology,* 12 (6), pp. 668-675

7. Baba, S., Osakabe, N., Yasuda, A., Natsume, M., Takizawa, T., Nakamura, T. & Terao, J. (2000), 'Bioavailability of (-)-epicatechin upon intake of chocolate and cocoa in human volunteers', *Free Radical Research*, 33 (5), pp. 635–41
8. Balzer, J., Rassaf, T., Heiss, C., Kleinbongard, P., Lauer, T., Merx, M., Heussen, N., Gross, H. B., Keen, C. L., Schroeter, H. & Kelm, M. (2008), 'Sustained Benefits in Vascular Function Through Flavanol-Containing Cocoa in Medicated Diabetic Patients', *Journal of the American College of Cardiology,* 51 (22), pp. 2141-4149
9. Benjamin, E.J., Larson, M.G., Keyes, M.J., et al. (2004), 'Clinical Correlates and Heritability of Flow-Mediated Dilation in the Community: The Framingham Heart Study', *Circulation*, 109 (5), pp. 613–619
10. Berry, N., Davison, K., Coates, A. M., Buckley, J. D. & Howe, P. R. C. (2010), 'Impact of cocoa flavanol consumption on blood pressure responsiveness to exercise', *British Journal of Nutrition,* 103, pp. 1480-1484
11. Bolton, E. & Rajkumar, C. (2011), 'The ageing cardiovascular system', *Clinical Gerontology,* 21, pp. 99-109
12. Brickman, A. M., Khan, U. A., Provenzano, F. A., Yeung, L-K., Suzuki, W., Schroeter, H., Wall, M., Sloan, R. P. & Small, S. A. (2014), 'Enhancing dentate gyrus function with dietary flavanols improves cognition in older adults', *Nature Neuroscience,* 17 (12), pp. 1798-1803
13. British Heart Foundation (2019) **UK Factsheet** [online]. Available from: www.bhf.org.uk/what-we-do/our-research/heart-statistics [Accessed 20th August 2019)

14. Cagnacci, A., Cannoletta, M., Palma, F., Zanin, R., Xholli, A. & Volpe, A. (2012), 'Menopausal symptoms and risk factors for cardiovascular disease in postmenopause', *Climacteric,* 15, pp. 157-162
15. Carter, S. E. and Gladwell, V. F. (2017), 'Effect of breaking up sedentary time with callisthenics on endothelial function', *Journal of Sports Sciences,* 35 (15), pp. 1508-151
16. Carter, S. E., Draijer, R., Holder, S. M., Brown, L., Thijssen, D. H. J. &Hopkins, N. D. (2018), 'Regular walking breaks prevent the decline in cerebral blood flow associated with prolonged sitting', *Journal of Applied Physiology,* 125 (3), pp. 790-798
17. Cassidy, A., Rimm, E. B., O'Reilly, É. J., Logroscino, G., Kay, C., Chiuve, S. E. & Rexrode, K. M. (2012), 'Dietary Flavonoids and Risk of Stroke in Women', *Stroke,* 43, pp. 946-951
18. Chambliss, K. L. & Shaul, P. W. (2002), 'Estrogen Modulation of Endothelial Nitric Oxide Synthase', *Endocrine Reviews,* 23 (5), pp. 665-686
19. Chen, Y. & Parrish, T. B. (2009), 'Caffeine dose effect on activation-induced BOLD and CBF responses', *NeuroImage,* 46, pp. 577-583
20. Choi, S. Y., Oh, B. H., Choi, D. J., Rhee, M. Y. & Park, S. (2013), 'Age-associated increase in arterial stiffness measured according to the cardio-ankle vascular index without blood pressure changes in healthy adults', *Journal of Atherosclerosis and Thrombosis,* 20 (12), pp. 911-923
21. Cifuentes-Gomez, T., Rodriguez-Mateos, A., Gonzalez-Salvador, I., et al. (2015), 'Factors Affecting the Absorption, Metabolism, and Excretion of Cocoa

Flavanols in Humans', *Journal of Agricultural and Food Chemistry*, 63 (35), pp. 7615–7623

22. Climie, R. E., Wheeler, M. J., Grace, M., Lambert, E. A., Cohen, N., Owen, N., Kingwell, B. A., Dunstan, D. W. & Green, D. J. (2018), 'Simple intermittent resistance activity mitigates the detrimental effect of prolonged unbroken sitting on arterial function in overweight and obese adults', *Journal of Applied Physiology,* 125, pp. 1787-1794
23. Commenges, D., Scotet, V., Renaud, S., Jacqmin-Gadda, H., Barberger-Gateau, P. & Dartiques, J.-F. (2000), 'Intake of flavonoids and risk of dementia', *European Journal of Epidemiology,* 16 (4), pp. 357-363
24. Cooper, K. A., Dovovan, J. L., Waterhouse, A. L. & Williamson, G. (2008), 'Cocoa and health: a decade of research', *British Journal of Nutrition*, 99 (1), pp. 1-11
25. Coverdale, N. S. (2015), 'Reactivity of the middle cerebral artery to carbon dioxide', *Electronic Thesis and Dissertation Repository,* 2885, pp. 1-144
26. Crozier, A., Jaganath, I. B. & Clifford, M. N. (2006), 'Phenols, Polyphenols and Tannins: An Overview', *Plant Secondary Metabolites: Occurrence, Structure and Role in the Human Diet,* November, pp. 1-24
27. Davison, K., Coates, A. M., Buckley, J. D. & Howe, P. R. C. (2008), 'Effect of cocoa flavanols and exercise on cardiometabolic risk factors in overweight and obese subjects', *International Journal of Obesity,* 32, pp. 1289-1296
28. Davison, K., Berry, N. M., Misan, G., Coates, A. M., Buckley, J. D. & Howe, P. R. C. (2010), 'Dose-related effects of flavanol-rich cocoa on blood pressure', *Journal of Human Hypertension,* 24 (9), pp. 568-576

29. Decroix, L., De Pauw, K., Van Cutsem, J., et al. (2018), 'Acute cocoa flavanols intake improves cerebral hemodynamics while maintaining brain activity and cognitive performance in moderate hypoxia', *Psychopharmacology*, 235 (9), pp. 2597–2608
30. Desideri, G., Kwik-Uribe, C., Grassi, D., et al. (2012), 'Benefits in cognitive function, blood pressure, and insulin resistance through cocoa flavanol consumption in elderly subjects with mild cognitive impairment: The cocoa, cognition, and aging (CoCoA) study', *Hypertension*, 60 (3), pp. 794–801
31. Doshi, S. N., Naka, K. K., Payne, N., Jones, C. J. H., Ashton, M., Lewis, M. J. & Goodfellow, J. (2001), 'Flow-mediated dilation following wrist and upper arm occlusion in humans: the contribution of nitric oxide', *Clinical Science,* 101 (6), pp. 629-635
32. Duarte, J., Franciso, V. & Perez-Vizcaino, F. (2014), 'Modulation of nitric oxide by flavonoid', *Food & Function,* 5, pp. 1653-1668
33. Engler, M. B. & Engler, M. M. (2004), *'The vasculoprotective effects of flavonoid-rich cocoa and chocolate',* Nutrition Research. 24, pp. 695-706
34. Estruch, R., Emilio, R., Salas-Salvadó, J., Covas, M-I., Corella, D., Arós, F., Gómez-Gracia, E., Ruiz-Gatiérrez, V., Fiol, M., Lapetra, J., Lamuela-Raventos, R. M., Serre-Majem, L., Pintó, X., Basore, J., Munoz, M. A., Sorlí, J. V., Martínez, J. A. & Martínez-González, M. A. (2013), 'Primary Prevention of Cardiovascular Disease with a Mediterranean Diet', *The New England Journal of Medicine,* 368 (14), pp. 1279-1290
35. Faridi, Z., Njike, V. Y., Dutta, S., Ali, A. & Katz, D. L. (2008), 'Acute dark chocolate and cocoa ingestion and endothelial function: a randomized

controlled crossover trial', *The American Journal of Clinical Nutrition,* 88, pp. 58-63

36. Farouque, H. M. O., Leung, M., Hope, S. A., Baldi, M., Schechter, C., Cameron, J. D. & Meredith, I. T. (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 (1), pp. 71-80
37. Field, D. T., Williams, C. M. & Butler, L. T. (2011), 'Consumption of cocoa flavanols results in an acute improvement in visual and cognitive functions', *Physiology & Behaviour,* 103, pp. 255-260
38. Fisher, N. D. L., Hughes, M., Gerhard-Herman, M. & Hollenberg, N. K. (2003), 'Flavanol-rich cocoa induces nitric-oxide-dependent vasodilation in healthy humans', *Journal of Hypertension,* 21, pp. 2281-2286
39. Fisher, N. D. L.,Hurwitz, S. & Hollenberg, N. K. (2012), 'HABITUAL FLAVONOID INTAKE AND ENDOTHELIAL FUNCTION IN HEALTHY HUMANS', *Journal American College of Nutrition,* 31 (4), pp. 275-279
40. Francis, S. T., Head, K., Morris, P. G., Macdonald, I. A. (2006), 'The Effect of Flavanol-rich Cocoa on the fMRI Response to a Cognitive Task in Healthy Young People', *Journal of Cardiovascular Pharmacology,* 47 (2), pp. S215-S220
41. Galleano, M., Oteiza, P. I. & Fraga, C. G. (2009), *'Cocoa, chocolate and cardiovascular disease',* Journal of Cardiovascular Pharmacology. 54 (6), pp. 483 -490

42. Gando, Y., Murakami, H., Kawakami, R., Yamamoto, K., Kawano, H., Tanaka, N., Sawada, S. S., Miyatake, N. & Miyachi, M. (2016), 'Cardiorespiratory Fitness Suppresses Age-Related Arterial Stiffening in Healthy Adults: A 2-Year Longitudinal Observational Study', *The Journal of Clinical Hypertension,* 18 (4), pp. 292-298
43. Geleijnse, J. M., Launer, L. J., van der Kuip, D. A. M., Hofman, A. & Witteman, J. C. M. (2002), 'Inverse association of tea and flavonoid intakes with incident myocardial infarction: the Rotterdam Study', *The American Journal of Clinical Nutrition,* 75, pp. 880-886
44. Goetz, M., Judd, S., Safford, M., Hartman, T., McClellan, W. and Vaccarino, V. (2015), 'Dietary Flavonoid Intake and Incident Coronary Heart Disease in the Reasons for Geographic and Racial Differences in Stroke Study (REGARDS)', *Annals of Epidemiology*, 25 (9), pp.715
45. Grassi, D., Necozione, S., Lippi, C., Croce, G., Valeri, L., Pasqualetti, P., Desideri, G., Blumberg, J. B. & Ferri, C. (2005a), 'Cocoa Reduces Blood Pressure and Insulin Resistance and Improves Endothelium-Dependent Vasodilation in Hypertensives', *Hypertension,* 46, pp. 398-405
46. Grassi, D., Lippi, C., Necozione, S., Desideri, G. & Ferri, C. (2005b), 'Short-term administration of dark chocolate is followed by a significant increase in insulin sensitivity and a decrease in blood pressure in healthy persons', The American Journal of Clinical Nutrition, 81, pp. 611-614
47. Grassi, D., Desideri, G., Necozione, S., Lippi, C., Casale, R., Properzi, G., Blumberg, J. B. & Ferri, C. (2008), '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, pp. 1671-1676

48. Halani, S., Kwinta, J. B., Golestani, A. M., Khatamian, Y. B. & Chen, J. J. (2015), 'Comparing cerebrovascular reactivity measured using BOLD and cerebral blood flow MRI: The effect of basal vascular tension on vasodilatory and vasoconstrictive reactivity', *Neuroimage,* 110, pp. 110-123
49. Harris, R.A., Nishiyama, S.K., Wray, D.W., et al. (2010), 'Ultrasound assessment of flow-mediated dilation', *Hypertension*, 55 (5), pp. 1075–1085
50. Haskell-Ramsay, C.F., Schmitt, J. and Actis-Goretta, L. (2018), 'The impact of epicatechin on human cognition: The role of cerebral blood flow', *Nutrients*, 10 (8), pp. 1–19
51. Hasse, C. G., Becka, M., Kuhlmann, J. & Wensing, G. (2005), 'Influences of caffeine, acetazolamide and cognitive stimulation on cerebral blood flow velocities', *Progress in Neuro-Psychopharmacology & Biological Psychiatry,* 29, pp. 549-556
52. Heiss, C., Dejam, A., Kleinbongard, P., et al. (2003), 'Vascular Effects of Cocoa Rich in Flavan-3-ols', *Journal of the American Medical Association*, 290 (8), pp. 1030–1031
53. Heiss, C., Kleinbongard, P., Dejam, A., Perre., S., Schroeter, H., Sies, H. & Kelm, M. (2005), 'Acute Consumption of Flavanol-Rich Cocoa and the Reversal of Endothelial Dysfunction in Smokers', *Journal of the American College of Cardiology,* 46 (7), pp. 1276-1283
54. Heiss, C., Finis, D., Kleinbongard, P., Hoffman, A., Rassaf, T., Kelm, M. & Sies, H. (2007), 'Sustained Increase in Flow-Mediated Dilation After Daily

Intake of High-Flavanol Cocoa Drink Over 1 Week', *Journal of Cardiovascular Pharmacology,* 49 (2), pp. 74-80

55. Heiss, C., Jahn, S., Taylor, M., Real, W. M., Angeli, F. S., Wong, M. L., Amabile, N., Prasad, M., Rassaf, T., Ottaviani, J. I., Mihardja, S., Keen, C. L., Springer, M. L., Boyle, A., Grossman, W., Glantz, S. A., Schroeter, H. & Yeghiazarians, Y. (2010), 'Improvement of endothelial function with dietary flavanols is associated with mobilization of circulating angiogenic cells in patients with coronary artery disease', *Journal of the American College of Cardiology,* 56 (3), pp. 218-224
56. Heiss, C., Sansone, R., Karimi, H., Krabbe, M., Schuler, D., Rodriquez-Mateos, A., Kraemer, T., Cortese-Krott, M. M., Kuhnle, G. G. C., Spencer, J. P. E., Schroeter, H., Merx, M. W. & Kelm, M. (2015), 'Impact of cocoa flavanol intake on age-dependent vascular stiffness in healthy men: a randomized, controlled, double-masked trial', *AGE,* 37 (56), pp. 1-12
57. Hermann, F., Spieker, L. E., Ruschitzka, F., Sudano, I., Hermann, M., Binggeli, C., Lu¨scher, T. F., Riesen, W., Noll, G. & Corti, R. (2006), "Dark chocolate improves endothelial and platelet function", *Heart*, 92(1), pp. 119-120
58. Hertog, M.G.L., Feskens, E.J.M., Hollman, P.C.H., et al. (1993), 'Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study', *Lancet*, 342
59. Hertog, M. G. L., Feskens, E. J. M. & Kromhout, D. (1997), 'Antioxidant flavanols and coronary heart disease risk', *The Lancet,* 349, pp. 699

60. Heyn, P., Abreu, B. C. & Ottenbacher, K. J. (2004), 'The Effects of Exercise Training on Elderly Persons With Cognitive Impairment and Dementia: A Meta-Analysis', *Archives of Physical Medicine and Rehabilitation,* 85, pp. 1694-704
61. Hollman, P. C. H. (2004), 'Absorption, Bioavailability, and Metabolism of Flavonoids', *Pharmaceutical Biology,* 42 (supplement), pp. 74-83
62. Holt, R. R., Lazarus, S. A., Sullards, M. C., Zhu, Q. Y., Schramm, D. D., Hammerstone, J. F., Fraga, C. G., Schmitz, H. H. & Keen, C. L. (2002), 'Procyanidin dimer B2 epicatechin-(4beta-8)-epicatechin in human plasma after the consumption of a flavanol-rich cocoa', *The American Journal of Clinical Nutrition*. 76 (4), pp. 798-804
63. Hooper, L., Kroon, P. A., Rimm, E. B., Cohn, J. S., Harvey, I., Le Cornu, K. A., Ryder, J. J., Hall, W. L. & Cassidy, A. (2008), 'Flavonoids, flavonoid-rich foods, and cardiovascular risk: a meta-analysis of randomized controlled trials', *The American Journal of Clinical Nutrition,* 88, pp. 38-50
64. Huxley, R. R. & Neil, H. A. W. (2003), 'The relation between dietary flavonol intake and coronary heart disease mortality: a meta-analysis of prospective cohort studies', *European Journal of Clinical Nutrition,* 57, pp. 904-908
65. Ide, K., Worthley, M., Anderson, T., Poulin, M. J. (2007), 'Effects of the nitric oxide synthase inhibitor L-NMMA on cerebrovascular and cardiovascular responses to hypoxia and hypercapnia in humans', *The Journal of Physiology,* 584 (1), pp. 321-332
66. Ito, H., Kanno, I., Ibaraki, M., Hatazawa, J. & Miura, S. (2003), 'Changes in Human Cerebral Blood Flow and Cerebral Blood Volume During Hypercapnia

and Hypocapnia Measured by Positron Emission Tomography', *Journal of Cerebral Blood Flow & Metabolism,* 23, pp. 665-670

67. Inaba, Y., Chen, J. A. & Bergmann, S. R. (2010), 'Prediction of future cardiovascular outcomes by flow-mediated vasodilatation of brachial artery: a meta-analysis', *The International Journal Cardiovascular Imaging,* 26, pp. 631-640
68. Jakovljevic, D. G. (2017), 'Physical activity and cardiovascular aging: Physiological and molecular insights', *Experimental Gerontology,* 109, pp. 67-74
69. Jalil, A. M. M. & Ismail, A. (2008), *'Polyphenols in Cocoa and Cocoa Products: Is There a Link between Antioxidant Properties and Health'*, Molecules. 13 (9), 2190-2219
70. Jankovic, N., Geelen, A., Streppel, M. T., de Groot, L. CPGM., Kiefte-de Jong, J. C., Orfanos, P., Bamia, C., Trichopoulou, A., Boffetta, P., Bobak, M., Pikkart, H., Kee, F., O'Doherty, M., Buckland, G., Woodside, J., Franco, O. H., Ikram, M. A., Struijk, E. A., Pajak, A., Malyutina, S., Kubinova, R., Wennberg, M., Park, Y., Bueno-de-Mesquita, H. B., Kampman, E. & Feskens, E. J. (2015), 'WHO guidelines for a healthy diet and mortality from cardiovascular disease in European and American elderly: the CHANCES project', *The American Journal of Clinical Nutrition,* 102, pp. 745-756
71. Kalmijn, S., Launer, L.J., Ott, A., et al. (1997), 'Dietary fat intake and the risk of incident dementia in the Rotterdam study', *Annals of Neurology*, 42 (5), pp. 776–782

72. Keli, S. O., Hertog, M. G., Feskens, E. J. & Kromhout, D. (1996), 'Dietary flavonoids, antioxidant vitamins, and incidence of stroke: the Zutphen study', *JAMA Internal Medicine, 156 (6), pp. 637-642*
73. Kerimi, A. & Williamson, G. (2015), 'The cardiovascular benefits of dark chocolate'*, Vascular Pharmacology*. 71, pp. 11-15
74. Klonizakis, M., Alkhatib, A. & Middleton, G. (2014), 'Long-term effects of an exercise and Mediterranean diet intervention in the vascular function of an older, healthy population', *Microvascular Research,* 95, pp. 103-107
75. Knekt, P., Järvinen, R., Reunanen, A. & Maatela, J. (1996), 'Flavonoid Intake And Coronary Mortality In Finland: A Cohort Study', *British Medical Journal,* 312 (7029), pp. 478-481
76. Knopman, D. S. (2006), 'Dementia and Cerebrovascular Disease', *Mayo Clinic Proceedings,* 81 (2), pp. 223-230
77. Lamport, D. J., Pal, D., Mountsiana, C., Field, D. T., Williams, C. M., Spencer, J. P. E. & Butler, L. T. (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, pp. 3227-3234
78. Lavi, S., Egbarya, R., Lavi, R., et al. (2003), 'Role of nitric oxide in the regulation of cerebral blood flow in humans: Chemoregulation versus mechanoregulation', *Circulation*, 107 (14), pp. 1901–1905
79. Letenneur, L., Proust-Lima, C., Le Gouge, A., Dartigues, J. F. & Barberger-Gateau, P. (2007), 'Flavonoid Intake and Cognitive Decline over a 10-Year Period', *American Journal of Epidemiology,* 165 (12), pp. 1364-1371

80. Lindauer, U., Kunz, A., Schuh-Hofer, S., Vogt, J., Dreier, J. P. & Dirnagl, U. (2001), 'Nitric oxide from perivascular nerves modulates cerebral arterial pH reactivity', *American Journal of Physiology- Heart and Circulatory Physiology,* 281, pp. H1353-H1363
81. Lindauer, U., Vogt, J., Schuh-Hofer, S., et al. (2003), 'Cerebrovascular vasodilation to extraluminal acidosis occurs via combined activation of ATP-sensitive and Ca2+-activated potassium channels', *Journal of Cerebral Blood Flow and Metabolism,* 23 (10), pp. 1227–1238
82. Macready, A. L., Kennedy, O. B., Ellis, J. A., Williams, C. M., Spencer, J. P. E. & Butler, L. T. (2009), 'Flavonoids and cognitive function: a review of human randomized controlled trial studies and recommendations for future studies', *Genes and Nutrition,* 4, pp. 227-242
83. Manach, C., Williamson, G., Morand, C., et al. (2005), 'Bioavailability and bio efficacy of polyphenols in humans. I. Review of 97 bioavailability studies', *The American journal of clinical nutrition*, 81 (1), pp. 230–242
84. Markos, F., Ruane O'Hora, T. and Noble, M.I.M. (2013), 'What is the mechanism of flow-mediated arterial dilatation', *Clinical and Experimental Pharmacology and Physiology*, 40 (8), pp. 489–494
85. Marsh, C. E., Carter, H. H., Guelfi, K. J., Smith, K. J., Pike, K. E., Naylor, L. H. & Green, D. J. (2017), 'Brachial and Cerebrovascular Functions Are Enhanced in Postmenopausal Women after Ingestion of Chocolate with a High Concentration of Cocoa', *The Journal of Nutrition,* pp. 1-7
86. Mastroiacovo, D., Kwik-Uribe, C., Grassi, D., et al. (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', *American Journal of Clinical Nutrition*, 101 (3), pp. 538–548

87. McFarlin, B. K., Venable, A. S., Henning, A. L., Prado, E. A., Sampson, J. N., Vingren, J. L. & Hill, D. W. (2015), 'Natural cocoa consumption: Potential to reduce atherogenic factors?', *The Journal of Nutritional Biochemistry,* 26 (6), pp. 626-632
88. Meadows, G.E., Kotajima, F., Vazir, A., et al. (2005), 'Overnight changes in the cerebral vascular response to isocapnic hypoxia and hypercapnia in healthy humans: Protection against stroke', *Stroke,* 36 (11), pp. 2367–2372
89. Mitchell, G. F., Parise, H., Benjamin, E. J., Larson, M. G., Keyes, M. J., Vita, J. A., Vasan, R. S. & Levy, D. (2004), 'Changes in Arterial Stiffness and Wave Reflection with Advancing Age in Healthy Men and Women: The Framingham Heart Study', *Hypertension,* 43 (6), pp. 1239-1245
90. Mokhtar, S. S. & Rasool, A. H. (2017), 'Plant-derived foods containing polyphenols with endothelial protective effects', *International Food Research* Journal. 24 (2), pp. 471-482
91. Molino, S., Dossena, M., Buonocore, D., Ferrari, F., Venturini, L., Ricevuti, G. & Verri, M. (2016), 'Polyphenols in dementia: From molecular basis to clinical trials'*, Life Sciences*. 161, pp. 69-77
92. Monahan, K. D., Feehan, R. P., Kunselmann, A. R., Preston, A. R., Miller, D. L. & Lott, M. E. J. (2011), 'Dose-dependent increases in flow-mediated dilation following acute cocoa ingestion in healthy older adults', *Journal of Applied Physiology,* 111 (6), 1568-1574

93. Mullen, W., Borges, G., Donovan, J. L., Edwards, C. A., Serafini, M., Lean, M. E. J. & Crozier, A. (2009), 'Milk decreases urinary excretion but not plasma pharmacokinetics of cocoa flavan-3-ol metabolites in humans', *The American Journal of Clinical Nutrition,* 89, pp. 1784-1791
94. Mursu, J., Voutilainen, S., Nurmi, T., et al. (2008), 'Flavonoid intake and the risk of ischaemic stroke and CVD mortality in middle-aged Finnish men: The Kuopio Ischaemic Heart Disease Risk Factor Study', *British Journal of Nutrition*, 100 (4), pp. 890–895
95. Ng, T-P., Feng, L., Niti, M., Kua, E-H. & Yap, K-B. (2008), 'Tea consumption and cognitive impairment and decline in older Chinese adults', *The American Journal of Clinical Nutrition,* 88, pp. 224-231
96. Njike, V. Y., Faridi, Z., Shuval, K., Dutta, S., Kay, C. D., West, S. G., Kris-Etherton, P. M. & Katz, D. L. (2011), 'Effects of sugar-sweetened and sugar-free cocoa on endothelial function in overweight adults', *International Journal of Cardiology,* 149 (1), pp. 83-88
97. Noon, J. P., Trischuk, T. C., Gaucher, S. A., Galante, S. & Scott, R. L. (2008), 'The effect of age and gender on arterial stiffness in healthy Caucasian Canadians', *Journal of Clinical Nursing,* 17, pp. 2311-2317
98. O'Rourke, M.F. & Hashimoto, J, (2007), 'Mechanical factors in arterial aging: a clinical perspective', *Journal of the American College of Cardiology,* 50, pp. 1–13
99. Ottaviani, J.I., Momma, T.Y., Kuhnle, G.K., et al. (2012), 'Structurally related (-)-epicatechin metabolites in humans: Assessment using de novo chemically

synthesized authentic standards', *Free Radical Biology and Medicine,* 52 (8), pp. 1403–1412

100. Padilla, J., Sheldon, R. D., Sitar, D. M. & Newcomer, S. C. (2009), 'Impact of acute exposure to increased hydrostatic pressure and reduced shear rate on conduit artery endothelial function: a limb-specific response', *American Journal of Physiology- Heart and Circulatory Physiology,* 297, pp. H1103-H1108
101. Pase, M.P., Scholey, A.B., Pipingas, A., et al. (2013), 'Cocoa polyphenols enhance positive mood states but not cognitive performance: A randomized, placebo-controlled trial', *Journal of Psychopharmacology*, 27 (5), pp. 451–458
102. Patik, J. C., Tucker, W. J., Curtis, B. M., Nelson, M. D., Nasirian, A., Park, S. & Brothers, R. M. (2018), 'Fast-food meal reduces peripheral artery endothelial function but not cerebral vascular hypercapnic reactivity in healthy young men', *Physiology Reports,* 6 (18), pp. 1-11
103. Pretnar-Oblak, J., Sabovic, M. & Zaletel, M. (2007), 'Associations between Systemic and Cerebral Endothelial Impairment Determined by Cerebrovascular Reactivity to L-Arginine', *Endothelium: Journal of Endothelial Cell Research,* 14 (2), pp. 73-80
104. Quinones, M., Miguel, M. & Aleixandre, A. (2013), *'Beneficial effects of polyphenols on cardiovascular disease',* Pharmacological Research, 68 (1), pp. 125-131
105. Rein, D., Holt, R. R., Keen, C. L., Lotito, S., Fraga, C. G. & Schmitz, H. H. (2000), 'Epicatechin in human plasma; In vivo determination and effect of

chocolate consumption on plasma oxidation status', *Journal of Nutrition,* 130 (8), pp. 2109S-2114S

106. Restaino, R. M., Holwerda, S. W., Credeur, D. P., Fadel, P. J. & Padilla, J. (2015), 'Impact of prolonged sitting on lower and upper limb micro- and macrovascular dilator function', *Experimental Physiology,* 100 (7), pp. 829-838
107. Richelle, M., Tavazzi, I., Enslen, M. & Offord, E. A. (1999), 'Plasma kinetics in man of epicatechin from black chocolate', *European Journal of Clinical Nutrition,* 53 (1), pp. 22-26
108. Rimbach, G., Melchin, M., Moehring, J. & Wagner, A. E. (2009), 'Polyphenols from Cocoa and Vascular Health—A Critical Review', *International Journal of Molecular Sciences,* 10, pp. 4290-4309
109. Rodriguez-Mateos, A., Hezel, M., Aydin, H., Kelm, M., Lundberg, J. O., Weitzberg, E., Spencer, J. P. E. & Heiss, C. (2015), 'Interactions between cocoa flavanols and inorganic nitrate: Additive effects on endothelial function at achievable dietary amounts', *Free Radical Biology and Medicine,* 80, pp. 121-128
110. Rosenblum, W.I., Nishimura, H. and Nelson, G.H. (1990), 'Endothelium-dependent L-Arg- and L-NMMA-sensitive mechanisms regulate tone of brain microvessels', *American Journal of Physiology - Heart and Circulatory Physiology,* 259 (5 28-5)
111. Safford, M.M., Brown, T.M., Muntner, P.M., et al. (2012), 'Association of race and sex with risk of incident acute coronary heart disease events', *JAMA - Journal of the American Medical Association*, 308 (17), pp. 1768–1774

112. Sánchez-Rabaneda, F., Jáuregui, O., Casals, I., Andrés-Lacueva, C., Izquierdo-Pulido, M., Lamuela-Raventós, R. M. (2003), 'Liquid chromatographic/electrospray ionization tandem mass spectrometric study of the phenolic composition of cocoa *(Theobroma cacao)*', *Journal of Mass Spectrometry,* 38, pp. 35-42

113. Sansone, R., Heiss, C., Karimi, H., Krabbe, M., Schuler, D., Rodriguez-Mateos, A., Kraemer, T., Cortese-Krott, M. M., Kuhnle, G. G. C., Spencer, J. P. E., Schroeter, H., Merx, M. W. & Kelm, M. (2015), 'Impact of cocoa flavanol intake on age-dependent vascular stiffness in healthy men: a randomized, controlled, double-masked trial', *AGE,* 37 (56), pp. 1-12

114. Sansone, R., Ottaviani, J. I., Rodriguez-Mateos, A., Heinen, Y., Noske, D., Spencer, J. P., Crozier, Merx, M. W., Kelm, M., Schroeter, H. & Heiss, C. (2017), 'Methylxanthines enhance the effects of cocoa flavanols on cardiovascular function: randomized, double-masked controlled studies', *The American Journal of Clinical Nutrition,* 105 (2), pp. 352-360

115. Schewe, T., Steffen, Y. & Sies, H. (2008), 'How do dietary flavanols improve vascular function? A position paper', *Archives of Biochemistry and Biophysics,* 476 (2), pp. 102–106

116. Schmetterer, L., Findl, O., Strenn, K., et al. (1997), 'Role of NO in the O 2 and CO 2 responsiveness of cerebral and ocular circulation in humans', *American Journal of Physiology - Regulatory Integrative and Comparative Physiology*, 273 (6 42-6)

117. Scholey, A. B., French, S. J., Morris, P. J., Kennedy, D. O., Milne, A. L. & Haskell, C. F. (2010), 'Consumption of cocoa flavanols results in acute

improvements in mood and cognitive performance during sustained mental effort', *Journal of Psychopharmacology,* 24 (10), pp. 1505-1514

118. Schramm, D. D., Karim, M., Schrader, H. R., Holt, R. R., Kirkpatrick, N. J., Polagruto, J. A., Ensunsa, J. L., Schmitz, H. H. & Keen, C. L. (2003), 'Food effects on the absorption and pharmacokinetics of cocoa flavanols', *Life Sciences.* 73 (7), pp. 857-869
119. Schroeter, H., Heiss, C., Balzer, J., Kleinbongard, P., Keen, C. L., Hollenberg, N. K., Sies, H., Kwik-Uribe, C., Schmitz, H. H. & 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 (4), pp. 1024-1029
120. Seals, D. R., DeSouza, C. A., Donato, A. J. & Tanaka, H. (2009), 'Habitual exercise and arterial aging', *Journal of Applied Physiology,* 105, pp. 1323-1332
121. Serafini, M. & Crozier, A. (2003), 'Milk and absorption of dietary flavanols', *Nature*, 426, pp. 788
122. Sesso, H. D., Gaziano, J. M., Liu, S. & Buring, J. E. (2003), 'Flavonoid intake and the risk of cardiovascular disease in women', *The American Journal of Clinical Nutrition,* 77, pp. 1400-1408
123. Shirai, K., Song, M., Suzuki, J., Kurosu, T., Oyama, T. & Nagayama, D. (2011), 'Contradictory effects of beta1- and alpha1- aderenergic receptor blockers on cardio-ankle vascular stiffness index (CAVI)--CAVI independent of blood pressure', *Journal of Atherosclerosis and Thrombosis,* 18 (1), pp. 49-55
124. Shrime, M. G., Bauer, S. R., McDonald, A. C., Chowdghury, N. H., Coltart, C. E. M. & Ding, E. L. (2011), 'Flavonoid-Rich Cocoa Consumption Affects

Multiple Cardiovascular Risk Factors in a Meta-Analysis of Short-Term Studies', *The Journal of Nutrition*, 141, pp.1982-1988

125. Small, S. A., Stern, Y., Tang, M. & Mayeux, R. (1999), 'Selective decline in memory function among healthy elderly', *Neurology,* 52 (7), pp. 1392-1396
126. Sokolov, A. N., Pavlova, M. A., Klosterhalfen, S. & Enck, P. (2013), 'Chocolate and the brain: Neurobiological impact of cocoa flavanols on cognition and behavior', *Neuroscience and Biobehavioral Reviews,* 37 (10), pp. 2445-2453
127. Sorond, F. A., Lipsitz, L. A., Hollenberg, N. K. & Fisher, N. D. L. (2008), 'Cerebral blood flow response to flavanol-rich cocoa in healthy elderly humans', *Neuropsychiatric Disease and Treatment,* 4 (2), pp. 433-440
128. Sorond, F.A., Hurwitz, S., Salat, D.H., et al. (2013), 'Neurovascular coupling, cerebral white matter integrity, and response to cocoa in older people', *Neurology*, 81 (10), pp. 904–909
129. Stroke Association (2018), **State of the nation; Stroke statistics** [online]. Available from: www.stroke.org.uk/sites/default/files/state_of_the_nation_2018 [Accessed 1st August 2018]
130. Taubert, D., Berkels, R., Roesen, R. & Klaus, W. (2003), 'Vascular Effects of Cocoa Rich in Flavan-3-ols', *The Journal of the American Medical Association,* 76, pp. 798-804
131. Thijssen, D. H. J., Maiorana, A. J., O'Driscoll, G., Cable, N. T., Hopman, M. T. E. & Green, D. J. (2010), 'Impact of inactivity and exercise on the vasculature in humans', *European Journal of Applied Physiology,* 108 (5), pp. 845-875

132. Thijssen, D.H.J., Black, M.A., Pyke, K.E., et al. (2011), 'Assessment of flow-mediated dilation in humans: A methodological and physiological guideline', *American Journal of Physiology - Heart and Circulatory Physiology,* 300 (1), pp. 2–12

133. Thilakarathna, S. H. & Rupasinghe, H. P. V. (2013), 'Flavonoid Bioavailability and Attempts for Bioavailability Enhancement', *Nutrients,* 5, pp. 3367-3387

134. Thosar, S. S., Bielko, S. L., Wiggins, C. C. & Wallace, J. P. (2014), 'Differences in brachial and femoral artery responses to prolonged sitting', *Cardiovascular Ultrasound,* 12 (50), pp. 1-7

135. Thosar, S. S., Bielko, S. L., Mather, K. J., Johnston, J. D., & Wallace, J. P. (2015), 'Effect of prolonged sitting and breaks in sitting time on endothelial function', *Medicine and Science in Sports and Exercise*, 47(4), pp. 843–849

136. Tsai, W.C., Li, Y.H., Lin, C.C., et al. (2004), 'Effects of oxidative stress on endothelial function after a high-fat meal', *Clinical Science*, 106 (3), pp. 315–319

137. Vaitkevicius, P. V., Fleg, J. L., Engel, J. H., O'Connor, F. C., Wright, J. G. & Lakatta, L. E. (1993), 'Effects of age and aerobic capacity on arterial stiffness in healthy adults', *Circulation,* 88 (1), pp. 1456-1462

138. Vita, J. A. (2005), 'Endothelial function and clinical outcome', *Heart (British Cardiac Society),* 91 (10), pp. 1278-1279

139. Vlachopoulos, C., Aznaouridis, K., Alexopoulos, N., Economou, E., Andreadou, I. & Stefanadis, C. (2005), 'Effect of Dark Chocolate on Arterial Function in Healthy Individuals', *American Journal of Hypertension,* 18, pp. 785-791

140. Vlachojannis, J., Erne, P., Zimmermann, B. & Chrubasik-Hausmann, S. (2016), 'The Impact of Cocoa Flavanols on Cardiovascular Health', *Phytotherapy Research,* 30 (10), pp. 1641-1657
141. Wang, Q., Paulson, O.B. and Lassen, N.A. (1992), 'Effect of nitric oxide blockade by NG-nitro-l-arginine on cerebral blood flow response to changes in carbon dioxide tension', *Journal of Cerebral Blood Flow and Metabolism*, 12 (6), pp. 947–953
142. Wang, J., Schramm, D., Holt, R. & Ensunsa, J. (2000), 'A dose-response effect from chocolate consumption on plasma epicatechin and oxidative damage', *The Journal of Nutrition,* 130 (8S), pp. 2115S-9S
143. Wang-Polagruto, J. F., Villablanca, A. C., Polagruto, J. A., Lee, L., Holt., R. R., Schrader, H. R., Ensunsa, J. L., Steinburg, F. M., Schmitz, H. H. & Keen, C. L. (2006), 'Chronic Consumption of Flavanol-rich Cocoa Improves Endothelial Function and Decreases Vascular Cell Adhesion Molecule in Hypercholesterolemic Postmenopausal Women', *Journal of Cardiovascular Pharmacology,* 47 (2), pp. S177-S186
144. Weinstein, J. R. & Anderson, S. (2010), 'The Aging Kidney: Physiological Changes', *Advances in Chronic Kidney Disease,* 17 (4), pp. 302-307
145. Westphal, S & Luley, C. (2011), 'Flavanol-rich cocoa ameliorates lipemia-induced endothelial dysfunction', *Heart Vessels,* 26, pp. 511-515
146. White, R.P., Deane, C., Vallance, P., et al. (1998), 'Nitric oxide synthase inhibition in humans reduces cerebral blood flow but not the hyperemic response to hypercapnia', *Stroke*, 29 (2), pp. 467–472

147. Widlansky, M. E., Gokce, N., Keaney, J. F. & Vita, J. A. (2003), 'The Clinical Implications of Endothelial Dysfunction', *Journal of the American College of Cardiology,* 42 (7), pp. 1149-1160

148. Willie, C. K., Tzeng, Y-C., Fisher, J. A. & Ainslie, P. N. (2014), 'Integrative regulation of human brain blood flow', *The Journal of Physiology,* 592 (5), pp. 841-859

149. Witte, D., Westerink, E., De Koning, E. J., Van der Graaf, Y., Grobbee, D. E. & Bots, M. L. (2005), 'Is the Association Between Flow-Mediated Dilation and Cardiovascular Risk Limited to Low-Risk Populations?', *Journal of the American College of Cardiology,* 45 (12), pp. 1987-1993

150. Wray, D. W. &Richardson, R. S. (2006), 'Aging, Exercise, and Limb Vascular Heterogeneity in Humans', *Medicine & Science in Sport & Exercise,* pp. 1804-1810

151. Xu, X., Wang, B., Ren, C., Hu, J., Greenberg, D. A., Chen, T., Xie, L. & Jin, K. (2017), 'Recent Progress in Vascular Aging: Mechanisms and Its Role in Age-related Diseases', *Aging and Disease,* 8 (4), pp. 486-505

152. Yamamoto, T., Takahashi, H., Suzuki, K., Hirano, A., Kamei, M., Goto, T., Takahashi, N. & Kawada, T. (2014), 'Theobromine enhances absorption of cacao polyphenol in rats', *Bioscience, Biotechnology and Biochemistry,* 78 (12), pp. 2059-2063

153. Yassa, M. A., Mattfeld, A. T., Stark, S. M. & Stark, C. E. L. (2011), 'Age-related memory deficits linked to circuit-specific disruptions in the hippocampus', *Proceedings of the National Academy of Sciences,* 108 (21), pp. 8873-8878

154. Yochum, L., Kushi, L. H., Meyer, K. & Folsom, A. R. (1999), 'Dietary Flavonoid Intake and Risk of Cardiovascular Disease in Postmenopausal Women', *American Journal of Epidemiology,* 149 (10), pp. 943-949

Appendices

Appendix A.1

Flavonoid rich foods to avoid for 24 hours prior to each study day

Please **EXCLUDE** these foods from your diet for **24 hours** prior to the study days:

- Any kind of fruit juice (orange, apple, grapefruit, berry etc.)

- Most Fruits, in particular: orange, grapefruit, berries, apple, pear, grapes, peach, apricot, nectarine, plum, any kind of exotic fruit.
- Most Vegetables, in particular: onion, leek, peppers, tomato, broccoli, aubergine, beetroot, green beans, carrots.
- Any pre-prepared food containing the above.
- Any soya containing-products
- Black , green tea, any kind of herbal tea or fruit tea
- Any kind of wine, beer, cider or any alcoholic beverages
- All high energy and/or caffeinated drinks, eg: Coca-Cola, Red Bull, Lucozade etc
- Olive oil
- All nuts
- Jams and preserves
- Pesto Sauce
- Dark or milk chocolate
- Cocoa/ Chocolate beverages
- Coffee (caffeinated or decaffeinated)

An indication of foods that you **CAN** eat during the pre-trial period is given below:

- Meat, poultry and fish
- Dairy products (milk, cheese, yogurt; not fruit containing yogurts)
- Bananas
- Croissants/ Pastries (not containing vegetables, fruits, fruit preserves or chocolate)
- Cabbage
- Cucumber
- Lettuce
- Potatoes, Rice and Pasta
- Sweetcorn
- Eggs and Butter

REMEMBER:

Please fast for 12 h before the study visit (approximately from 8 pm the day before). During this period, you should only consume water.

Please do not exercise for 24 h before the study visit.

Please do not consume alcohol for 24 h before the study visit.

Appendix A.2

24-HOUR RECALL

Participant ID _____

This is a recall of food and drink consumed in the last 24 hours. Give Date and day (yesterday) _____

1. What was the first thing you had to eat or drink when you got out of bed yesterday?

_____

_____

_____

2. What did you have to eat or drink at mid-morning?

_____

_____

3. What did you have to eat or drink during lunchtime?

_____

_____

_____

4. What did you have to eat or drink in the afternoon between lunchtime and your evening meal?

_____

_____

_____

5. What did you have to eat and drink for your evening meal?

_____

_____

_____

6. Did you have anything to eat or drink after your dinner or before you went to bed?

_____

_____

7. Are there any snacks you had during the day you forgot to mention? (circle your response)

YES / NO

8. Have you taken any medication or supplements in the last 24 hours, please report here:

_____

_____

9. Have you taken any anti-inflammatory medication or painkillers in the last 24 hours? (circle your response)

YES / NO

if yes, please list here (including dose):

_____

Researcher: _____ Date: _____

Appendix A.3

The University of Birmingham

School of Sport, Exercise and Rehabilitation Sciences

General Health and Lifestyle Questionnaire

Name: .....

Address: .....

.....

.....

Phone:.....

Name of the responsible investigator for the study:

.....

Please answer the following questions. If you have any doubts or difficulty with the questions, please ask the investigator for guidance. These questions are to determine whether the proposed exercise is appropriate for you. Your answers will be kept strictly confidential.

1.	You are.....	Male	Female
2.	What is your exact date of birth? Day..... Month.....Year..20..... So your age is..... Years		
3.	When did you last see your doctor? In the: Last week..... Last month..... Last six months..... Year..... More than a year.....		
4.	Have you been diagnosed as suffering from heart disease, stroke or any other disease of the circulation?	YES	NO
5.	Have you been diagnosed as suffering from any respiratory disease or condition?	YES	NO
6.	Do you suffer from asthma?		
7.	Has your doctor ever said you have diabetes?		

8.	Has your doctor ever said you have high blood pressure?	YES	NO
9.	Has your doctor (or anyone else) said that you have a raised blood cholesterol?	YES	NO
10.	Are you currently taking any medication? If yes, please give details:_____ _____	YES	NO
11.	Do you suffer from any other illness? If yes, please give details:_____ _____	YES	NO
12.	Have you had a cold or feverish illness in the last month?	YES	NO
13.	Do you ever lose balance because of dizziness, or do you ever lose consciousness?	YES	NO
14.	Do you smoke?	YES	NO
15.	Do you drink alcohol? If yes, approx. how many units per week do you drink? _____	YES	NO
16.	Have you ever had viral hepatitis?	YES	NO

17.	If you are female, to your knowledge, are you pregnant?	YES	NO
18.	If you are female, do you take contraceptive pills or are you on any other form of hormonal contraception? If yes, what hormonal contraception are you on? _____	YES	NO
19.	If you are female, when was the last time you had your period? (if you know the day of your menstrual cycle you are on, please state here) _____		
20.	Do you have any known food allergies?	YES	NO
21.	Do you take any form of dietary supplement e.g. fish oils, vitamins or minerals? If yes, please give details:_____ _____	YES	NO

22.	Are you currently on a weight reducing or other diets? If yes, please give details:_____ _____	YES	NO
23.	Do you exercise regularly or take part in team sports? If yes, which form of exercise, how often (times per week), and at what intensity (light, moderate, vigorous)? _____ _____	YES	NO

I have completed the questionnaire to the best of my knowledge and any questions I had have been answered to my full satisfaction.

Signed: .....

Date: .....

Appendix A.4

ADDITIONAL INFORMATION AND SCREENING FOR THE USE OF COCOA

Please answer these questions to confirm your eligibility to take part in this study:

Are you allergic to any of the following?

- Cocoa YES/NO
- Caffein YES/NO
- Saccharose YES/NO
- Fructose YES/NO

Are you allergic to other medications/supplements?

_____

Do you understand that if you experience any side effects of this supplement you should report this to Dr Samuel Lucas, Dr Catarina Rendeiro or Rosie Pritchard?

_____

I have completed the questionnaire to the best of my knowledge and any questions I had have been answered to my full satisfaction.

Signed: .....

Date: .....

Appendix A.5

Participant Characteristics

All participants included within this study were informed of the procedures and requirements of the study and provided written informed consent at least 48 hours prior to inclusion within the study. Participants were screened at least 48 hours prior to their first data collection visit for; cardiovascular, respiratory or neurovascular diseases, high blood pressure, raised blood cholesterol, medication usage, smoking or allergies to cocoa. Included within the study were nine young healthy male (age 20 ± 3yrs) participants (mean ± SD). Younger menstruating females were excluded from this study due to known alterations in hormonal profile throughout the month, which have been shown to influence the vasculature.

Experimental Conditions

The protocol of this experiment as discussed below was conducted within a temperature-controlled laboratory (~22°C) at the University of Birmingham, School of Sport, Exercise and Rehabilitation Sciences. An acute randomized, placebo-controlled, double-blinded, cross-over human study design was used. All participants visited the laboratory on three occasions, the first for a familiarization visit, done at any time of day, and the following two for data collection done at the same time of day starting at between 6:30-8:00am with a minimum of 4 days in-between these visits. Participants were instructed to avoid certain foods and beverages high in polyphenols (see appendix A.1; E-Flavonoid rich foods to avoid for 24 hours prior to each study day_ERN_17_1755), beverages high in caffeine, alcohol or exercise 24 hours prior to visiting the laboratory on data collection sessions, they were also

asked to complete a 12 hour overnight fast. To try and measure adherence to this avoidance protocol a dietary recall questionnaire was utilised at the beginning of each visit (see appendix **A.2**; 24h Hour Recall Questionnaire), if participants had not adhered, they were requested to visit the laboratory on another day to collect data after adhering to the avoidance list. Participants were provided Buxton® water throughout the laboratory visits, due to low levels of nitrite within this bottled water. The study was approved by the University of Birmingham Ethics Board (ERN_17-1591) and conformed to the standards set by the Declaration of Helsinki.

Data Acquisition

During imaging of the Carotid and the Internal Carotid Arteries physiological data was recorded via a Terason ultrasound machine (Usmart 3300 NexGen Ultrasound; Terason, United States) onto a PC laptop (Fujitsu Lifebook A series, Japan), displayed and analysed in real time via QUIPU Cardiovascular Suite software (Smart Medical, United Kingdom).

Measurement

Duplex Doppler Ultrasound

Using a 15-4Mhz (15L4 Smart MarK™) transducer attached to a Terason Duplex Doppler ultrasound system (Usmart 3300 NexGen Ultrasound; Terason, United States) participants right Carotid and Internal Carotid arteries were imaged during a supine resting baseline. The Duplex Doppler allowed for continuous measurement of both blood flow velocity and artery diameter. Once the researcher found and optimised the image for each artery, firstly the Carotid and then the Internal Carotid, the researcher held the transducer in place.

This study utilised automatic edge-detection and wall-tracking software (Cardio-vascular Suite, Quipu). The image on the Terason Duplex Doppler system was fed into the software via a video converter (Epiphan AV.io HD Frame Grabber, Quipu). Within this software, a region of interest (ROI) box was drawn manually by the researcher within the optimum area on the artery, allowing the software to frame-by-frame detect the edges of the arterial walls within this ROI. A second ROI is then selected around the pulse-wave velocity recording, allowing the software to detect the edges of the pulse wave form giving a measurement of velocity.

Experimental Protocol

Carotid and Internal Carotid Artery Baseline Measurements

Within this study the participants lay supine with their heads slightly tilted backwards and to the left, to allow for extension of the right side of the neck. Prior to data collection, 20 minutes of supine rest was used to allow for the participants vital signs to stabilise and the researcher to find the optimal image. Once the Terason Duplex Doppler ultrasound machine and Cardio-Vascular Suite software was set up, 2 minutes of continuous baseline data was recorded, first from the Carotid Artery and then from the Internal Carotid Artery. During this 2-minute time frame, continuous recording of blood flow velocity and arterial diameter was taken.

Data Analysis

Carotid and Internal Carotid Artery Diameter and Blood Flow Velocity

Assessment of each Carotid and Internal Carotid artery video was done by running the analysis of each video once, after choosing the optimum edge detection section (high clarity and contrast of arterial walls) to get a continuous measure of both

baseline arterial diameter and blood flow velocity. The reliability and accuracy of the arterial wall edge detection was done by the researcher scrutinizing the edge detection throughout running of the video, and utilising techniques such as using landmarks to evaluate any movement of the artery. Once each video analyse was determined reliable, arterial diameter and blood flow velocity were recorded.

Statistical Analysis

Comparisons between the interventions were done by running 2-way repeated measures ANOVAs (IBM SPSS Statistics Data Editor, version 23), for all outcome measures. Values are reported as means ± SD or as means alongside individual data points, significance values were only considered statistically significant if $P < 0.05$. If interaction main effects were statistically significant syntax post hoc analysis was run and corrected using Bonferroni corrections.

Carotid and ICA Results

Carotid and Internal Carotid Artery Diameter Data

Carotid Artery diameter significantly increased from pre to post-ingestion in both the low flavanol condition (6.06 ± 0.30 → 6.38 ± 0.28 mm, $F(1,6)= 16.765$, $p = 0.006$) and the high flavanol condition (6.17 ± 0.33 → 6.32 ± 0.25 mm, $F(1,6)= 16.765$, $p = 0.006$), however there was no difference between the conditions ($F(1,6)= 0.134$, $p = 0.727$; see **Fig. A1**). Internal Carotid Artery diameter also increased from pre to post-ingestion in both the low flavanol condition (4.92 ± 0.52 → 5.21 ± 0.64 mm, $F(1,6)= 5.405$, $p = 0.059$) and the high flavanol condition (4.77 ± 0.63 → 5.23 ± 0.71 mm, $F(1,6)= 5.405$, $p = 0.059$), however this change did not quite reach significance. There

was no difference between the two conditions in the change from pre to post-ingestion ($F(1,6)= 0.547$, $p = 0.487$; see **Fig. A1**).

Carotid and Internal Carotid Velocity Data

Carotid Artery velocity decreased from pre to post-ingestion in both the low flavanol condition ($42 \pm 7 \rightarrow 34 \pm 8$ cm·s^{-1}, $F(1,6)= 23.301$, $p = 0.003$) and the high flavanol condition ($40 \pm 7 \rightarrow 34 \pm 9$ cm·s^{-1}, $F(1,6)= 23.301$, $p = 0.003$), however there was no difference in this change between the two conditions ($F(1,6)= 0.221$, $p = 0.655$; see **Fig. A2**). Internal Carotid Artery velocity significantly decreased from pre to post-ingestion in both the low flavanol condition ($40 \pm 10 \rightarrow 24 \pm 5$ cm·s^{-1}, $F(1,4)= 9.249$, $p = 0.038$) and the high flavanol condition ($33 \pm 16 \rightarrow 32 \pm 6$ cm·s^{-1}, $F(1,4)= 9.249$, $p = 0.038$). This decrease in velocity within the Internal Carotid Artery from pre to post-ingestion was not different between the two conditions ($F(1,4)= 0.046$, $p = 0.841$; see **Fig. A2**).

Carotid and Internal Carotid Flow Rate Data

Carotid Artery blood flow rate decreased from pre to post-ingestion in both the low flavanol condition ($725 \pm 123 \rightarrow 659 \pm 120$ cm·s^{-1}, $F(1,6)= 9.064$, $p = 0.024$) and the high flavanol condition ($718 \pm 138 \rightarrow 634 \pm 184$ cm·s^{-1}, $F(1,6)= 9.064$, $p = 0.024$), however there was no difference in this change between the two conditions ($F(1,6)= 0.066$, $p = 0.805$; see **Fig. A2**). There was no change in blood flow rate within the Internal Carotid Artery from pre to post-ingestion in either condition ($F(1,4)= 1.351$, $p = 0.310$) and there was no difference between the two conditions ($F(1,4)= 0.087$, $p = 0.783$).

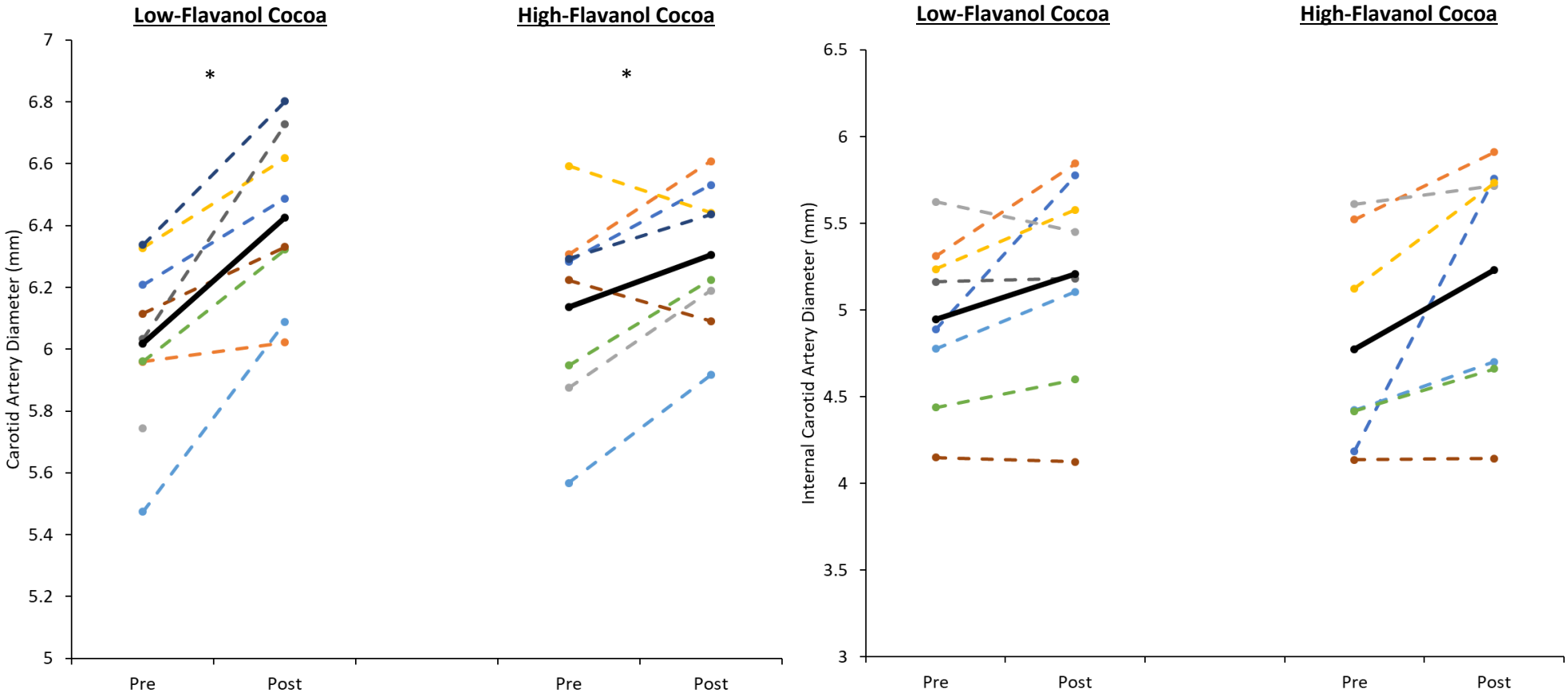


Figure A1. Shows Carotid Artery and Internal Carotid Artery Diameter pre-ingestion and post-ingestion of both low flavanol and high flavanol cocoa. All individual data is displayed including the means (as shown by the black lines). Main effect significance; * P<0.05, comparison between time points (pre vs post), † P<0.05, comparison between conditions (low flavanol and high flavanol cocoa). Interaction effect significance; ⁋ P<0.05, condition (low flavanol and high flavanol cocoa.) and time interaction (pre vs post), ¥ P<0.05, time (pre) and condition interaction (low flavanol and high flavanol cocoa), Ω P<0.05, time (post) and condition interaction (low flavanol and high flavanol cocoa).

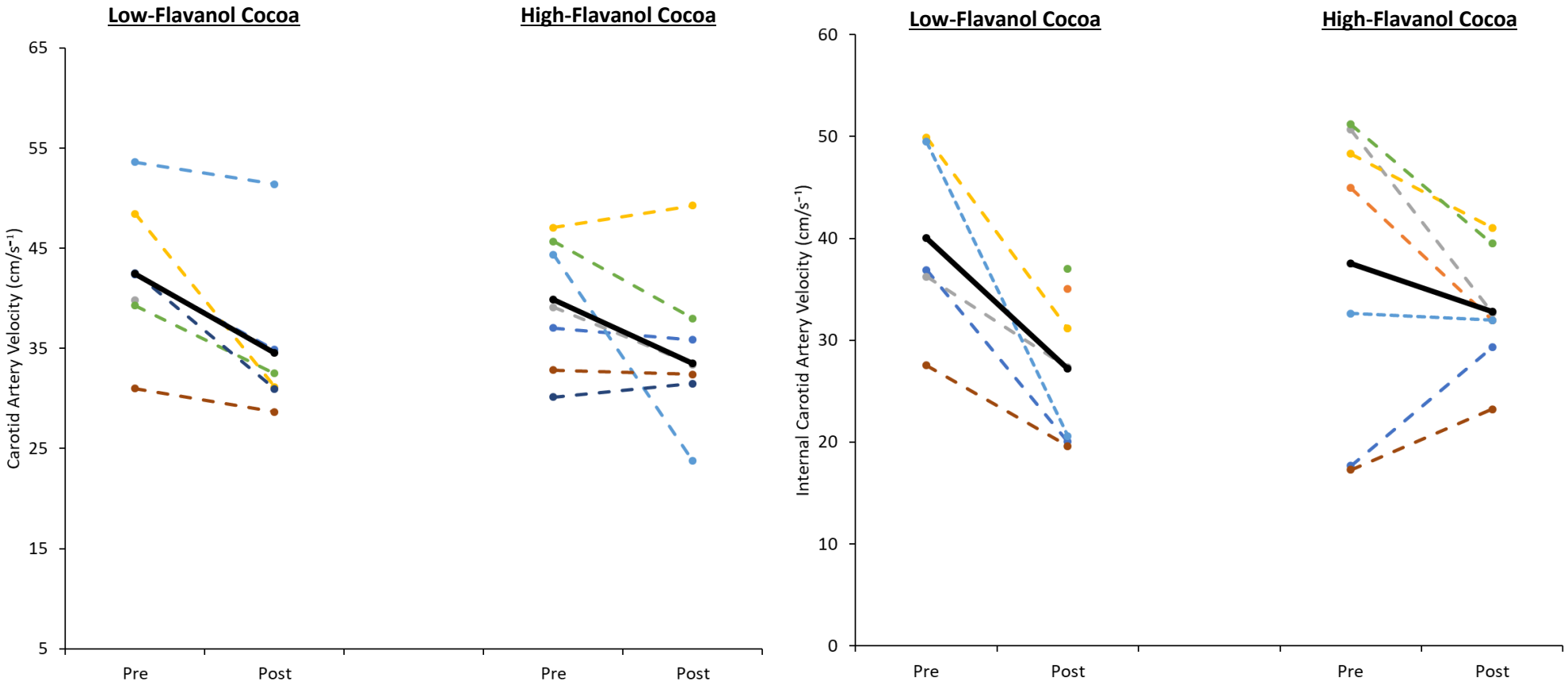


Figure A2. Shows Carotid Artery and Internal Carotid Artery Velocity pre-ingestion and post-ingestion of both (low flavanol and high flavanol cocoa. All individual data is displayed including the means (as shown by the black lines). Main effect significance; * P<0.05, comparison between time points (pre vs post), † P<0.05, comparison between conditions (low flavanol and high flavanol cocoa). Interaction effect significance; ⁋ P<0.05, condition (low flavanol and high flavanol cocoa) and time interaction (pre vs post), ¥ P<0.05, time (pre) and condition interaction (low flavanol and high flavanol cocoa), Ω P<0.05, time (post) and condition interaction (low flavanol and high flavanol cocoa).