University of Reading

Investigating the Effects of Wild Blueberry Polyphenols on Cognitive Function and Cardiovascular Health

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Declaration of Original Authorship

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

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Abstract

Scientific evidence suggests that wild blueberry (WBB) polyphenols elicit benefit for cognitive function in humans. In this thesis, the effects of acute WBB consumption will be explored in participants across different age groups. The aim is to understand how cognitive function is affected in the immediate period post-dosing and to examine how different age groups show sensitivity to WBB in different cognitive domains. Whilst acute effects are interesting, repeated daily dosing is required to have long-term impact and to delay cognitive decline. To explore this further, the consumption of WBB for three months will be explored in a healthy ageing population. The aim of this is to further our understanding related to the cognitive effects of chronic WBB consumption, and to understand which cognitive domains show sensitivity following chronic consumption.

This thesis will also investigate how vascular and cerebral blood flow parameters are affected following acute consumption across the life-course and following chronic consumption in healthy older adults. The aim of this is to elucidate the mechanisms of action behind observed cognitive effects. Furthermore, the assessment of polyphenol metabolites in plasma and urinary samples will be measured to help provide evidence into the bioavailability of metabolites in the human body. Lastly, recent investigations are beginning to demonstrate that polyphenols impact the gut microbiome. To explore this further, the microbial composition following three months consumption of WBB in healthy older adults will be assessed

Benefits for episodic memory, visuo-spatial working memory, and executive function were found following acute consumption across different age groups. Secondly, chronic WBB consumption demonstrated improvements for episodic memory and executive function in healthy older adults. No treatment-related effects were observed for parameters of cerebral blood flow in either study. Both acute and chronic WBB consumption revealed increases in the level of metabolites, when compared to placebo consumption. However, analyses of microbiome revealed no significant differences in alpha or beta diversity following WBB consumption.

As a whole, the results observed from this thesis support the current evidence for WBB and cognitive function. The implications of these findings are of great importance and may be translated into our everyday lives as a way of enhancing cognitive function.

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Abbreviations List

AD	Alzheimer's Disease
ADME	Absorption, Distribution, Metabolism, Excretion
ASL	Arterial Spin Labelling
BBB	The Blood-Brain Barrier
BDNF	Brain Derived Neurotrophic Factor
CBFV	Cerebral Blood Flow Velocity
BMI	Body Mass Index
BOLD	Blood Oxygenation Level Dependent
CBF	Cerebral Blood Flow
CHD	Coronary Heart Disease
CNS	Central Nervous System
CREB	cAMP-Response Element-Binding Protein
CVD	Cardiovascular Disease
EEG	Electroencephalography
ERK1/2	Extracellular Signal-Related Kinase
FETA	The FFQ EPIC Tool for Analysis
FFQ	Food Frequency Questionnaire
FMD	Flow Mediated Dilation
fMRI	Functional Magnetic Resonance Imaging
GABA	Gamma-Aminobutyric Acid
Hb	Haemoglobin
HDL	High Density Lipoprotein
HPLC-DAD	High Performance Liquid Chromatography with Diode-Array Detection
HPLC-MS	High Performance Liquid Chromatography-Mass Spectrometry
IPAQ	International Physical Activity Questionnaire
LDL	Low Density Lipoprotein
LMM	Linear Mixed Modelling
LPS	Lipopolysaccharide
MANT	Modified Attention Network Task
MAO	Monoamine Oxidase
MCA	Middle Cerebral Artery
MCI	Mild Cognitive Impairment
MET	Metabolic Equivalent Task
MFV	Mean Blood Flow Velocity
MMSE	Mini Mental State Examination
MOA	Mechanism Of Action
MWM	Morris Water Maze
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NCDs	Non-Communicable Diseases
NGF	Plasma Nerve Growth Factor
NIRS	Near Infrared Spectroscopy
NO	Nitric Oxide
NVC	Neurovascular Coupling
ORAC	Oxygen Radical Absorbance
Pi	Pulsatility Index
PWV	Pulse Wave Velocity
RCT	Randomized Controlled Trial
ROS	Reactive Oxygen Species
RT	Reaction Time
SST	Steady-State Probe Topography
TCD	Transcranial Doppler Ultrasound
TNF-α	Tumour Necrosis Factor
TST	Task-Switching Test
WBB	Wild Blueberry
UPLC-MS	Ultra-High Performance Liquid Chromatography- Mass Spectrometry

Chapter One

Introduction

1.1 Human Health and Diet

Human health is defined by a number of factors; including genetic, physiological, behavioural and environmental factors. The latest World Health Organization (WHO) report stated that 71% of deaths worldwide are due to non-communicable diseases (NCDs) such as dementia and cardiovascular diseases (WHO, 2021). NCDs are not caused by pathogenic organisms but instead are driven in part by the presence of behavioural factors throughout the lifespan such as smoking, excessive alcohol consumption and poor diet (Hunter et al., 2013).

It is well known that the consumption of fruit and vegetables is vital for maintenance of health. Current UK guidelines recommends that we eat five 80g portions of fruits and vegetables per day (NHS, 2018), although some research suggests that 10 portions a day may be better (Aune et al., 2017). This is not a surprise considering that fruit and vegetables are high in a range of macro- and micronutrients such as fibre, vitamins and minerals which are critical for our immune system, growth, repair, and optimal functioning.

Copious amounts of research support the notion that a healthy balanced diet, rich in fruit and vegetables, is vital for optimal human health and is important in reducing the risk of developing NCDs, particularly as we age. Numerous studies have demonstrated that people whose habitual diets are high in fruit and vegetables suffer less from chronic illnesses compared to those who consume little amounts. Data derived from the Health

Survey for England (HSE) found a correlation between the number of fruit and vegetables consumed and mortality from cancer and CVD, with increased benefits observed amongst people who consumed 7 portions or more (Oyebode et al., 2014). The evidence provided by various epidemiological studies, which will be later described in this chapter in more detail, demonstrate that diet greatly influences our health but more specifically our cognitive health.

One particular area of research in human ageing which has received significant attention is in the field of cognitive health. Research has attempted to show how we can use dietary strategies to delay the onset of neurodegenerative diseases and/or delay the progression of disease trajectory such as dementia. Dementia is an umbrella term for different syndromes including, but not exclusive to; memory loss, difficulty with language and changes in mood, to name a few (NHS, 2020). Examples of dementia include Alzheimer's disease (AD), Vascular dementia and dementia with Lewy bodies (Alzheimer's Research UK). Data suggests that 1 in 6 people over 80 years old are affected by dementia and figures predict that by 2025 more than one million people may be affected (NHS, 2020).

Various pharmaceutical products exist for the purpose of helping dementia sufferers cope with symptoms, but no cure exists. Growing evidence in literature suggests that plant-derived compounds called polyphenols may positively influence cognitive health and delay cognitive ageing; meaning that these compounds may be a strong candidate for delaying or preventing the incidence of neurological conditions as we age

1.2 Polyphenolic Compounds

Plants produce primary and secondary metabolites. Primary metabolites are produced for growth and developmental processes whilst secondary metabolites are produced for adaptation and survival purposes (Zaynab et al., 2019). For example, secondary metabolites may function to counteract predation by herbivores through the production of bitter tasting molecules throughout the plant (Singh et al., 2021), to protect against pathogenic and fungal infections (Levin, 1976), mechanical damage, improving adaption to abiotic stresses such as UV light, drought and temperature (Schäfer et al., 2009) and ameliorating pollination through the production of colourful pigmentation (Stalikas et al. 2007). Examples of secondary metabolites include the terpenoids, steroids, alkaloids and polyphenols (Kessler and Kaskle, 2018).

As well as increasing the chances of a plants' survival, polyphenols have gained significant interest in the past two decades for their beneficial effects in humans and animals. Polyphenolic compounds are found in all plants and exist throughout all plant tissues including the leaves, stem, roots, flowers, and fruits. Over 8000 polyphenolic compounds have been discovered thus far (Tsao, 2010); all of which are classified according to their chemical structures. Polyphenolic compounds contain aromatic rings with hydroxyl rings attached to the aromatic rings. There are two main categories of polyphenols: flavonoids and non-flavonoids. To better understand the mechanisms of action of these compounds it is important to first understand their chemistry. Structurally, flavonoids consist of two benzene rings (ring A and ring B) and a pyrene ring (ring C) connecting the two aromatic rings together whereas non-flavonoids consist of one benzene aromatic ring (Figure 1.1).

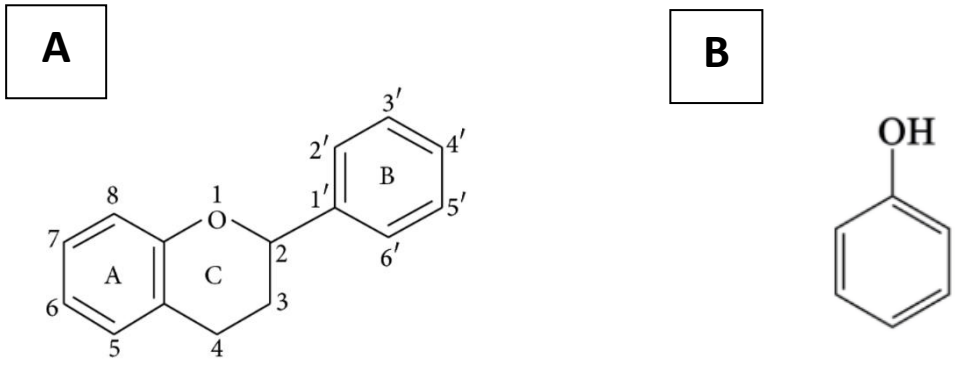


Figure 1. 1 A) Basic structure of flavonoid compound, adapted from Kumar et al. (2013); B) Basic structure of a non-flavonoid compound, adapted from Li et al. (2018)

1.2.1 Flavonoids

Over 4,000 different types of flavonoids have been discovered to date (Harborne and Williams, 2000). Within this there are 6 different classes of flavonoids (Figure 1.2) categorized based on their chemical properties. An example of this is the oxidation of the carbon-hydrogen (C-H) bond into carbon-hydroxyl (C-OH) bond (Spencer et al., 2009). In humans, the six classes of flavonoids have been documented to provide extensive benefits to health when consumed, of which I will be describing in more detail in section 1.5 of this chapter.

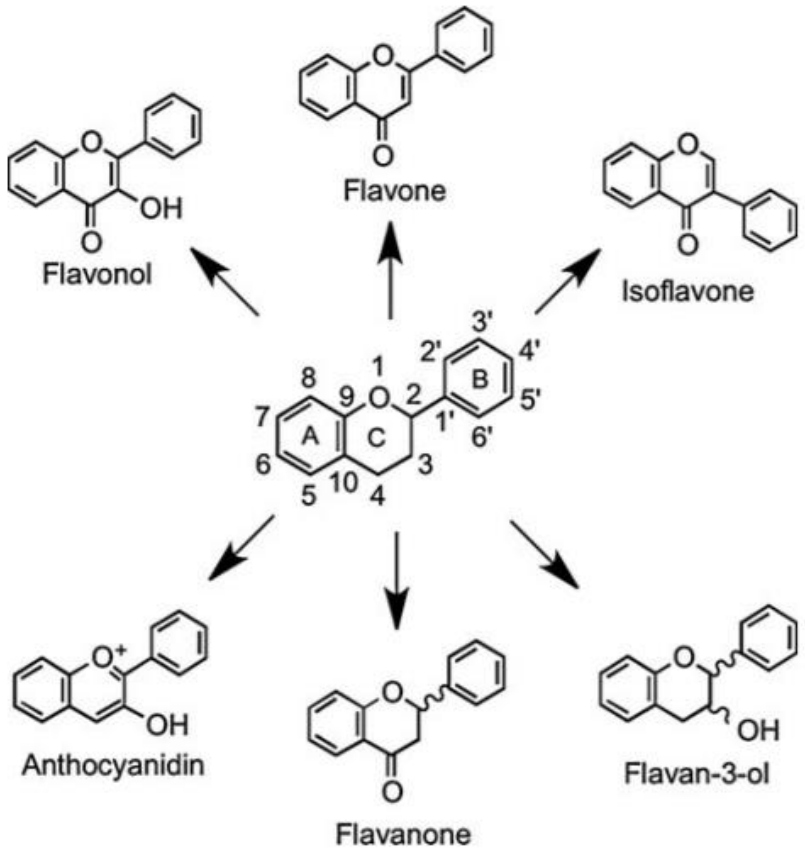


Figure 1. 2 Six classes of flavonoids and their chemical structures. Adapted from Del Rio et al. (2013)

1.2.1.1 Flavones

Flavones (molecular formula: $C_{15}H_{10}O_2$) have the simplest structure and are characterised by two carbon rings joined by a double bond and a benzene ring on the second position. Sub-classes of flavones include apigenin and luteolin. Flavones are particularly abundant in herbs such as parsley and in chamomile flower (Hostetler, 2017).

1.2.1.2 Flavonols

Flavonols (molecular formula: $C_{15}H_{10}O_3$) have a similar chemical structure to flavones but have an extra hydroxyl group. Sub-classes include quercetin and kaempferol. Flavonols are found in an array of fruit and vegetables including onions and kale, as well as tea and red wine (Aherne et al., 2012).

1.2.1.3 Isoflavones

Isoflavones (molecular formula: $C_{15}H_{10}O_2$). Unlike the other 5 flavonoid subgroups, the phenyl group on the benzene ring is on position 3, not position 2. Legumes are abundant in isoflavones, with soybeans being the richest source. Sub-classes of isoflavones include genistein and daidzein (Setchell et al., 1999).

1.2.1.4 Flavan-3-ols

Flavan-3-ols (molecular formula: $C_{15}H_{14}O_5$) are highly reactive due to the occurrence of a hydroxyl group (-OH) on the pyrene ring. Sub-classes include catechin, epicatechin, gallocatechin and theavin which is highly abundant in teas, apples and cocoa (Kuhnle, 2018).

1.2.1.5 Flavanones

Flavanones (molecular formula: $C_{15}H_{12}O_2$). Structurally, flavanones differ from flavones whereby they have a saturated (single) bond between the second carbon ring and the benzene ring. Sub-classes include hesperetin and naringenin and they exist in high quantities across all citrus fruits such as grapefruit, oranges and lemons (Barecca et al., 2017).

1.2.1.6 Anthocyanidins

Anthocyanidins (molecular structure= $C_{15}H_{11}O^{+}$). The glycosylated form of anthocyanidin is called anthocyanins. Structurally they do not contain a covalently-bonded oxygen on the 4th position. Sub-classes include cyanidin, delphinidin, pelargonidin, peonidin, petunidin, and malvidin (Khoo et al., 2017) (Table 1.1). Anthocyanins are primarily found in highly pigmented plant sources such as berries and colourful flowers. The name is of Greek origin with 'anthos' meaning flower and 'kianos' meaning blue (Castañeda-Ovando, 2008). The pigmentation is dependent on its chemical pH, with acidic and alkaline solutions resulting in different colours (Khoo et al., 2017).

Table 1. 1 Anthocyanidins and their corresponding colour and examples of where they are found in nature

Anthocyanidin	**Colour**	**Example in Nature**
Cyanidin	Red	Raspberries
Delphinidin	Pink-purple	Pomegranate
Pelargonidin	Orange-red	Plums, strawberries
Peonidin	Magenta	Cranberries
Petunidin	Purple	Purple flowers, blackcurrants
Malvidin	Red-purple	Grapes, blueberries

1.2.2 Non-Flavonoids

Among the major classes of non-flavonoids documented for nutritional importance, phenolic acids are the most abundant class found in the human diet (Singla et al., 2019). Two sub-classes of phenolic acids exist, namely the cinnamic acid and benzoic acids and their corresponding derivatives. Common examples include gallic acid, a derivative of hydroxybenzoic acid highly abundant in tea or chlorogenic acid, a derivative of hydroxycinnamic acid, which is abundant in coffee and blueberries (Manach et al., 2004).

Stilbenes are found across many different types of fruit and vegetables. Examples of common stilbenes include resveratrol and piceattannol which is abundant in fruits such as grapes (Reinsilo et al., 2015). Finally, lignans are found in relatively high concentrations in grains and seeds, with particularly rich sources found in flaxseeds and sesame seeds (Rodriguez-Garcia, 2019) (Figure 1.3).

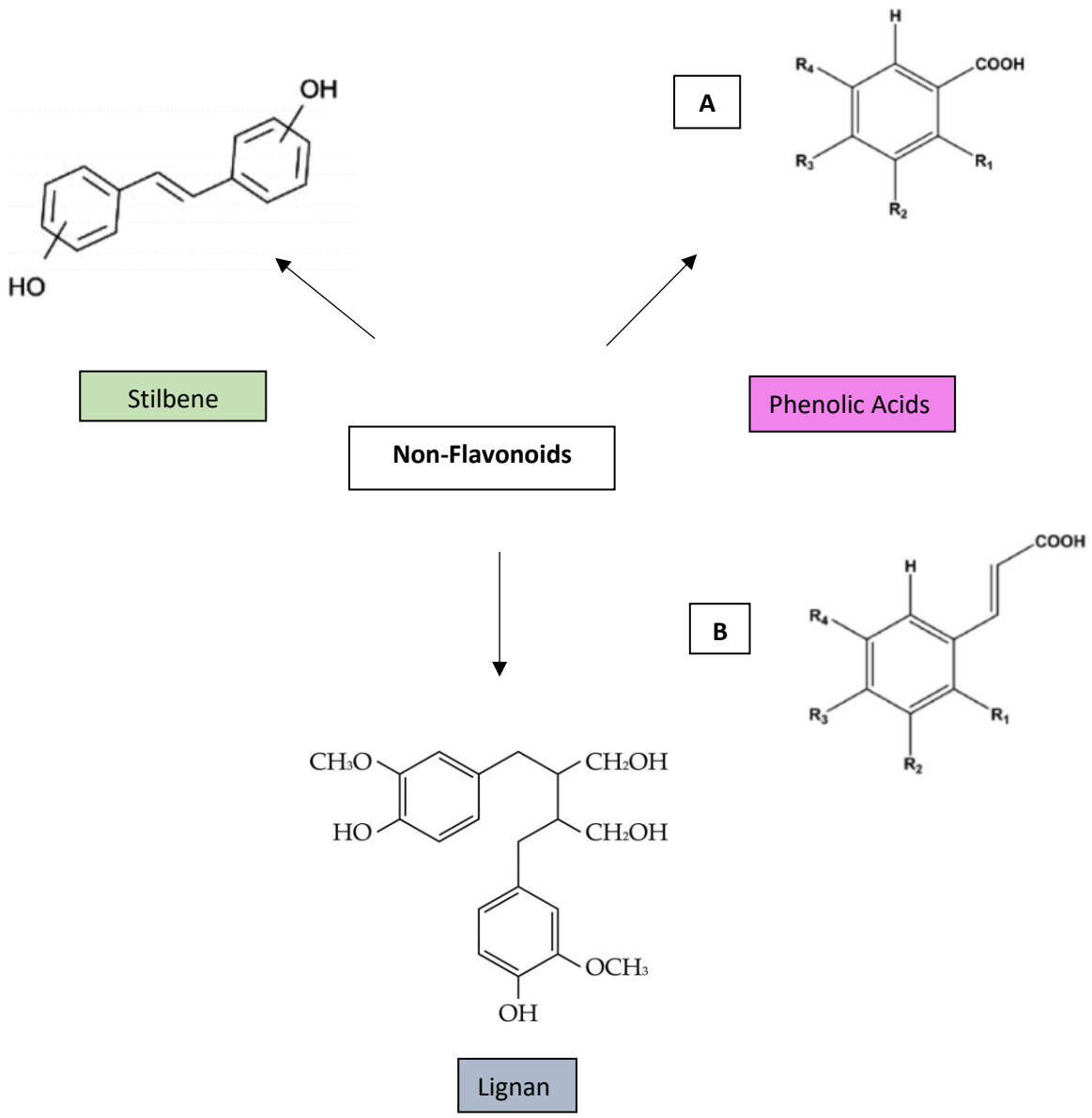


Figure 1. 3 Basic chemical structure of non-flavonoids including a stillbene (adapted from Singla et al., 2019), phenolic acid derivates a) hydrobenzoic acids and b) hydroxycinnamic acid adapted from Stalikas, (2007) and example lignan compound adapted from Mrduljas et al. (2017)

1.3 Absorption, Distribution, Metabolism and Excretion (ADME) of Flavonoids

The ADME of flavonoids in the human is highly complex. Flavonoids are metabolised in the gastrointestinal tract by phase I and II enzymes and by the gut microbiome, and involves many different processes (Del Rio et al., 2013; Scalbert et al., 2002).

Bioavailability refers to the existence of nutrients or non-nutrient such as polyphenols, in a form which can be used by the body for physiological purposes or in a form that is stored for future use, instead of excreted (Bohn, 2014). The bioavailability therefore is very much dependent of the rate of absorption of polyphenols in the body. The absorption process can be affected by various factors. Firstly, different types of polyphenols exist during different developmental stages of a plant (Stalikas, 2007). For example, in blueberries, it has been shown that flavonols, flavanol-3-ols and phenolic acids were found in high contents in unripe green berries, whereas anthocyanins levels were highest in ripe berries with their developed purple colour (Gibson et al., 2013). Secondly, growing conditions including temperature, water availability and nutrient content of the soil greatly influences polyphenol quantity in plants. It has been documented that when the plant experiences abiotic stress, such as drought, the production of polyphenols is increased. These secondary metabolites offer the plants more protection in order to increase the chances of survival (Thakur et al., 2019). Other factors that can affect the polyphenol content in plants include the ripeness of the fruit at the time of harvest, processing methods such as freeze-drying or cooking, and storage conditions post-harvest (Manach et al., 2004). The variety of the crop can also influence polyphenol content with different cultivars containing differing amounts of polyphenolic compounds (Matthes et al., 2009). In addition to this, the chemical structure of polyphenols can affect their hydrophilicity and therefore their bioavailability in the body (Mrduljas, 2017). One way

in which the chemical structure of polyphenols can affect the bioavailability in humans is that they cannot be absorbed by the body when they exist in their glycosylated form, instead they must be hydrolyzed into their aglycone form to be absorbed efficiently. This means that the number and type of sugar molecule attached can influence the rate at which they are absorbed in the small intestine (D'Archivio, 2010). Moreover, the number of hydroxyl rings attached to the aromatic ring also influences the bioavailability of the compound due to the requirements of further metabolism of hydroxyl rings prior to absorption. Compounds with no hydroxyl rings may be able to be absorbed by the body without the need of hydroxylation in the small intestine (Bohn, 2014). It may also influence the bioactivity of polyphenols (Heim et al., 2002).

The Blood-Brain Barrier (BBB) is a layer composed of three different cell types dividing the vascular system and the vertebral regions. The BBB influences what reaches brain circulation and also plays a protective role by preventing xenobiotic substances such as toxins, from entering the brain and in turn allowing beneficial compounds such as nutrients and glucose (Faria et al., 2012). It has been reported that flavonoids may be able to exert their antioxidant and anti-inflammatory effects in the central nervous system (CNS), by permeating through the BBB, which is highly selective and facilitates compounds of certain chemical properties only (Vauzour, 2012). Nevertheless, this may not be the only mechanism, as flavonoids may have indirect effects without the need of entering the BBB. As mentioned before, the bioavailability of flavonoids is dependent on their hydrophilicity. To understand whether flavonoid metabolites are able to cross the BBB, a number of in-vitro and in-vivo studies have been conducted with inconclusive findings. Faria et al. (2011) demonstrated that catechin and epicatechin compounds do cross the BBB during an in-vitro investigation, whilst naringin (Peng et al., 1998) and anthocyanins (Milbury and Kalt, 2010) were similarly found to cross

the BBB in in-vivo studies conducted in rodents. However, questions remain about whether the levels of flavonoids found to have crossed the BBB are sufficient to have produced antioxidant and anti-inflammatory effects. Importantly, studies assessing the bioavailability of flavonoids in the brain is limited, due to the difficulty in accurately measuring flavonoids, as well as their chemical instability, thus evidence regarding flavonoid concentration in brain tissues should be taken cautiously.

Most flavonoids present in food have a sugar molecule such as glucose or galactose attached via a glycosidic bond (Heim et al., 2002). Following the ingestion of flavonoids, the absorption of some constituents into the bloodstream take place in the small intestine. The enzyme lactase phloridizin hydrolase, which resides in brush border of the small intestine, hydrolyses the glycosidic bond to form aglycones (Del Rio, 2013). Aglycones, which have increase lipophilicity compared to its glycoside form may then enter epithelial cells via passive diffusion. Alternatively, glucosides may be cleaved into aglycones via the action of cytosolic b-glucosidase enzymes in the epithelial cells via the sodium dependent glucose transporter 1 (Del Rio et al., 2013).

Before the aglycones enter the circulatory system, phase II metabolism occurs whereby enzymes sulfotransferases, uridine-5'-diphosphate glucuronosyltransferases, and catechol-*O*-methyltransferases form sulfate, glucuronide and in some cases methylated metabolites into sulfate, glucuronide or methylated metabolites (Del Rio et al., 2013). Some of these metabolites may travel back to the small intestinal lumen and some metabolised compounds may travel through the portal vein to the liver. In the liver these are hydrolysed further into polar compounds and transported into the bloodstream whereby small amounts of the metabolised compounds may be recycled back to the intestinal lumen. These metabolites,

which do not get absorbed into the bloodstream, are transported to the colon where they are extensively metabolised by the gut microbiota. Here, bacterial enzymes metabolize the compounds into smaller phenolic compounds, which facilitates their ability to be re-absorbed into the liver where they may undergo further metabolism. Subsequently, most of these metabolites are absorbed into the bloodstream and transported to the kidneys where they are excreted via urine.

Numerous studies have been conducted investigating the absorption and metabolism of flavonoids following consumption of flavonoid-rich foods and a detailed review is beyond the scope of this thesis. Nevertheless, literature suggests that factors such as gender (Del Bo et al., 2019), and age (Alkhaldy et al., 2019) influenced mainly by habitual diet (Del Bo et al., 2019) may all result in variations in the metabolism process, indicating that the ADME of flavonoids may be highly variable and personalised.

1.4 Evidence for Flavonoids and Health

1.4.1 Mechanisms of Action (MOA) of Flavonoids

The past two decades has seen an ever-growing interest into the effects of flavonoids in the human body. Copious amounts of research ranging from rodent studies, epidemiological and human RCTs suggest that flavonoids anti-inflammatory, anti-microbial and anti-oxidant properties may exert benefits and protection against chronic illnesses including cardiovascular and neurodegenerative diseases. The process of inflammation is a common occurrence across multiple chronic diseases including cardiovascular health, dementia, cancer and asthma (Maleki et al., 2019). It has been reported that flavonoids may reduce the progression of inflammation through mechanisms such as reducing the activity of protein kinases which are involved in transduction during cell inflammation, (Maleki et al., 2019). In addition to this, their chemical structure gives flavonoids their antioxidant properties due their ability to scavenge free-radicals by donating a hydrogen atom (Al-Sehemi & Irfan, 2017), although whether the antioxidant capacity of flavonoids in vitro is relevant in human health is still a matter of extensive debate (Hollman et al., 2011).

1.4.1.1 The Effects of Flavonoids on Neuroinflammation

Flavonoids have been actively studied for their neuroprotective effects mediated by a number of physiological processes. One of these includes the role of flavonoids in the reduction of neuroinflammation in the brain. Neuroinflammation is often a natural and normal occurrence in healthy people; for example, due to an infection. However, persistent, and accelerated neuroinflammation may eventually contribute towards the progression of neurodegenerative

disorders such as Alzheimer's disease (Spencer et al., 2012). Immune cells in the CNS and microglial cells, play an important role as first responders against pathogenic attack as well as removal of toxin accumulation in the brain (Spencer et al., 2009). However, over activation of the microglial cells due to increased pathogenic response may cause harmful side-effects by increasing levels of nitric oxide (NO) (Spencer et al., 2009). As a result, this may lead to an increase in reactive oxygen species (ROS) and inflammation-causing cytokines (Spencer et al., 2009). This may consequently lead to apoptosis, which as a result may lead to neurodegeneration. Flavonoids have been extensively studied for their potential to reduce neuroinflammation through a number of suggested mechanisms of action. The role of flavonoids in reducing neuroinflammation is complex. *In vitro* investigations have shown that flavonols, which possess antioxidant properties, are able to reduce apoptosis and may therefore reduce the negative effects of oxidative damage (Kang et al., 2004). Similarly, studies conducted in animal models demonstrate that flavonoids are able to mitigate effects that lead to apoptosis (Nakayama, 2011; Hwang et al. 2012). Regular consumption of antioxidant flavonoids may therefore minimise neuro-inflammation by reducing ROS activity, however it has to be noted that metabolism was not taken into account in these in-vitro and animal studies.

To understand whether polyphenolic metabolites in the blood could mediate anti-inflammatory effects, Shukitt-Hale et al. (2019) investigated the effects of anthocyanins on inflammation, measured via the production of NO and TNF-α induced by an endotoxin known as lipopolysaccharide (LPS). This was assessed in the brains of rats with different levels of cognition, fed on a blueberry intervention versus rats who were on a control diet. The results found significantly lower NO levels amongst the blueberry fed rats with observed lower cognitive function compared to the control group. This was also observed for the production

of TNF-α, which was significantly lower post-test compared to pre-tests for the blueberry supplemented rats with lower and average cognition compared to placebo treated rats with poor and average cognition. Reasons for this may be due to increased inflammation associated with poorer cognitive functioning. Nevertheless, a reduction of TNF-α was also observed for the control group who exhibited poor cognitive performance. This reduction in lower inflammatory markers was accompanied by improved cognitive performance amongst rats with poorer cognition, but not amongst the rats who displayed high cognitive performance. This indicates that blueberry anthocyanins may reduce inflammation and improve cognition in cognitively compromised animals. Evidence for the effect of neuroinflammation in humans is limited, with one study thus far having collectively analysed the effect of WBB on cognition, CBF and biomarkers of inflammation (Bowtell et al. (2017) However in this case no treatment-related effects were found for anti-inflammatory biomarkers in serum. Therefore, more research is required to understand whether these mechanisms of action relating to inflammation could also be translated to humans; especially those with MCI who are generally at risk of cognitive decline.

1.4.1.2 Anti-Cholinesterase Activity

Although not extensive, some studies have looked at the potential for flavonoids to exert anti-acetylcholinesterase activity in the CNS. Acetylcholinesterase is an enzyme that breaks down acetylcholine, a neurotransmitter which plays a vital role in learning and memory (Hasselmo, 2006). Therefore, a reduction or inhibition of the activity of acetylcholinesterase boosts levels of acetylcholine in the brain and produces beneficial effects on cognitive functioning. For example, galantamine, an acetylcholinesterase inhibitor, is used as a frontline treatment in patients with AD to manage symptoms and unfavourable symptoms associated with the

disease (Trinh et al., 2003). With respect to the flavonoids, a number of *in vitro* studies suggest that the hydroxyl group on the 'A' ring of flavonoids may exert anti-cholinesterase activity and may therefore be a mechanism through which flavonoids boost cognitive performance, particularly in individuals with dementia or MCI (Khan et al, 2018).

1.4.1.3 Effects of Flavonoids on Neuronal Signalling

Studies have also suggested that flavonoids are able to stimulate the production of new neurones (Spencer, 2010; Cichon et al., 2020) and modulate cell signalling (Williams et al., 2004). Cyclic AMP Response Element Binding protein (CREB) plays an important role in learning and memory formation. This is documented via animal studies which demonstrate a deficit in memory when CREB levels are lowered (Guzowski and McGaugh, 1997) or deactivated (Kida et al., 2002). Furthermore, Williams et al. (2008) demonstrated that rats who received a blueberry supplementation had higher CREB levels and significantly improved spatial memory after 12 weeks consumption. Increases in brain derived neurotrophic factor (BDNF) and extracellular signal-related kinase (ERK1/2) was also observed during this investigation. BDNF is a signalling protein that play a role in neuronal plasticity (Bramham and Messaoudi, 2005). The increase in BDNF levels and ERK1/2 following flavonoid consumption is also supported by animal investigations conducted by Rendeiro et al. (2012). Interestingly, the increase of BDNF was observed in the hippocampus, a region of the brain known for memory formation and the effective learning of new information.

1.4.1.4 Flavonoids and Cardiovascular disease (CVD)

CVD is reported to be the number one cause of death around the world and encompasses a range of disorders including coronary heart disease, cerebrovascular disease, and peripheral

artery disease (WHO, 2021). Flavonoids have been investigated for their ability to ameliorate cardiovascular health and protect against CVD through a variety of mechanism. Firstly, epidemiological studies demonstrate that a diet rich in fruit and vegetables, and therefore flavonoids, reduces the incidence of mortality from heart diseases. Key et al. (1996) conducted an observational study looking at people's dietary habits and mortality outcome. 11,000 volunteers took part in this 17-year long study, with results revealing that a consumption of fruit and vegetables daily resulted in a significant reduction of death due to either ischemia heart disease or cerebrovascular disease. Risannen et al. (2003) also observed similar results in a 12.8 year long follow up study, with findings showing that men between the ages of 42-60 years of age, who consumed diets high in fruit, vegetable were at a lower risk of cardiovascular related mortality. Jennings et al. (2012) observed through a cross-sectional study that women who were regular consumers of fruit and vegetables had a lower central blood pressure and lower arterial stiffness. Once analysed by flavonoid type, the researchers found that this was associated with the consumption of anthocyanins and flavones specifically. In support of this, a dose-response meta-analyses of 39 prospective cohort studies recently conducted by Micek et al. (2021) revealed that specific flavonoids play a pivotal role in this decrease, with anthocyanins and flavan-3-ols shown to decrease the risk of CVD. They also found that flavonols and flavones exhibited important roles in reducing coronary heart disease (CHD), that flavanones were associated with lower risks of stroke incidence and catechins displayed effectiveness against all disease outcomes (Micek et al., 2021).

Nevertheless, although these epidemiological studies provide insight into the relationships between polyphenols and vascular health, they do not take into account additional factors which may also influence the progression of vascular disease. Therefore, in order to

understand the effects of polyphenols on cardiovascular health further, it is recommended that randomized controlled trials are conducted.

Several RCTs in humans looking at the effects of flavonoids in vascular outcomes suggest that flavonoids may decrease the levels of low-density lipoprotein (LDL)-cholesterol. This is demonstrated in a meta-analysis of 45 RCTs whereby patients in the flavonoid intervention group had lower levels of LDL post intervention versus the control group (Luis et al., 2018).

Blood vessels are lined with endothelial cells, referred to as the endothelium. This endothelium layer plays an important role in optimal vascular function as it plays a role in blood flow, regulation of clotting and pressure (Rajedran et al., 2013). Endothelial function can be assessed clinically using an ultrasound technique referred to as flow-mediated dilation (FMD), a validated gold standard assessment. Studies looking at the effects of flavonoids on FMD generally show that flavonoids may significantly improve FMD. For instance, Rodriguez-Mateos et al. (2013) observed significant improvements in FMD after consumption of blueberry flavonoids. This increase was accompanied by a reduction in Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a producer of free radicals, which gives us a potential mechanistic insight behind improvements in endothelial function.

Lastly, it is widely documented that poor vascular health correlates with the incidence of developing neurological disorders as age increases (Satizabal et al., 2016) and that poorer cognitive function may be associated with poorer cardiovascular health (Taylor and Macqueen, 2007). Although reasons for this is still unclear, evidence suggests that poor arterial health leads to a decrease in circulation, including cerebral blood flow (CBF) (Pase et al., 2017), and as a result limits the amount of oxygen, nutrients and energy to the brain. This negatively impacts the neurological functions, and chronically may lead to pathological

changes which may causes neuro-vascular disorders such as vascular dementia (O'Brien and Thomas, 2015). Furthermore, it is known that CBF naturally decreases as we age for reasons which could be related to cardiovascular dysfunctions as ageing progresses, including arterial stiffening and elevated blood pressure (Tarumi & Zhang, 2018).

1.4.1.5 Flavonoids & Glucose Metabolism

Observational studies have shown that flavonoid consumption may be associated with lower insulin resistance, an incidence often associated with type 2-diabetes (Jennings et al., 2013). This is supported by a meta-analysis conducted by Guo et al. (2016) revealing that the risk of developing type-2 diabetes was significantly reduced with a diet rich in anthocyanins and berries. In terms of randomized controlled trials with human subjects, Whyte et al. (2020) observed a reduction in glucose and insulin for 2hrs following a meal in middle-aged participants who consumed a wild blueberry beverage (475mg anthocyanins) together with the meal, versus a placebo. Similar observations have also been observed in Bell et al.'s (2017) study whereby they found that a blueberry treatment containing 724mg anthocyanins resulted in elevated blood glucose levels for 120 minutes. Metabolically speaking, this is important in reducing the incidence of hypoglycaemia, which is when the blood sugar level drops below a healthy level (Amiel, 2021). Mechanisms behind these effects of flavonoids on glucose metabolism include flavonoids' potential ability to control glucose homeostasis which play a role in insulin sensitivity (Russo et al., 2019) and also flavonoid's potential ability to protect insulin-secreting beta-cells (Ghorbani et al., 2019). The ability to regulate glucose metabolism may play an important role in the prevention of certain vascular disorders such as type-2 diabetes.

1.4.1.6 Flavonoids for Cerebral Blood Flow (CBF)

To date, a small number of studies have looked at the effects of flavonoids on CBF in humans. For instance, Lamport et al. (2016) investigated the effects of a flavanone-rich citrus juice on CBF measured using arterial spin-labelling (ASL) in a group of 16 young healthy adults and found that regional perfusion was significantly greater for the citrus juice treatment group compared to the control group at 2 hours post-consumption. More recently, Jackson et al. (2020) investigated the effects of three phenolic-rich extracts a) blueberry b) apple c) coffee berry containing beetroot, ginseng, and sage, on cognitive function and CBF in a group of healthy adults aged between 18-49 years old. The researchers employed near-infrared spectroscopy (NIRS) as a measure of CBF and found significant positive effects for total haemoglobin for the apple extract. In addition to this, significant positive effect for oxygen saturation was found for all 3 treatment groups compared to placebo, suggesting that polyphenols have the ability to enhance CBF. Moreover, Francis et al. (2006) observed blood oxygenation level-dependent signalling (BOLD) increases via fMRI following 5 days consumption of a high flavanol cocoa drink compared to a low flavanol cocoa drink in the right hemisphere region in a group of young females aged 18-30 years old. Francis et al. (2006) also observed a peak in CBF using arterial spin-labelling (ASL) at 2hrs post consumption following the high flavanol cocoa drink but not the low flavanol cocoa beverage.

Effects for CBF in older adults have also been observed in the study by Lamport et al. (2015) who found a significant increase in regional perfusion measured via ASL following acute cocoa consumption with a high flavanol content versus a low-flavanol treatment) in a group of adults aged 55–65 years old. Similarly, Sorond et al. (2008) looked at the effects of flavanol-rich cocoa supplementation on cerebral blood flow in a group of older adults aged between

59-83 years old. Here, the researchers divided the recruited participants into two groups and conducted two studies with different study designs. One group was involved in a 2 week-long study looking at how dietary consumption of flavanol-rich cocoa affected CBF velocity (group A). The other group participated in a placebo controlled parallel trial look at how CBF is affected after daily consumption of flavanol rich cocoa versus a flavanol poor cocoa intake (group B). Results found that for group A, CBF velocity was significantly increased at 1 week and 2 weeks' time point, compared to baseline, indicating that flavanols may enhance CBF when consumed daily, whereas no effects of treatment were seen for the poor flavanol group.

Using blueberry polyphenols as their investigations, Bowtell et al. (2017) observed increased cerebral perfusion detected using ASL in healthy young adults following 12 weeks consumption of blueberry versus placebo. Furthermore, previous studies using fMRI have demonstrated significant increases in neural activity during a working memory task following a daily consumption of WBB for 4 months relative to placebo, in older adults with MCI (Boespflug et al., 2018).

These studies suggest that flavonoids may positively affect cerebral blood flow. It is believed that improvements in cerebral blood flow may be mediated by improvements in peripheral blood flow. Furthermore, cerebral blood flow has been proposed as a potential mechanism of action behind changes in cognitive function, nevertheless further research is required to determine these effects. For this reason, the effects of flavonoids on cerebral blood flow, alongside cognitive function will be explored throughout this thesis.

1.5 Flavonoids for Cognitive Function

In recent years, flavonoids have gained interest for their potential benefits to cognitive health. Epidemiological studies have given us an insight into the correlations that exist between the consumption of fruit and vegetables and cognitive function.

1.5.1 Cognitive Function

Cognitive functioning includes the process related to learning, memory and reasoning. Different domains of cognitive functioning exist within these processes which range from processes such as motor skills to memory (Harvey, 2019). These domains can be divided further into sub-domains such as decision making and language. These subdomains often are interrelated within other domains such as language which is involved in semantic memory and processing speed which is involved in executive functioning and attention (Harvey, 2019) (Figure 1.4).

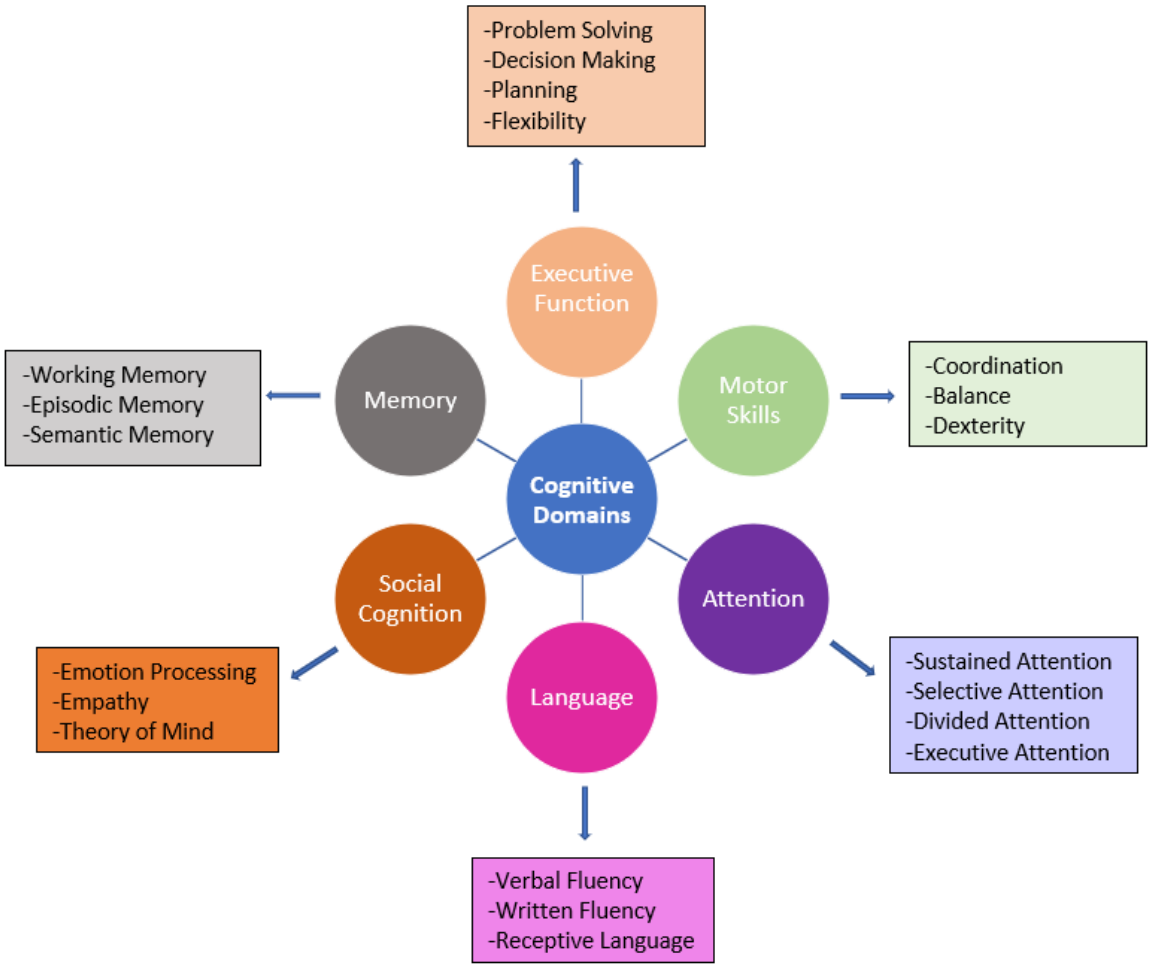


Figure 1. 4 The six main domains which play a role in cognitive function and their subsequent sub-domains. Information sourced from Harvey (2019) and Sachdev et al. (2014)

When it comes to cognitive testing in nutritional psychology, cognitive testing batteries have been developed to test the effects of nutrients on domains in order to better understand sensitivity of the tasks towards the effects of nutritional interventions (see review by de Jager et al., 2014). In the following section I will be providing the evidence in support of flavonoids influencing cognitive function in humans, including RCTs which have utilised different types of flavonoid-rich interventions, tested across different ages and with varying intervention

lengths. The aim of this is to understand if and how domains are affected following flavonoid intake.

Observations from epidemiological studies suggest that consumption of foods that contain high flavonoid content may offer cognitive and neuroprotective properties. For instance, Letenneur et al. (2006) prospectively examined the dietary habits and cognitive function of 1,640 participants with a mean baseline age of 77 over a ten year period. Following baseline assessments, the participants were assessed at 3, 5, 8, 10, and 13 years. At each time point, the participants conducted the same psychometric tests (the Mini-Mental State Examination (MMSE), Benton's Visual Test, and the Isaacs Set Test) to determine cognitive functioning. The researchers used data between year 3 and year 13 (forming a total of 10 years) to observe how cognition evolved. The researchers also calculated flavonoid intake based on the consumption of fruit and vegetables. The results of this 10-year observation revealed that firstly, flavonoid levels positively correlated with the consumption of fruit and vegetables. Secondly, cognitive function at baseline was better for amongst individuals who consumed larger amounts of fruit and vegetables. Lastly, the cognitive decline at the 10 years timepoint was less amongst participants who habitually consumed high flavonoid intakes compared to those who had low flavonoid consumption. This demonstrates that a diet higher in fruit and vegetables, therefore high in flavonoids, may be associated with better cognitive progression as we age.

These observations are supported by the findings of Devore et al. (2012) who looked at the effects of health using data from the Nurses' Health Study which included responses from food frequency questionnaires (FFQ) and cognition collected every 4 years from participants aged 70 years and above. Cognition was assessed using 6 different cognitive tasks, with the

results added up into composite scores to reflect overall cognitive function. Results revealed that reduced cognitive decline was associated with a high total consumption of flavonoids. Interestingly, Devore et al. (2012) also observed that consumption of blueberries and strawberries consumption specifically is associated with delaying cognitive ageing by 2.5 years, which may give us an insight into the cognitive effects of specific flavonoids that exist in high quantities in berries, such as anthocyanins. These observations suggests that flavonoids may offer neuroprotective properties, which consequently may decrease the rate of cognitive ageing. In the following section the acute and chronic effects of flavonoid-rich intervention in humans by randomized, controlled trials will be reviewed.

1.5.2 Cocoa Flavonoids and Cognitive function

1.5.2.1 Cocoa Flavonoids and Cognitive function: Acute Studies

Investigations looking at the acute effects of cocoa on cognitive function have brought mixed findings thus far. For instance, Scholey et al. (2010) found significant improvements following cocoa consumption in healthy young adults. Here, the researchers conducted an acute double blind, placebo-controlled crossover trial testing the cognitive effects of three different doses (0, 520 and 994mg) using a cognitive testing battery including the serials subtraction task assessing working memory, and the Rapid Visual Information Processing (RVIP) task which assesses sustained attention. Results revealed significant increase in correct responses during a Serial 3s Subtraction Task assessing working memory following acute cocoa consumption of the drink containing 520mg and 994mg flavanol, and significant improvements to reaction time following a 994mg flavanol drink. However, although reaction time benefits were seen following consumption of the high dose, it was found that the numbers of errors produced also increased in this case, indicating that a trade-off between speed and accuracy exists for

the high-flavanol treatment, therefore further research looking at the effects of different flavanol doses should be conducted.

Using similar doses to Scholey et al., Decroix et al. (2016) examined executive functioning in a group of 12 healthy men assessed as physically fit and active but found no acute improvements in the executive function task. In support of Scholey's findings, Field et al. (2011) observed significant improvement in correct scores during a visuospatial working memory task in a group of young adults, following dark chocolate containing 773 mg of flavanols relative to white chocolate consumption containing negligible amounts of flavanols. It is worth mentioning that some methodological limitations in this study do exist, as firstly this was a single-blind crossover trial, and the treatments in this study were not matched for caffeine, sugar or theobromine. Caffeine and theobromine are known for various pharmacological effects, which may stimulate the central nervous system, as well for their potential neuroprotective properties (Franco et al., 2015). In addition to this, acute sugar consumption may positively affect cognitive function (Van der Zwaluw et al., 2014; Giles et al., 2018). This means that the effects seen for visuospatial working memory could have been mediated by caffeine, theobromine or sugar. The trend for improvement for the working memory domain following acute cocoa consumption continues with the findings observed in the study by Massee et al. (2015) study who investigated both the acute (2hr post intervention) and chronic (30-day daily consumption) effects of 250mg flavanols versus placebo in a group of 38 young adults. The study used a cognitive testing battery looking at working memory, recognition memory, attention, executive functioning, and reaction time. Results revealed that following the acute dosing regimen, cocoa flavanols significantly improved performance during a serial subtraction task assessing working memory. It is also worth noting that the dose used in Massee's study was relatively lower compared to the other

studies which have observed effects for the working memory domain thus far, which may suggest that even low doses of cocoa flavanols may be effective enough for working memory benefits to be seen. Nevertheless, it should be taken into consideration that, similarly to the study by Field et al. (2011), the control was not matched for caffeine levels, therefore it cannot be ruled out that caffeine may have also affected cognition in this case.

Working memory was assessed further in Grassi et al.'s (2016) study who investigated whether acute cocoa flavonoids consumption could mitigate the effects of sleep deprivation on cognitive function. Here, the researchers conducted a double blind, crossover RCT in a group of 32 young volunteers who experienced sleep deprivation prior to the testing session. Sleep deprivation in this case was induced as part of the testing procedure by continuously keeping the participants awake in the test laboratory throughout the night. The participants were administered the interventions in the morning which included a high flavanol cocoa (520mg) treatment versus a low-flavanol cocoa treatment (88.5mg). Cognitive tests were administered 2 hr post consumption of treatment and consisted of tasks assessing sustained attention and working memory. Unlike Scholey et al. (2010), Field et al. (2011) and Massee et al. (2015) who observed benefits of acute cocoa consumption for the working memory domain, the results revealed no significant main effect of treatment for cognitive function. However, it should be remembered that the study sample is not comparable to other study populations mentioned thus far, as participants in the study by Grassi et al. (2016) was subject to sleep deprivation. However, the researchers did find a significant post-hoc interaction whereby sleep deprived females performed better following the high-flavanol treatment, relative to the sleep-deprived females who consumed the low-flavanol treatment. Although further research is recommended, it does demonstrate that acute cocoa consumption may maintain cognitive function following sleep deprivation.

Pase et al. (2013) did not find any improvements in cognitive function after testing the acute effects of a 500 mg, 250 mg or 0 mg flavanol intervention on cognitive performance in healthy adults aged 40–65 years old following 30 days consumption. Acute testing time points included 1, 2.5 hour, and 4 hours post consumption. A testing battery looking at an array of domains including working memory, episodic memory, attention and reaction time was used but no significant differences between treatment were observed. Nevertheless, it is worth noting that a lunch was administered prior to the 2.5 hour, and 4 hours testing time points, therefore the effects of the lunch, in terms of calorific energy it provided, may have masked the effects of cocoa flavanols on cognitive function.

To my knowledge Calderón-Garcidueñas et al. (2013) conducted the first study investigating the cognitive effects of cocoa in children, who were living in urban areas exposed to high air pollution. Here, they looked at 680mg of cocoa flavanols administered daily to 15 children for a range of 9-24 days. Short-term working memory was measured using object and letters span tasks at baseline and 4 hr after the last consumption of their allocated cocoa intervention. Fifteen out of the 18 children tested showed a significant improvement on an individual level in the memory tasks, but this was not enough for the overall group change to be significant. Nevertheless, a few issues were detected with the study design including a lack of a control to compare against the effects of cocoa and the inconsistency in the number of days the intervention was consumed. Additionally, the children were allowed to add sugar of any quantity, to the test drink. Literature suggests that sugar may improve cognitive function, in children and young adults (Bellisle, 2004; Giles et al., 2018). This may therefore render the results inconclusive.

Very recently, Lamport et al. (2020) conducted an acute parallel RCT looking at whether dark chocolate versus white chocolate had any effects on episodic memory, which has not yet been explored, in a group of young people. In this study, the dark chocolate intervention contained 83mg of flavonoids per serving, flavanol content not specified. Although the flavonoid content is lower relatively to previous studies, it represents a realistic quantity, of which can be translated into everyday consumption. Lamport et al. (2020) found that after 2 hours of consumption, dark chocolate led to significant improvements across 6 out of the 8 measures of the episodic memory task. This study suggests that dark chocolate may acutely improve episodic memory in university-aged people, a cognitive domain which is particularly important for learning and information retention. Nevertheless, it must be noted that the caffeine and theobromine levels were not quantified in either treatment, which means that the potential effect of theobromine and/or caffeine on cognition cannot be disregarded.

Overall, beneficial effects were seen for working memory following acute cocoa consumption of an extensive range of doses. This demonstrates that further testing is required to understand what dose is optimal for cognitive effects to be observed, especially since lower doses have also been observed for episodic memory improvements (Lamport et al., 2020). Lastly, there is little existing evidence for the effects of cocoa for young children, and no studies looking at the acute cognitive effects of cocoa have yet been conducted in older adults. Future research exploring acute cocoa effects on cognitive measures across a wide range of ages is recommended.

1.5.2.2 Cocoa Flavonoids and Cognitive Function: Chronic Studies

As outlined above, investigations into acute effects of cocoa have demonstrated cocoa's ability to enhance cognition. Observations form epidemiological studies also suggest that long-term consumption of flavonoids offer cognitive and neuroprotective properties. To investigate this further, various RCTs have been conducted to understand whether chronic cocoa consumption offers any benefits to cognitive function. For instance, Francis et al. (2006), conducted a crossover study to assess executive function in a group of females aged 18-30 years. Here, the participants consumed either a high flavanol cocoa drink containing 172 mg flavanols or a low flavanol cocoa drink containing 13mg flavanols. However, following 5 days consumption of both treatments, no significant effects of treatment were observed for cognitive function. Reason for the lack of observed treatment effects may be due to an insufficient number of days of treatment consumption (5 days).

To assess whether longer period of cocoa consumption may enhance cognition, Crews et al. (2008) conducted a parallel, double blind study whereby participants aged 60 years or older consumed either a supplement containing 754.71mg of cocoa proanthocyanins (flavanol content not specified) or matched placebo, daily for six weeks. Cognitive function was assessed using the Mini-Mental State Examination (MMSE) which consists of tasks assessing domains including working memory, episodic memory, visuo-spatial memory, language, and attention. However, the study did not find any significant beneficial effects of treatment for cognitive function. The researchers suggested that the lack of findings may be attributed to the high level of education amongst the study cohort, short duration of treatment consumption and an already healthy population. Other contributing reasons may be that the MMSE is not sufficiently sensitive to detect effects of a short-term nutritional intervention,

leading to ceiling effects. In support of this, Camfield et al. (2012) assessed spatial working memory in a group of 63 adults aged between 40-65 years who consumed either a high flavanol cocoa drink (500mg), a medium flavanol drink (250 mg) or a placebo (0 mg flavanol) drink daily for 30 days. A cognitive battery assessing working memory was administered at baseline and 30 days post consumption; however, findings revealed no differences between any treatment groups on the accuracy scores or reaction time scores. These findings by Camfield et al. (2012), are in line with Francis et al. (2006) and Crews et al. (2008 which suggests that the working memory test used in Crews et al.'s (2008) or Camfield et al.'s (2012) study was not sufficiently sensitive to detect changes in cognitive function following chronic consumption of cocoa flavonoids.

In support of this, Massee et al. (2015) did not find any effects of treatment on cognition after 30 days consumption of a 250mg flavanols cocoa treatment versus placebo in a group of 38 young adults. The study utilized a cognitive testing battery looking at working memory, recognition memory, attention, executive functioning, and reaction time. Interestingly, Massee et al. (2015) did find effects following the acute dosing regimen for working memory, as mentioned in section 1.5.2.1.

Sorond et al. (2013) conducted a double blind RCT in a group of 60 healthy older adults with a mean age of 72 years. Here they looked at whether a high flavanol cocoa (609 mg) versus flavanol-poor cocoa (13 mg) consumed daily for 30 days could affect cognitive functioning. Cognition was tested using the MMSE, also used in Crews et al. (2008) study, and the Trail Making Test, with the overall domains assessed including working memory, episodic memory, visuo-spatial memory, language and attention. Adults in this cohort included healthy adults and adults with impaired neurovascular coupling (NVC). For cognitive function, they found

improved psychomotor speed after cocoa in participants who had compromised NVC, but this effect was not seen for the placebo group. The findings are promising and suggest that cocoa flavonoids may improve cognitive health amongst older individuals with impaired NVC, however, it should be taken into consideration that the level of caffeine and theobromine were not quantified in this study.

In support of this, Desideri et al. (2012) investigated the effects of 8-weeks daily consumption of cocoa flavanols or placebo, in a group of 90 older adults experiencing mild cognitive impairment (MCI). This was a double blind, parallel study where the subjects were grouped into either a high flavanol group (990mg), a medium flavanol group (520mg) or a low flavanol group (45 mg), all of which were matched for caffeine and theobromine. The cognitive testing battery consisted of tasks assessing executive function, memory and reaction time. The findings revealed that after 8 weeks of daily consumption of their allocated intervention, significant improvements were observed for the 990mg and 520mg flavanol cocoa on tasks assessing executive functioning. However, the researchers questioned whether the memory tasks were sufficiently sensitive for any effects to be observed after consumption of cocoa flavanols as no effects of treatment were observed for this domain.

Mastroiacovo et al. (2015) adopted a similar study design to Desideri et al. (2012) and conducted a dose-dependent, double-blind study investigating the effects of a cocoa treatment containing 993 mg, 520 mg or 48 mg flavanols on the cognition of a group of 90 healthy older individuals. The effects were measured at baseline and 8 weeks using a battery which includes an array of tasks measuring different domains including memory and reaction time, as well as a composite score of overall cognitive function. Results revealed that after 8 weeks consumption, participants who consumed the 993mg and 520mg flavanol

interventions daily, completed tasks significantly quicker than participants consuming 48mg cocoa flavanol treatment. They also found significant improvements in verbal fluency scores across all three treatment groups, but the magnitude of improvement was less for the 48mg flavanol group. In terms of the global cognitive function, Mastroiacovo et al. (2015) found significant improvements compared to baseline for the high and intermediate flavanol groups and not for the low flavanol group, which suggests that cocoa flavanols may have the ability of improving cognitive function in healthy older adults.

The trend of cognitive improvement post consumption of cocoa flavanols continues with the findings observed by Neshatdoust et al. (2016) who investigated the chronic effects of a high-flavanol cocoa beverage containing 494mg of flavanols versus a low-flavanol cocoa beverage containing 23mg in a group of 40 healthy older individuals. Cognitive domains tested included episodic memory, working memory, executive function, attention and psychomotor speed. A global cognitive score was also calculated by combining the scores for tasks assessing executive function. After 12 weeks of consumption, participants who consumed the high flavanol cocoa treatment had a significantly higher global cognitive score compared to the participants in the low flavanol treatment group.

Along with cognitive function, many of the acute and chronic cocoa studies also assessed other parameters to understand the potential mechanisms of action behind observed cognitive effects. For instance, Field et al. (2011) conducted tests of visual function 2 hours post consumption, which was chosen following the findings by Francis et al. (2006). The study findings revealed a significant improvement in visuospatial working memory, and although inconclusive, suggests that cocoa flavanols may enhance visual ability by increasing blood flow to the retina. Mirroring the findings of Francis et al. (2006), the two studies by Grassi et al.

(2016) and Decroix et al. (2016) revealed no significant effects of treatment for cognitive function, but physiological effects were found following acute cocoa consumption. Grassi et al. (2016) observed a correlation for improvements in performance during a working memory task and improvements in flow-mediated dilation (FMD), and Decroix et al. (2016) observed increased cerebral oxygenation following acute cocoa flavanol consumption. In addition to this, Brickman et al. (2014) observed enhanced neural activity via fMRI following a high flavanol (900mg) cocoa drink relative to a low flavanol drink in healthy older adults. These observations may suggest a potential mechanism of action for cognitive enhancements via improvements in vascular functioning.

For vascular outcomes observed for chronic dosing regimen, Crews et al. (2008) did not observe any effects for blood pressure or blood lipids, following consumption of 775mg of cocoa procyanidin versus in healthy older adults following 6 weeks consumption. However, Desideri et al. (2012), who also considered the effects of cocoa flavanols on blood pressure and blood lipids did observe significant improvements following 520mg and 990mg flavanol cocoa compared to low flavanol in older adults with MCI following 8 weeks consumption, an intervention period slightly longer than Crews et al. (2008). Interestingly, a significant reduction in insulin resistance was also observed alongside improvements in cognition. This may suggest that metabolic function and glucose control may play a role in cognitive function which is an observation also observed by Whyte et al. (2020) following an anthocyanin-rich WBB intervention in middle-aged adults.

1.5.3 Citrus Flavonoids for Cognitive function

Although not as extensively as cocoa, a few studies have been conducted looking at the effects of citrus flavanones on cognitive function. Studies looking at the mechanistic effects of citrus flavanones suggest that they may be effective at preventing neuronal apoptosis (Vauzour et al., 2007) and neuroinflammation (Vafeiadou et al., 2009). Studies using animal models demonstrated that hesperidin, a citrus flavanone, is able to protect neuronal health by preventing the activity of toxins that inflict damaging neuronal changes (Menze et al., 2012) and physiological changes associated with neurodegenerative disease (Kumar and Kumar, 2010).

To understand whether these benefits to neuronal health translate to human cognition following flavanone consumption, Lamport et al. (2016) looked at the cognitive effects of acute flavanone intervention. Furthermore, as described in section 1.4.1.5, flavonoids may enhance cerebral blood flow, which may lead to positive effects for cognition. Here, the researchers assessed the effects of a high flavone citrus beverage containing 70.5 mg of flavonoids versus a placebo containing nil amounts, on cognition in a group of 24 young adults with a mean age of 22 years. The cognitive testing battery consisted of tasks assessing immediate and delayed memory function, working memory, psychomotor speed and executive function. CBF was measured using arterial spin labelling (ASL) through fMRI. Blood pressure was also assessed for vascular effects following flavanones consumption to be understood. Results revealed that the high flavanone beverage resulted in significant improvements in performance on the task assessing executive function at 2 hours compared to baseline, however no other significance differences were observed for the other cognitive tasks. For CBF, the ASL images revealed significant increased blood flow to the areas of the

right hemisphere, at 2h post consumption of the high flavanone drink, which interestingly correlates with the positive effects observed for executive functioning, which may suggest that the effects of flavanols are time-dependent. However, no effects were observed between the two treatments for global perfusion or blood pressure, which may indicate that the increase of CBF in right hemisphere maybe be at the expense of other cerebral regions.

It is worth mentioning that the flavonoids dose used in this study was relatively low which may suggest that a higher flavonoid quantity and/or longer intervention periods may be required for flavanones to elicit vascular benefits. Overall, these findings suggest that citrus flavanones may enhance executive functioning and CBF, giving an insight into the possible mechanisms of action behind improvements in cognitive function.

Alharbi et al. (2016) continued the investigation of acute flavanones consumption on cognitive function, but this time looked at the effects in a slightly older population aged 30-65 years. After administering either an orange fruit drink containing 272 mg flavonoids (exact flavanone concentration not reported) or a placebo, the participants underwent a cognitive testing battery looking at an array of cognitive domains including working memory, episodic memory, attention, and psychomotor speed. The effects on cognition were assessed at different time-points including 2 and 6 hours. Similarly, to Mastroiacovo et al. (2015) the cognitive scores were combined to form a composite score reflecting global cognitive function. Results revealed a significant main effect of treatment for the high-flavanone drink, compared to the control, at 2 hours post-consumption on a psychomotor task, whereby psychomotor speed was significantly improved following the orange juice drink. This is in line with Lamport et al.'s (2016) study who also observed cognitive improvements at this time-point. Interestingly, Alharbi et al. (2016) also observed significant improvements in scores

assessing attention and executive function at 6 hours post consumption following the high-flavanone drink, giving us further insight into the effects the flavanones, including the time-dependent effects of flavanones for cognition. However, it should be taken into consideration that the flavonoid content in Alharbi et al.'s (2016) study was almost 4 times higher than the content used in Lamport et al.'s (2016) study. Although not conclusive, as the effects at 6 hours was not measured in Lamport et al.'s study (2016), it may suggest that higher doses may elicit cognitive benefits for 6 hours post-consumption, however additional research is needed to understand the time-dependent effects of flavanones further.

To understand whether citrus flavanones may have any benefits on the cognitive function of healthy older adults, Kean et al. (2015) conducted a chronic study whereby 37 adults aged 60-81 years old were administered either a daily citrus-based drink containing 305mg of flavanones or a control drink containing 37mg. Cognitive tests assessing episodic memory and executive function were administered at baseline and 8 weeks post consumption. Global cognitive function was calculated by converting the means of all test scores and combining this to make one overall composite score. Blood pressure was also assessed to establish whether flavanones may have positive benefits for vascular health. Findings revealed improved global cognitive functioning for the high flavanone treatment group compared to low flavanone treatment group, indicating that chronic consumption of flavanones may have benefits for cognitive functioning in this age group. No effects of treatment were found for blood pressure, which is in line with Lamport et al.'s (2016) study who also did not observe any vascular effects following an acute dosing regimen.

1.5.4 Cherry Flavonoids for Cognitive Function

Keane et al. (2016) investigated whether cherry polyphenols may have an acute effect on the cognitive functioning and CBF in a group of healthy middle-aged people. Cognition was tested via a selection of tasks assessing domains such as attention, working memory, executive functioning, and reaction time. Blood pressure was assessed, as well as CBF was assessed using NIRS and transcranial doppler ultrasound sequentially to cognitive testing. Treatment beverage was reported to contain 68mg/l of anthocyanins. Results revealed that after acute consumption there was no significant effect of treatment for any of the cognitive outcomes and no change in cerebral blood flow measured via doppler ultrasound. The researchers did, however, find significant changes in brain activity from NIRS indicated by increased oxygenated and total haemoglobin in the prefrontal cortex region, after consumption of cherry polyphenols versus placebo. Systolic blood pressure also improved significantly for the cherry treatment group compared to placebo. This indicates that improved physiological effect post consumption of cherry polyphenols may be resulting in improved blood flow activity detected via NIRS. Reasons for observed differences in CBF detected via NIRS versus TCD may be due to technique differences, whereby NIRS detected oxygenated and deoxygenated blood, and TCD measures cerebral blood flow velocity, which is the rate at which the blood flows to the brain.

To investigate whether cherry flavonoids may enhance cognition in older adults with MCI, Caldwell et al. (2017) conducted a 12 week long parallel RCT whereby 45 participants with MCI received a daily administration of either cherry juice containing 138mg of total anthocyanins, or an apple juice placebo drink containing only trace amounts of anthocyanins.

Here, the researchers used a testing battery comprising of 7 different cognitive tasks to assess memory (recall, working and semantic memory) and executive functioning. A positive effect of treatment was observed following both 6- and 12-weeks consumption of cherry juice, for episodic memory and executive function, with performances for participants in the cherry intervention group improving significantly relative to control. In terms of the vascular parameters assessed, a significant improvement in systolic blood pressure was observed for the cherry treatment group, but this was not the case for the placebo group. These findings are promising and suggest that cherry polyphenols may enhance cognitive benefits in individuals with MCI.

To understand whether similar findings are observed amongst healthy older adults, Chai et al. (2019) conducted a trial looking at cherry polyphenols on cognitive function in a group of 37 adults aged 65-80 years old with no reported memory complaints. In brief, the participants consumed a treatment containing 480 mg total polyphenols or placebo daily for 12 weeks. Attention, spatial working memory and episodic visual memory, reaction time and short-term working memory was assessed at baseline and after 12 weeks. Findings revealed that after 12 weeks consumption, adults in the cherry treatment group showed significant improvements in performance on tasks measuring working memory and attention, as well as a reduction in the errors produced during a visual memory task. No improvements were seen in psychomotor functioning. This suggests that cherry polyphenols may enhance memory and the learning of novel information in healthy older adults following chronic consumption, however further research investigating whether acute consumption delivers similar results is needed.

1.5.5 Berry Flavonoids for Cognitive Function

In this section I will be reviewing the effects of grape, blackcurrants, and mixed berry polyphenols on cognitive function in humans. In my experimental investigations, I will be using wild blueberry polyphenols to understand their effect on cognitive function in humans. Therefore, in chapter two I will be providing a detailed review of the literature looking at blueberry polyphenols for cognitive function.

1.5.5.1 Grape Flavonoids for Cognitive Function

Before the effects of grape polyphenols on cognitive function had been studied, a handful of publications suggest that they may exert benefits for the cardiovascular system in humans (Lekakis et al., 2005). Further studies also demonstrated improvements to cognitive function after supplementation with grape polyphenols in animal models (Shukitt-Hale et al., 2006). To test whether these effects could be translated to humans, Hendrickson and Mattes (2008) completed the first trial assessing the acute effects of grape polyphenols on cognitive function in a group of 35 young adults who smoked cigarettes. This population was chosen due to the known negative effects of cigarettes including increased oxidative stress. However, the smokers were refrained from smoking during the study days. Participants received either a grape juice drink or matched placebo beverage in a double blind, crossover RCT after the consumption of a lunch meal, with the aim of assessing whether grape polyphenols may improve cognition during the 'post lunch dip.' In addition to this, the 'post-lunch' dip may be greater when participants are not able to smoke. The dosage amounts for the grape juice treatment group were personalised; this is different to other studies whereby a fixed dose was administered. In this study each participant received 10 ml of grape juice per kilogram of body weight. The grape juice contained 2,100 mg per litre of total polyphenols. Cognitive

testing involved a task assessing implicit memory and took place after a standardised lunch plus treatment consumption. Results revealed no effects of grape juice for implicit memory. Reasons for this lack of significance could be the lack of sensitivity of the task in this age group, or the intervention length, whereby a longer-term supplementation of grape polyphenols may be needed for effects to be observed. The researchers also commented on the possibility of the implicit memory domain not being sensitive enough to the effects of grape flavonoids. Hendrickson and Mattes (2008) suggested the need for the assessment of a wide range of cognitive domains, such as executive functioning which has been previously shown to be sensitive to the effects of flavonoids, as well as testing the effects in a non-smoking cohort.

A previous study by Shukitt-Hale et al. (2006) with aged rodents showed that supplementation with grape juice demonstrated improved performance on motor function and improved working memory compared to aged rats supplemented with placebo treatment. To understand whether grape juice possesses similar properties in aged adults, Krikorian et al. (2010) conducted the first RCT investigating grape polyphenol versus a placebo on cognitive function in 12 older adults with reported memory impairment but not a full dementia diagnosis. Here, the researchers administered a daily drink of either concord grape juice or a placebo beverage for 12 weeks. Polyphenol content was not reported but the specific volumes were administered according to body weight as in Hendrickson and Mattes' (2008) study. A cognitive battery comprising of word listening tasks assessing episodic memory was administered, and body anthropometrics and blood glucose were assessed. Results revealed a significant effect of treatment for the grape intervention group on the performance of word recall compared to the placebo group, whereby the participants who consumed grape juice for 12 weeks performed significantly better for total acquisition relative to the placebo treated group. Although not significant, a trend towards better performance

was also observed for delayed recall for the grape treated group versus the placebo treated group (p=0.10). No effects were seen for glucose levels; however, fasting insulin levels were significantly higher following grape juice consumption which was unexpected as the baseline levels were not significantly different between groups. Resultantly, research into the effects of grape polyphenols on insulin levels warrants further research. Overall, the study suggests that grape polyphenols may have benefits for episodic memory in older adults with MCI after a daily consumption for 12 weeks. This also demonstrates that a longer intervention period, such as in this study, may be required for any effects to be observed and may explain the lack of significant findings in Hendrickson and Mattes' (2008) study. Krikorian et al. (2010) also suggested that the lack of vascular effect observed in this case may be due to the small sample size, and that studies using a larger study population should be conducted.

Nevertheless, the results in the study by Krikorian et al. (2010) were promising. Therefore, to continue this investigation, Krikorian et al. (2012) conducted a similar study in older adults with MCI but used almost double the sample size (n=21) and with a longer intervention duration period of 16 weeks, which is 4 weeks longer than their previous trial. The tasks and dosing protocol used were identical to their previous study (Krikorian et al., 2010) however the grape juice contained 2,091mg per litre of total polyphenols. In addition to this, fMRI analysis was conducted during task performance in order to investigate the effects of grape polyphenols on CBF. The findings revealed no significant improvements in any of the cognitive outcomes for either treatment group, which was surprising as they originally found positive effects for word recall tasks in their previous study. Although there were no improvements in accuracy this time round, the results did show that the number of errors made was significantly higher for the placebo group relative to the grape treatment groups, which suggest that grape polyphenols may reduce the effects of interference during cognitive tasks

in older adults with MCI. Overall, the two different studies have shown effects on different cognitive aspects which may imply that different intervention lengths may lead to different cognitive effects. The fMRI analysis showed increased neuronal activity in the right cortical region, which is similar to the findings observed by Lamport et al. (2016) after supplementation with citrus flavanones and Francis et al. (2006) after supplementation with cocoa, however further research is needed to understand the mechanisms behind the effects of flavonoids for the right hemispheric lateralisation.

In 2016 Lamport et al. published a paper looking at the effects of a grape juice containing 777mg of polyphenols versus placebo, on the cognition and mood of 26 working mothers between the ages of 40-50. Test drinks were consumed for a duration of 12 weeks. The cognitive testing battery comprised of tasks assessing episodic memory, psychomotor skills and executive functioning. Driving performance was also assessed via a simulation. The findings revealed a significant improvement for immediate recall after grape juice consumption during a visual spatial learning test (VSLT), indicating an enhancement of short-term spatial memory. Significant improvements were also observed after consumption of grape juice for driving performance. No other significant effects of treatments were observed for any of the other cognitive tasks, or blood pressure. Ultimately, this was the first study to show that grape polyphenols may have the potential to improve immediate recall memory in middle-aged people with busy lifestyles.

To build on the existing data for the cognitive effects of acute grape consumption thus far, Haskell-Ramsay et al. (2017) conducted a double blind, crossover RCT looking at this effect in 20 young adults with a mean age of 21.5 years. Previous investigation by Hendrickson and Mattes (2008) have only considered implicit memory, however, here the researchers used a

comprehensive battery using tasks which have shown previous sensitivity assessing a wide range of cognitive domains including episodic memory, working memory, attention, and reaction time. The grape juice treatment was reported to contain 1681.7 mg per litre of total polyphenols, which is lower compared to other existing grape RCTs.

Findings revealed significant reduction in reaction time during an attention task after consumption of grape polyphenols. No other significant effects of treatment were observed for the remaining cognitive tasks. This is in line with previous investigation using cocoa flavanols which also observed significant improvement to reaction time during an attention task following acute consumption in young adults (Scholey et al., 2010). However, this effect for improved reaction time was not seen following a chronic dosing in the study by Lamport et al. (2016) in middle-aged females. This may indicate that flavonoids may be particularly sensitive to this cognitive domain in younger adults, or following an acute dosing compared to daily chronic consumption. These findings also illustrate the importance of task selection when conducting cognitive investigations into nutritional products. The tasks selected by Haskell-Ramsay et al. (2017) have previously shown sensitivity to nutritional products, whereas implicit memory, measured by Hendrickson and Mattes (2008) has not demonstrated any prior sensitivity to acute dosing with grape treatment versus placebo.

1.5.5.2 Blackcurrant Flavonoids for Cognitive Function

Watson et al. (2015) investigated whether two different blackcurrant supplementations had any acute effects for cognitive function, when compared to a placebo beverage. For this they conducted a double blind, placebo-controlled crossover trial looking at the effects in 36 young adults with a mean age of 24.8. Here, the researchers considered the effects of two different blackcurrant treatments, one of which was a standard juice providing a mean of 599.3 mg of

total polyphenols and one of which was a blackcurrant extract, providing a mean of 590 mg total polyphenols, versus a placebo. Assessment of both intervention drinks revealed similar anthocyanin and total polyphenols content, but differing polyphenol profile, which might help us understand whether different constituents play a role on the outcome of the variables measured in this study. The dosing quantity was administered according to the participant's body weight, with the average dosing calculated to provide 599.3 mg of total polyphenols. The cognitive battery administered comprised of a range of tasks assessing domains including attention, working memory and executive functioning. Monoamine oxidase-B (MAO-B) is an enzyme known to break down and therefore, negatively affect the activity of neurotransmitters including dopamine and serotonin, examples which are vital for optimal cognitive function and neuronal health (Foley et al., 1999). In-vitro studies suggest that polyphenols may reduce the activity of MAO (Dreiseitel, 2009). Reducing the activity of MAO may offer neuroprotective effects by limiting hydrogen peroxide production during metabolism of monoamines (Watson et al., 2015). In order to understand this effect, Watson et al. (2015) collected blood samples from a subgroup of participants at 60- and 150-minutes post treatment consumption, in order for the levels of monoamine oxidase-B (MAO-B) as well as glucose levels to be assessed. Results revealed a significant improvement in a task assessing working memory and attention after consumption of the blackcurrant extract intervention compared to placebo, however this was not observed following the juice consumption. On the contrary, significant improvements in reaction time were observed following the juice consumption versus placebo and not for the blackcurrant extract versus placebo. There was also a significant effect of treatment observed for the MAO-B inhibition whereby the activity of MAO-B enzyme was reduced after consumption of the blackcurrant juice, but not for the blackcurrant extract. For plasma glucose levels, observations revealed a peak at 60 minutes

post consumption and 150 minutes post consumption for the blackcurrant juice compared to the extract or placebo, suggesting that the juice was more effective at attenuating the glucose level. These findings suggest that different constituents may lead to different effects via mechanisms of action which are unique to certain polyphenolic compounds. Overall, the effects observed are promising and suggest that anthocyanin rich fruits such as blackcurrants have the potential to improve some aspects of cognition such as working memory, attention, and reaction time, and also modulate MAO activity. It also highlights the need to explore whether different polyphenolic profiles, which exists in nutritional intervention, leads to different results, which may tell us whether a melange of certain flavonoids are required for optimal effects.

In 2018 Watson et al. conducted a pilot study investigating the acute cognitive impact and effect on brain waves activity of a blackcurrant beverage containing 500mg of polyphenols versus a placebo. This follows on from a feasibility study conducted by Okello et al. (2016) who previously found increased brain wave activities, notably theta waves, at 1 hour post green and black tea consumption in healthy adults. Watson et al. (2018) recruited 9 university-aged volunteers and brain activity was assessed using electroencephalography (EEG) during the completion of cognitive tasks, before and after supplementation of each treatment. Cognitive domains assessed here included sustained attention, and reaction time, domains which have been previously shown to be sensitive to flavonoid intervention. EEG is a non-invasive technique where brain waves are monitored across using multiple sensors placed around the head. Different brain waves frequencies are related to different state of minds including alertness, attentiveness (Okello et al. 2016). However, results revealed no effect of treatment for any of the cognitive outcomes. There were no significant differences

observed for EEG either; however, the authors did see a trend for increased brain activity following consumption of the blackcurrant treatment.

1.5.5.3 Mixed Berry Flavonoids for Cognitive Function

Studies using different extracts from the same fruit have resulted in different effects, such as in the study by Watson et al. (2015), whereby two different blackcurrant extracts were investigated, which suggested that different constituents may lead to different effects via different mechanisms of action. Using a mixed berry intervention therefore has an advantage as different constituents, and doses of these constituents which exist in different berries are included. To my knowledge, Nilsson et al. (2017) conducted the first study exploring the effects of a mixed berry intervention in a randomized, double-blind, crossover trial. This was conducted in a group of 40 middle aged adults (50-70 years old). The interventions comprised of a mixed fruit beverage containing blueberries, elderberries, lingonberries, strawberries and tomatoes, giving a total of 795 mg of total polyphenols per serving. Each intervention was consumed daily for 5 weeks and measures of cognitive function including verbal working memory, attention, reaction time were assessed at baseline and 5 weeks post consumption. In addition to this, measures of vascular health were assessed with blood pressure as well as measurements of glucose, insulin, low density (LDL) and high-density lipoprotein (HDL) levels in the blood. Significantly improved performance on a test assessing working memory was seen for the mixed berry beverage versus placebo. No significant effects of treatment were observed on any other aspect of cognition. For vascular health, the group consuming the mixed berry intervention showed a significant reduction in total cholesterol levels and LDL levels, with no changes in insulin levels being observed. Blood glucose remained unchanged for the mixed berries group; however significantly higher glucose levels were observed

following placebo intake. For vascular health, a reduction in cholesterol, particularly 'bad' cholesterol plus insulin control is promising, but further investigation is required to understand the cardiovascular effects of flavonoids in older adults.

Bensalem et al. (2019) completed a 6 months chronic RCT investigating the effects of a mixed blueberry and grape intervention (258mg flavonoids) in a group of older adults aged 60-70 years old identified as having mild memory decline. The researchers aimed to test the effects of people with varying cognitive decline. Due to the nature of cognitive decline, and its variability from person to person, the researchers identified a subset of these participants as having more cognitive decline than the remainder of the cohort and were referred to as 'decliners'. In this study, cognition was assessed using tasks assessing episodic memory, working memory and learning. Results revealed no effect of treatment on working memory performance following mixed berry intervention compared to placebo. However, a significant increase in word recall performance was observed amongst older adults who were experiencing cognitive decline. This is in line with results from previous literature (Caldwell et al.,2017; Kent et al., 2017; Desideri et al., 2012; Krikorian et al., 2010; Lamport et al., 2016) which support the beneficial effects of flavonoids in participants with MCI.

Similarly, Philip et al. (2019) investigated whether grape and blueberry extracts affected cognitive and vascular health acutely in young adults. Here a treatment containing 600mg of these extracts was administered in a group of 30 young adults. Working memory and attention, alongside FMD and blood pressure, was measured to assess the effects on cognitive and vascular functioning. Significant improvements in performance during the serial 3s subtraction task assessing working memory were seen following the flavonoid-rich extract, relative to placebo, which supports the findings by Scholey et al. (2010) who also observed

significant improvement during a serial 3s task, but not a serial 7s task, following acute cocoa consumption containing 552 or 994 mg flavanols, relative to placebo. No effects were observed for any other cognitive domains in this instance, and no beneficial effects of treatment were observed for vascular health. It is noteworthy however that extracts are not fundamentally equivalent to fresh or freeze-dried whole fruits, therefore different phenolic profiles may provide different effects. Studies using extracts as interventions have resulted in different effects when compared to the fresh fruit equivalent, such as in the study by Watson et al. (2015) whereby two different blackcurrant interventions, one being an extract, has led to different observed results, which suggested that different constituents may lead to different effects via mechanisms of action. Ultimately, research which have used extracts, such as in the study by Philip et al. (2019) should be repeated using whole fruit to assess whether the observations seen for extracts are repeated, this will mean that findings will therefore be more translatable to the general population.

Finally, Whyte et al. (2019) performed a single-blind parallel trial assessing the impact of a beverage consisting of blueberry, strawberry, raspberry, and blackberry, which provided 570mg total polyphenols versus a placebo, on executive functioning over the course of 6 hours immediately after dosing. Here the study population consisted of 40 young adults aged between 20-30 who underwent cognitive testing consisting of two tasks, one of which assessed selective attention and one which measured executive function and took place at 2, 4 and 6 hours post treatment consumption.

Findings revealed an overall significant effect of treatment for the attention task, whereby participants who consumed the mixed berry beverage resulted in significant improvements to overall performance. Interestingly, a treatment x session post-hoc analysis revealed

increased accuracy for the mixed berry treatment group relative to placebo at the 6 hours' time-point. This is in line with previous studies by Alharbi et al. (2016) who also observed significant improvements to cognitive function following 272 mg consumption of orange flavanones, giving us further insight into the potential time-dependent effects of flavonoids. Reaction time was also significantly better following mixed berry treatment relative to placebo, with post-hoc analysis revealing significant faster reaction time at 2 and 4 hours, and a trend for significantly quicker performance at 6 hours. Overall, these findings suggest that berry flavonoids can enhance and sustain cognitive function over the course of 6 hours, a finding which can be implemented in everyday lives to benefit studying, for example.

1.5.5.4 Conclusion of Literature Looking at The Effects of Flavonoids For Cognition

Although not conclusive, the evidence gathered thus far suggests that flavonoids may have benefits for cognitive function, both acutely and chronically. Improvements have been observed across multiple age groups, but predominantly in older age groups. Nevertheless, there is currently a lack of investigations in younger age categories including teenagers, young adults and middle-aged adults therefore further investigations into the cognitive effects in youngers adults is needed before it can be concluded that older adults are benefitted the most.

In terms of intervention types, copious amounts of research have been conducted using cocoa as a source of polyphenols followed by berries and a small amount looking at citrus flavonoids and cherry flavonoids. Nevertheless, benefits have been seen generally across all intervention types, indicating that polyphenolic compounds from a range of sources may contribute to improvements in cognition. In terms of dosage, significant improvements have been observed

across a range of flavonoid doses which suggests that higher doses are not necessarily needed for improvements in cognition to be seen. Furthermore, improvements in vascular health following flavonoid consumption may give us important insights into the mechanism of actions. Further research is recommended to understand the effects of flavonoids on cerebral blood flow and the effect this has on cognitive function. Lastly, taking into consideration that the gut microbiota may influence the ADME of polyphenols, further research is recommended to look at the role of polyphenols and the microbiome. There is increasing evidence to suggest that a bidirectional relationship between (poly)phenols that make it to the large intestine intact, and the gut microbiota exists (Shorrtt et al., 2018). Furthermore, increasing evidence suggest that the microbiome plays an important role in health (Jovel et al.,2018) including cognitive function (Magnusson et al., 2015) and vascular health (Tang et al., 2017). This area of research will be explored further in this thesis.

In the next chapter I will reviewing the effects of blueberries on cognitive function in humans. Blueberries will be the main focus of this thesis as the interventions used in the experimental chapters featured in this thesis will be using blueberries as intervention type. Following on from the review in chapter 2, the research gap which this thesis will aim to address will be covered chapter 3.

Chapter Two

Systematic review of the effects of blueberry on cognitive performance as we age

Work within this chapter has previously been published as:

(1) Hein, S., Whyte, A. R., Wood, E., Rodriguez-Mateos, A., & Williams, C. M. (2019). Systematic review of the effects of blueberry on cognitive performance as we age. The Journals of Gerontology: Series A, 74(7), 984-995.

(2) Whyte, A. R., Hein, S., Wood, E., Rodriguez-Mateos, A., & Williams, C. M. (2020). Response to Comments From Brydges & Gaeta and Vorland et al. With Respect to Hein et al. (2019)“Systematic Review of the Effects of Blueberry on Cognitive Performance as We Age”. The Journals of Gerontology: Series A, 75(8), e27-e29.

2.1 Introduction

Blueberries have been the subject of a number of health-related research studies in recent years with supplementation showing reduced risks for metabolic syndrome, cancer, cardiovascular disease and also cognitive decline (McAnulty et al., 2017). Mechanistically, initial research (Barnham et al., 2004; Giacalone et al., 2011) focused on the antioxidant properties of flavonoids and their ability to combat oxidative stress (OS). However, recent studies have suggested a number of other mechanisms by which flavonoid-rich interventions may promote cognitive health (for a review see Spencer, 2009). Indeed, recent mechanistic research has shown that the health benefits of blueberries may be ascribed to their particularly high flavonoid content. As can be seen from Table 2.1 blueberries are particularly high in anthocyanins along with lower amounts of flavanols and flavonols, all of which are flavonoid subclasses (Rodriguez-Mateos et al., 2012). They also contain small quantities of phenolic acids, in particular chlorogenic acid (Rodriguez-Mateos et al., 2012). Although there is evidence of higher anthocyanin content in other berries such as chokeberries, the ease of blueberry availability and also their relatively better palatability make them ideal candidates for flavonoid-rich intervention studies. Previous studies have documented that lowbush (typically called 'wild') and highbush blueberry varieties differ in taste, size and flavonoid content. Lowbush blueberries tend to be smaller, have a more intense flavour, and are usually found growing wild in colder and harsher climates whilst high-bush blueberries tend to be bigger and grow in abundance. For this reason, highbush blueberries tend to be the first choice for commercial cultivation, however High-Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS) has revealed that the lowbush variety contains approximately three times the number of phenolic compounds found in highbush varieties (Kalt et al., 2001; Kang

et al., 2015). Many different factors could account for these differences, such as cultivar, cultivation practise, environmental growing conditions, processing and storage (Cardenosa et al., 2016; Castrejon et al., 2008; Rodriguez-Mateos et al., 2013; Skrovankova et al., 2015).

Table 2. 1 Comparison of anthocyanidin content (mg/100g FW) in different berries retrieved from USDA Database for the Flavonoid Content of Selected Foods Release 3.1 (2014)

Flavonoid subclass	**Content of flavonoids in some common berries (raw form) (mg/100g of fresh weight)**						
	Blueberries (highbush)	**Blackberries**	**Blackcurrant**	**Chokeberry**	**Grapes**	**Raspberries**	**Strawberries**
Anthocyanidins							
Cyanidin	8.46	99.5	62.46	344.07	1.16	45.77	1.68
Delphinidin	35.43	0.00	89.62	0.65	2.27	1.32	0.31
Malvidin	67.59	0.00	n/a	1.22	39.00	0.13	0.01
Pelargonidin	0.00	0.45	1.17	0.98	0.02	0.98	24.85
Peonidin	20.29	0.21	0.66	0.08	3.62	0.12	0.05
Petunidin	31.53	0.00	3.87	2.79	1.97	0.31	0.11
Total	**163.3**	**100.16**	**157.78**	**349.79**	**48.04**	**48.63**	**27.01**
Flavan-3-ols							
(-)-Epicatechin	0.62	4.66	0.47	n/a	0.96	3.52	0.42
(-)-Epicatechin 3-gallate	0.00	0.00	0.00	n/a	0.17	0.00	0.15
(-)-Epigallocatechin	0.66	0.10	0.00	n/a	0.08	0.46	0.78
(-)-Epigallocatechin 3-gallate	0.00	0.68	0.00	n/a	0.00	0.54	0.11
(+)-Catechin	5.29	37.06	0.70	n/a	0.82	1.31	3.11
(+)-Gallocatechin	0.12	0.00	0.00	n/a	0.00	0.00	0.03
Total	**6.69**	**42.5**	**1.17**	**n/a**	**2.03**	**5.83**	**4.6**
Flavonones							
Hesperetin	0.00	0.00	n/a	n/a	n/a	0.00	0.00
Naringenin	0.00	0.00	n/a	n/a	n/a	0.00	0.26
Total	0.00	0.00	n/a	n/a	n/a	0.00	0.26
Flavones							
Apigenin	0.00	0.00	0.00	n/a	0.00	0.00	0.00
Luteolin	0.20	0.00	0.00	n/a	1.30	0.00	0.00
Total	**0.20**	**n/a**	**0.00**	**n/a**	**1.30**	**0.00**	**0.26**
Flavonols							
Isorhamnetin	n/a	n/a	0.12	n/a	n/a	0.00	0.00
Kaempferol	1.66	0.27	0.71	0.34	0.00	0.06	0.50
Myricetin	1.30	0.67	6.18	0.00	0.01	0.00	0.04
Quercetin	7.67	3.58	4.45	18.53	1.04	1.05	1.11
Total	**10.63**	**4.52**	**11.46**	**18.87**	**1.05**	**1.11**	**1.65**

In chapter 1, I outlined the impact of a number of flavonoid compounds on cognition and reviewed potential mechanisms of action. Here in Chapter 2, I will systematically review the evidence from randomised controlled trials for the impact of blueberries alone on cognition. In recent years, blueberries have gained significant attention for their ability to promote better cognitive performance and also contribute to a delay in cognitive decline as we age. As mentioned in chapter 1, epidemiological studies suggest that intake of flavonoid-rich foods, such as blueberries, ameliorates cognitive decline during ageing.

In addition to this epidemiological data, various pre-clinical animal studies have been conducted to investigate the effects of flavonoids derived from berries on cognition. In one of the first studies of its kind, Joseph et al. (1999) found blueberry, strawberry, or spinach supplementation for 8 weeks in mice with neurodegeneration resulted in a reversal of neuronal ageing which they attributed to a reduction in oxidative stress damage. Whilst this was one of the first studies to document a potential mechanism of action for positive cognitive effects following blueberry supplementation, other possible mechanisms have also been described in recent years including the flavonoid-induced upregulation of neuronal signalling proteins. For example, work by Williams et al., (2008) found that 18-month old rats supplemented for 12 weeks with blueberry showed elevated hippocampal levels of cAMP-response element-binding protein (CREB), extracellular signal-related kinase ERK1/2, and brain-derived neurotrophic factor (BDNF) in comparison to age-matched controls. Importantly, the alteration in these signalling proteins was accompanied by better performance on a spatial working memory cross maze task. In a follow-up study, it was demonstrated that the beneficial effects of blueberry supplementation could also be found on spatial working-memory tasks in younger rats aged 2 months, again accompanied by increased hippocampal levels of BDNF, CREB and ERK1/2 activation (Rendeiro et al., 2012).

BDNF seems to be a particularly important potential mechanism of action for blueberry supplementation. In humans BDNF levels are known to decrease across the day (Begliuomini et al., 2008; Pluchino et al., 2009). However, research by Dodd (2012) has found that, in both younger (18 – 25 yrs) and older (62 – 73 yrs) adults, plasma levels of BDNF are maintained following blueberry intervention compared to placebo treatments where BDNF levels decrease. Furthermore, BDNF is thought to play a critical role in the delay of ageing by improving hippocampal plasticity as well as increasing neurogenesis and long-term memory (Cunha et al., 2010). Therefore, these pre-clinical studies (Begliuomini et al., 2008; Pluchino et al., 2009) and the findings by Dodd (2012) suggest a possible mechanism of action whereby blueberry intervention contributes to the maintenance of BDNF availability which may be critical for cognitive function.

Pre-clinical studies have also provided evidence that blueberry flavonoids may exert a positive effect on neuroinflammation. For example, Shukitt-Hale et al., (2008) found that, following infusion of kainic acid (KA) to the hippocampus which provided a model of ageing, the 4-month-old rats supplemented with blueberry performed better on a Morris water maze task and showed a reduced inflammatory response to the KA insult. Finally, Casadesus et al. (2004) found that, when compared to placebo group, there was increased hippocampal neurogenesis alongside improved spatial memory performance in aged rats following 8 weeks blueberry supplementation.

Taken as whole there are a number of possible mechanisms underpinning the beneficial cognitive effects of blueberry intervention, these include antioxidant and anti-inflammatory actions, up-regulation of neuronal signalling proteins, and stimulation of neurogenesis (see Chapter 1 for further descriptions). Further details of pre-clinical studies which have

considered the potential mechanisms of action underpinning blueberry intervention can be found in reviews by Miller et al. (2012) and Pribis and Shukitt-Hale (2014). However, evidence would suggest that, irrespective of mechanism of action, the positive effects of blueberry intervention can be found primarily in the hippocampus, a brain area critical for optimal memory function (Williams et al., 2008; Casadesus et al., 2004; Andres-Lacueva et al., 2005; Willis et al., 2005).

From the pre-clinical research described above, there is good evidence from both a mechanistic and behavioural level to suggest that blueberry intervention should facilitate improved cognitive performance in clinical trials. Therefore, in this chapter I will assess the evidence from both acute and chronic intervention studies for the beneficial effects of blueberry on human cognitive functioning across the lifespan. The domains of cognitive function found to be sensitive to blueberry interventions will be identified and based on the reported research, cognitive areas which have yet to be considered will be highlighted. Tentative recommendations regarding future research directions will also be made.

2.2 Method

An electronic search of PubMed, Google Scholar and Web of Science was conducted using the search terms Blueberr* and/or Berr*, Anthocyan* and/or Flavonoid*, Cognit* and/or Polyphenol* and/or Memory and Executive function. The studies selected for inclusion were all subject to peer- and/or editorial-review, and for this reason, conference abstracts have been omitted. Papers published in the English language, with no restriction on publication date, were selected, and subsequently the bibliography of each paper was scanned to reveal further possible papers. The search and update of new papers was completed in August 2021.

The following inclusion and exclusion criteria were implemented, as well as the use of a decision tree (Figure 2.1):

- *Inclusion:* Human studies, participants of all ages, healthy participants/participants with MCI, studies measuring the effect of blueberries on cognitive function, cognition measured using appropriate cognitive tasks, all forms of blueberry treatment including juice, fresh, powder, extract, and smoothie.
- *Exclusion criteria*: Epidemiological studies, participants with neurodegenerative diseases such as Alzheimer's, animal studies, studies using more than just blueberries e.g., mixed berry drink.

Study selection was initially performed by SH, and excluded papers were independently verified by AW. Initially, papers were excluded based on the title if it was evident that the research fell outside of the inclusion criteria specified- e.g., animal studies. All studies of potential interest were then shortlisted before reading the full publications to decipher eligibility for inclusion. Data extraction was conducted independently by SH and AW to allow extraction of key information regarding design, sample characteristics, intervention/ exposure/ compliance, outcome measures and reported results. Data not related to cognition was ignored and not included. For data synthesis, extracted data was placed into tabular form to aid comparison of study characteristics and guide the grouping of studies for the narrative synthesis. Effect sizes were calculated using the means and standard deviation using the original study data or calculated using mean change scores and standard deviations from published data. A few Cohens' D effect size were pre-calculated and derived straight from the publication.

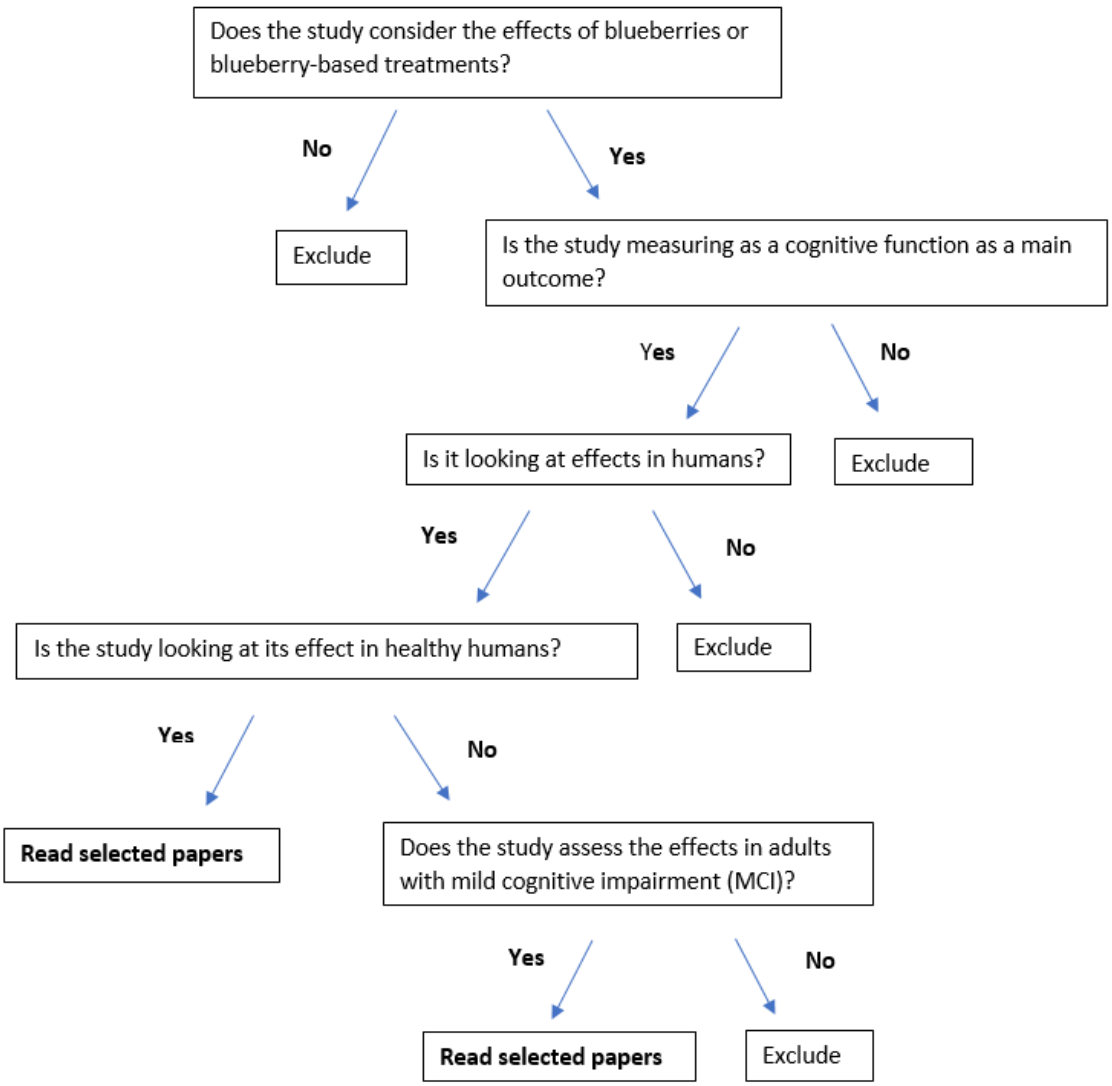


Figure 2. 1 A decision tree outlining the process of identifying eligible papers

2.3 Results

In total, 15 studies considering the cognitive effects of blueberry intervention were found (summarised in Tables 3-6). The primary cognitive domains considered were episodic memory (EM), working memory (WM), executive function (EF), and psychomotor function (PF), although a wide range of different tasks were used to test these cognitive domains. No studies were found considering the effect of blueberry interventions on young adults with the research to date focusing on children aged 7 – 10, middle-aged adults, healthy older adults aged 60+, or older adults exhibiting symptoms of Mild Cognitive Impairment (MCI).

Table 2. 2 Episodic memory measures of cognitive function used in blueberry studies

Task	Description	Cognitive process tested
Rey's Auditory Verbal Learning Task (RAVLT)	A list of set words is presented which the participants are asked to recall. This is repeated 5 times using the same list of words (List A). A second and different list of words is presented (list B), and the participants are required to recall as many words as they remember from this second list. Straight after, the participants are asked to recall as many words as possible from the original list (list A). After about 25 minutes, the participants are asked once more to recall the words from the original list and lastly presented with a word recognition task where they were presented with words from both lists A and B in addition to random words and asked to identify only the words from list A.	Immediate and delayed episodic memory, proactive and retroactive interference, word acquisition, total learning, and word recognition.
Object Location/Recognition Task	Participants are shown a set of pictures for a set amount of time. After a break, the participants are shown a new set of pictures, alongside some of the original pictures, and they have to indicate the pictures which they think are new and which pictures are from the original set. In some Object recognition task versions, the participants are shown the original pictures again but with some of the pictures moved around and the participants have to indicate which picture they think has not been moved.	Spatial memory
California Verbal Learning Test, 2nd ed. (CVLT-II)	Participants are shown a list of semantic words and asked to recall them 5 times. A second interference list is presented, which the participants have to recall before the initial list is tested again. After 20 mins the participants are shown a list of words containing words from both the original and interference list and the participants are asked to distinguish and respond to words from the original list.	Episodic memory
Virtual version of the Morris Water Maze (vMWM)	An adaptation of a physical task used with rodents. Participants are placed in a virtual pool and required to identify and memorise the location of a hidden platform under different conditions.	Spatial cognition
International shopping list task	The participant must try and remember as many words as possible from a list of words that they hear.	Verbal learning and delayed recall
Verbal paired associate learning test	The aim of this task is to learn pair of semantically linked words that have different and unrelated meanings.	Memory
Corsi block tapping task	The participant is shown a collection of blocks on the screen. The blocks are highlighted in a specific sequence and the participant are required to repeat that sequence.	Visual memory span
Hopkins Verbal Learning Test	The participant is read a list of 12 words, and then is required to memorise and recall as many words as they can remember. This is repeated 3 times. Afterwards the participant is read a list of 24 words which includes words from the first list and also distractor words and has to indicate by responding ‘yes or ‘no’ whether that word appeared in the word list or not.	Verbal learning and long-term memory

2.3.1 The Effects of Blueberry Polyphenols in Children

Six studies have investigated the effect of blueberry interventions on the cognitive function of children aged between 7 – 10 years of age (Table 2). Five of the studies considered the acute effects of blueberry intervention and one study considered a chronic repeated administration design of 4 weeks.

The first study considering the acute effects of blueberry intervention on children was performed by Whyte and Williams (2015). In this crossover trial, a blueberry-based drink was administered to a group of 14 children aged 8-10 years old. The drink consisted of 200g fresh highbush blueberries, blended with milk, giving a reported total anthocyanin concentration of 143 mg. This was a randomised crossover study design, with a 7-day washout period between the two study days. Baseline testing was not employed in this study but instead, on each study day, the cognition of each child was tested at 2 hours post consumption of either the blueberry or placebo drink. The cognitive tasks used in this study included the Go-NoGo, Stroop, Auditory Verbal Learning Task (AVLT), Object Location Task, and Visual N-back.

The study yielded no significant effects for accuracy and reaction times (RT) of blueberry intervention on any of the outcome measures in the Go-NoGo, Stroop, N-back or the object location task. However, ANOVA analysis of the AVLT revealed a significant benefit of blueberry, in comparison to placebo, for the main effect across short and long delayed word recall. Further post-hoc analysis revealed a positive trend for better recall following blueberry after a 25-minute delay indicating a sensitivity to delayed recall following blueberry intervention in children. In terms of proactive interference (PI) there was evidence that performance was less affected following the placebo drink compared to blueberry. However, when the interference recall list performance was directly compared no significant difference

was found between treatment groups, leading the authors to conclude that this effect was more likely an artefact of the PI calculation when applied across two separate test sessions.

In a follow-up, within-subject RCT, Whyte et al. (2016) considered the effects of two wild blueberry interventions of 15 and 30g (anthocyanin content of 127mg and 253mg respectively), or matched placebo on cognitive performance in 7 – 10 yr old children. On each study day cognitive tests were performed at baseline, then 1.5, 3, and 6 hrs following intervention. The four cognitive tasks used were the AVLT (as above), Modified flanker Task (MFT), Go-NoGo (as above), and Picture Matching Task (PMT). Analysis revealed dose response effects for both memory and EF measures. Memory effects included a significant interaction for the AVLT measure of final acquisition of the word list, with post-hoc analysis revealing significantly better 30g WBB performance at 1.5 hours in comparison to placebo. Additionally, for delayed word recognition, although there was a decrease in performance across the test day for all three treatments, there was a significant main effect of dose with the placebo performing least well overall. Furthermore, post-hoc analysis revealed the difference between placebo vs 15g WBB and between placebo vs 30g WBB to be greatest at the 6hr time point. Executive function effects included a significant effect 3 hours after the intervention on the incongruent trials of the flanker task where, compared to baseline performance, 30g WBB performance improved, placebo performance deteriorated, and there was no change for the 15g treatment. Analysis of the data also revealed significant linear trends for Final Acquisition, Word Recognition, Incongruent MANT trials, and Picture Name Matching Trials, with the placebo performing least well, followed by the 15g WBB and then the 30g WBB performing best in all cases. Given this evidence of a dose response effect, a non-parametric Page's test was conducted on combined scores from all tasks and session revealing a monotonic increase in cognitive performance in relation to WBB dose.

A study by Whyte et al. (2017) focused on the cognitive effects of a 30g WBB treatment (containing 253 mg anthocyanins) coinciding with the 3hr time point at which positive cognitive EF effects were found in the previous Whyte et al. (2016) paper. The aims of the study were to explore the effects of varying demand on cognitive performance following a flavonoid rich WBB intervention. This study looked at the effects in in children aged 7-10 and employed the Modified Attention Network Task (MANT), an executive function task that can be manipulated to vary cognitive demand/load across a number of different factors such as congruency, visual load, distractor noise, target duration, and target cueing. The results revealed that following WBB intervention, there was a significant global effect whereby children responded to the stimuli significantly faster, compared to placebo. Furthermore, it was found that WBB cognitive performance was better in comparison to placebo at the slower 500ms presentation rate during the more cognitively demanding, high visual load incongruent trails, supporting the study's hypothesis. However, cues alerting the appearance of the target al.so facilitated significantly better WBB performance in comparison to placebo. In contrast to the earlier findings of Whyte et al. (2016) no effects on accuracy were found for this EF task.

Barfoot et al. (2018) looked at the acute effects (2 hrs) of blueberries on executive function using the MANT on a group of 54 children aged 7-10. In this study they employed a single-blind, parallel-groups design. As well as executive function, they tested the effect on verbal memory using AVLT and reading efficiency using Test of Word Reading Efficiency-2 (TOWRE-2). Mirroring the findings of Whyte et al. (2016) executive function benefits were found with significantly faster reaction times on the MANT following WBB treatment, although on this occasion, the benefits were found on the trials presented at the faster 120 ms rate with no loss in accuracy. Furthermore, similar to the AVLT final acquisition and delayed recall benefits

found by Whyte et al. (2016), total acquisition performance was improved following the blueberry intervention, with significant improvements seen on the short delay trials. There were no effects seen for any of the TOWRE-2 parameters.

More recently, Whyte et al. (2020) conducted an acute double-blind crossover RCT looking at the effects of a WBB intervention (253mg anthocyanins) on cognitive function in a group of 18 children aged between 7-10 years old. Here the researchers assessed two different outcomes; the effect of WBB on episodic and visuo-spatial working memory 75 minutes post-consumption, and the effects of a blueberry intervention on executive function 3 hours post consumption. These post-intervention time-points were chosen due to previous literature indicating that the effects of blueberry polyphenols on those specific cognitive domains are most sensitive at these time points (Whyte et al., 2015; Whyte et al., 2016). Results found no significant effect of treatment on memory, although reaction time was improved for the blueberry group ($p=0.042$) during The Visuo-Spatial Grid Task assessing memory function. The same result was observed for the tasks assessing executive function, with improvement in reaction time observed following blueberry intervention but no effect of treatment on accuracy scores during the congruent trials of the Attention Network Task assessing executive function. These results suggests that blueberry polyphenols may improve response time in young children, however further research is needed to investigate the effects on episodic memory and executive functioning in this instance.

Finally, Barfoot et al. (2021) completed a pilot study assessing whether a blueberry intervention comprising of 253 mg anthocyanins consumed daily for 4 weeks had any effects on cognitive function in a group of 15 children aged between 7-10, which to my knowledge is the first chronic trial conducted in children. Tasks administered included Rey's Auditory-

Verbal Learning Task assessing episodic memory, and Modified Attention Network Task assessing executive function. Results found significant improvements in accuracy scores for the blueberry group compared to placebo during the MANT, whereby blueberry-treated participants were significantly more accurate than placebo-treated participants during the cognitively demanding incongruent trials. Results also found that reaction time remained unaffected during the most cognitively demanding aspects of the tasks for the blueberry group, whereas for the placebo group, performance was significantly slower. However, no effects of treatment were seen for episodic memory. These observations are in line with previous acute studies conducted by Whyte et al. (2017) and Barfoot et al. (2019) who also assessed cognitive benefits during tasks with high cognitive load. Moreover, it should be noted that the observations are not in line with the findings by Whyte et al. (2020) who observed reaction time benefits during congruent trial, whereas Barfoot observed WBB benefits during the more cognitively demanding incongruent trials. However, it should be taken into consideration the ANT utilised by Whyte et al. (2020) did not include variables which increases the demand, such as load and noise, compared to the MANT, which may therefore deem the task less sensitive to effects of WBB.

In summary, six studies have considered the impact of blueberry interventions on cognitive performance in children. These studies have found evidence that executive function and delayed memory performance are positively affected by blueberry treatment in comparison to placebo. It should be noted that, to date, only a narrow age range, between 7 – 10 yrs has been considered with effects in infants and teenagers yet to be explored.

Table 2. 3 Key studies investigating the effects of blueberry supplementation on cognition in healthy children.

Author(s)	Treatment	Total Anthocyanin (mg)	Total Polyphenol (mg)	Study Design	Study Type	Measurement Time Points	Size (n)	Age Range (years)	Cognitive Tests (Cognitive Domain Tested in Parenthesis)	Key Findings	Effect Size (Cohen's d)
Whyte & Williams (2015)	200g fresh BB	143	N/A	Placebo-controlled crossover design	Acute	2 hrs	14	8-10	**(1)** Go-NoGo (EF) **(2)** Stroop (EF) **(3)** Rey's Auditory Verbal Learning Task (AVLT) (EM) **(4)** Object Location Task (EM) **(5)** Visual N-back (WM)	AVLT: Delayed Recall (main effect of ANOVA on recalls 6 & 7) • BB vs Placebo (p=0 .038) AVLT: Proactive Interference • BB vs Placebo (p=0 .043[a]) [a]Better placebo performance.	Recall 6: 0.449* Recall 7: 0.551* 0.601*
Whyte, Schafer & Williams (2016)	Freeze dried BB powder **(1)** 15 g (120 g FE) **(2)** 30g (240 g FE)	**(1)** 127 **(2)** 253	N/A	Double blind, placebo-controlled crossover design	Acute	1.15, 3, & 6 hrs	21	8-10	**(1)** Auditory-Verbal Learning Task (EM) **(2)** Modified Flanker Task (MFT) (EF) **(3)** Go-NoGo (EF) **(4)** Picture Matching Task (PMT) (EF)	AVLT: Final Acquisition at 1.15hr • 30g BB vs Placebo (p=.023) AVLT: Delayed Word Recognition at 6hrs • 15g BB vs Placebo (p=.038) MFT: Incongruent Trial Accuracy at 3hrs • 30 g BB vs Placebo (p=0.035)	0.646* 0.597* 0.605*
Whyte, Schafer & Williams (2017)	Freeze dried BB powder, 30g (240 g FE)	253	N/A	Double blind, placebo-controlled crossov	Acute	3 hrs	21	7-10	**(1)** Modified Attention Network Task (MANT) (EF)	MANT: Reaction Time • BB vs Placebo (p =0 .048)	0.453*

				er design						
Barfoot et al. (2018)	Freeze dried BB powder, 30g (240 g FE)	253	N/A	Randomised, single-blind, parallel-groups design	Acute	2 hrs	54	7-10	**(1)** Rey's Auditory-Verbal Learning Task (EM) **(2)** Modified Attention Network Task (MANT) (EF) **(3)** Test of Word Reading Efficiency-2; TOWRE-2 (reading efficiency)	AVLT: Total acquisition performance • BB vs Placebo (p= 0.035) AVLT: Short delay recall • BB vs Placebo (p=0.04) MANT: Reaction time • Treatment x Target Time interaction (p=0.018).	0.485** 0.418** 0.062***
Whyte et al. (2020)	Freeze dried BB powder, 30g	253	N/A	Double blind, placebo-controlled crossover design	Acute	1.15 & 3 hrs	35	7-10	**(1)** Rey's Auditory-Verbal Learning Task (EM) **(2)** Picture Recognition Task (EM) **(3)** The Brown Peterson Task (PI) **(4)** The Visuo-Spatial Grid Task (WM) **(5)** The Attention Network Task (EF) **(6)** The Stop-Go Task (EF) **(7)** The Switching Task (EF)	The Visuo-Spatial Grid Task: Reaction Time • BB vs Placebo (p=0.042) The Attention Network Task: Reaction time • Treatment x congruence interaction (p=0.049)	-
Barfoot et al. (2021)	Freeze dried BB powder, 3.3 g 240 g FE)	253	766	Single blind, placebo control parallel design	Chronic	Baseline & 4 weeks	15	7-10	**(1)** Rey's Auditory-Verbal Learning Task (EM) **(2)** Modified Attention Network Task (EF)	Modified Attention Network Task: • Drink × Congruency interaction (p< 0.01), • Drink × Load (p= 0.03), • Drink × Target time (p<0.01) MANT: Reaction time	-

										• Slower performance on high load versus medium load incongruent trials (p=0.01)[b] [b]Significantly worse placebo performance	

Note. BB = Blueberry, FE = Fresh Equivalent; EM = Episodic Memory, PI=Proactive Interference, EF = Executive Function, WM = Working Memory, PF = Psychomotor Function. * Calculated using means and SDs from original study data, ** calculated using mean change scores and SDs from published data, ***Effect size reported here relates to main effect of treatment calculated using mean change scores and SDs from published data.

2.3.2 The Effects of Blueberry Polyphenols in Middle-Aged Adults

To my knowledge, Whyte et al. (2021) completed the first study to be published looking at the effects of a blueberry polyphenols on cognitive function in middle-aged adults (table 2.4). Here, 35 adults aged between 40-65 years old completed this crossover trial, receiving either a blueberry (475mg anthocyanins) or placebo at each study day. Cognition was measured at 5 different time points following consumption of treatment. Cognitive tasks included Rey's Auditory Verbal Learning Task assessing episodic memory, Modified Attention Network Task assessing executive function, and the Cued Go/No-Go task assessing executive function**.** It is expected that cognitive performance decreases over the day, however results revealed beneficial effects on performance for AVLT Word Recognition, MANT, and Go-NoGo, whereby the accuracy levels significantly decreased for the placebo group post-consumption, but the blueberry intervention group-maintained performance levels for cognition aspect of the task. Overall, these early findings into the cognitive effects of WBB polyphenols in middle-aged adults provide a promising insight into the potential benefits, and further research is warranted.

Table 2. 4 Key studies investigating the effects of blueberry supplementation on cognition in healthy middle-aged adults

Author(s)	Treatment	Total Anthocyanin (mg)	Total Polyphenol (mg)	Study Design	Study Type	Measurement Time Points	Size (n)	Age Range (years)	Cognitive Tests (Cognitive Domain Tested in Parenthesis)	Key Findings	Effect Size (Cohen's d)
Whyte et al. (2021)	25 g freeze-dried BB powder (~ 1-cup fresh weight)	475	725	Double blind, placebo-controlled crossover design	Acute	2,4,6,8 hrs	35	40-65	**(1)** Rey's Auditory Verbal Learning Task (AVLT) (EM) **(2)** Modified Attention Network Task (EF) **(3)** The Cued Go/No-Go task (EF)	AVLT: Word recognition • Significant decrease in accuracy scores for placebo between baseline and 2 hrs (p = 0.015), 6 hrs (p = 0.02) & 8 hrs (p = 0.043). Accuracy for BB condition remain unaffected. MANT: • Beverage × Congruency × Load interaction (P= 0.044) revealed decrease accuracy scores for placebo at 2hr and 6 hrs. The Cued Go/No-Go task (EF) • Significant difference between WBB & placebo during invalid Go cues (p=0.018). • Reaction time: Quicker performance during the Go trials after BB consumption (p=0.049)	

Note. BB = Blueberry, FE = Fresh Equivalent; EM = Episodic Memory, EF = Executive Function, WM = Working Memory, PF = Psychomotor Function

2.3.3 The Effects of Blueberry Polyphenols in Healthy Older Adults

Five studies have been conducted looking at the effects of blueberry on cognition in healthy older adults. This research is in line with a wider and growing body of research considering the delay of cognitive decline in older adults (Boespflug et al., 2018; Krikorian et al., 2010; McNamara et al., 2018). Previous findings from pre-clinical experiments looking at the effects of blueberry on the cognition of aged animals showed a positive change in working memory performance as well as improved mobility in aged rats (Joseph et al., 1999; Andres-Lacueva, 2005). Building on these findings, Miller et al. (2018) considered the effect of a 24g freeze dried blueberry (19.2 mg/g anthocyanins, equivalent to 460 mg anthocyanins daily) intervention on the cognitive performance and mobility of adults aged 60-75 years old for a total of three months (n=37). The study consisted of a parallel design, and measurements were taken at day 1, day 45, and day 90. Note that, for the purpose this review, only cognitive outcomes will be discussed. The cognitive tasks included in this study were the Task-Switching Test (TST), Trail Making Test (TMT), California Verbal Learning Test (CVLT), Digit Span (DS) Task, a virtual version of the Morris Water Maze (vMWM), and Attention Network Task (ANT).

Results for TST generated no significant differences between treatments for reaction times. In terms of accuracy, a reduction in switch cost was indicated whereby there was a significant visit by treatment interaction with participants in the blueberry condition showing a reduction in switch trial errors over the test visits in comparison to placebo. In the CVLT, participants improved significantly on the number of words correctly recalled regardless of treatment. However, there was a significant visit by treatment interaction with participants in the blueberry group making fewer repetition errors on day 90 than they did on day 0. Participants in the placebo group showed the opposite pattern making more repetition errors at day 90.

There were no significant treatment related effects of blueberry on any of the other stated outcome measures.

Schrager, Hilton, Gould and Kelly (2015) considered the positive effects of blueberry intervention on motor and psychomotor function along with tests of executive function. Twenty unblinded participants were randomly allocated to either a daily regimen of 2 cups of blueberries (n = 13) or a carrot juice placebo (n = 7) for 6 weeks. The polyphenol and anthocyanin content of the blueberry intervention were not stated. The cognitive tasks used were the Simple Reaction Time, TMT B, and Dual-Task Adaptive Gait Test (DTAG). Further measures of grip strength, gait speed and adaptive gait were also recorded. Analysis of the results revealed a significant improvement in executive function following blueberry treatment with participants performing less step errors during the DTAG in comparison to the placebo condition. There were no other treatment related effects for the cognitive tasks in this study, however the participants also showed improved motor function related to increased gait speed following blueberry intervention. It should be noted that there are possible issues with the control drink used in this study. Although not a rich source of flavonoids, carrots are abundant in carotenoids and other polyphenolic compounds. Research has shown that the carotenoids, lutein and zeaxanthin, are associated with improved cognitive function (Hammond et al., 2017; Saint et al., 2018) whilst long-term supplementation with beta-carotene influences cognition (Grodstein et al., 2007). Besides these issues with components in the placebo treatment that are known to influence cognition themselves, participants were also unblinded to the treatment they received. Given these concerns over the design, some caution should be employed in consideration of the findings here.

Bowtell et al. (2017) looked at the effects of blueberry supplementation on cerebral blood flow, with cognition as a secondary outcome. The study adopted a parallel, double-blind design, testing the effects in 26 healthy adults, with an average age of 68 yrs, after 12 weeks of supplementation. The cognitive battery consisted of 6 tasks: 1) Detection task, 2) Groton maze timed chase test, 3) Groton maze learning test, 4) Identification task, 5) International shopping list task, and 6) 1-back and 2-back task. To measure brain activation, functional magnetic resonance imaging (fMRI) was performed whilst the participants conducted a numerical Stroop task. Arterial spin labelling (ASL) measures of brain perfusion were also gathered whilst the participant was in a rested state. Analysis revealed no significant treatment-related effects on the Detection Task, Groton Maze tests, Identification task, shopping list task, and Stroop test, however, there was a trend towards better performance in the 1-back test and trends for improved reaction time and accuracy on the 2-back test. Although there were no significant treatment-related behavioural effects whilst performing the response interference Stroop task, fMRI analysis revealed significant increases in activation of a number of task related areas (Brodman areas 4, 6, 10, 21, 40, 44, 45, precuneus, anterior cingulate, insula, and thalamus) in comparison to baseline. No such effects were found for the placebo. Furthermore, the resting state ASL analysis revealed increased grey matter perfusion in the parietal and occipital lobes following blueberry whereas, again, no effect was found for the placebo.

A study by Whyte et al. (2018) compared three blueberry treatments stabilised with L-cysteine and L-glutathione with placebo on measures of cognitive function, cardiovascular function and mood. A total of 112 healthy, older participants completed the 6-month long study consuming two capsules of their allocated treatment per day, with testing occurring at baseline, 3 months and 6 months. The participants were randomised into 4 treatment groups

consisting of placebo, 500 mg wild blueberry powder (WBB), 1000 mg WBB, and 111 mg wild blueberry extract (WBE) containing 0, 1.35, 2.7 and 7 mg anthocyanin content and 0, 35, 70 and 50 mg polyphenols, respectively. Although the anthocyanin content is lower than what would be present from a single serving of fresh blueberries, all treatments had L-cysteine and L-glutathione added to them. This helped facilitate the stabilisation of their anthocyanin content and, in turn, allow a higher rate of absorption than might be possible via general habitual intake or at doses used in previous studies. In terms of cognitive testing, the primary outcome measure was episodic memory, via three different tasks including the RAVLT, Object Recognition Task, and Corsi Block task. The secondary cognitive outcome measures tested executive function, attention and working memory. Tasks included the Serial 3s and 7s, Sternberg Memory Scanning Task, MANT, and Stroop Task.

Linear mixed model analysis revealed that for the word recognition measure of the AVLT, there was a significant treatment by time interaction with post hoc analysis finding improvement after supplementation with WBE compared to the placebo after 3 months, but not after 6 months. There were no significant differences for the other blueberry treatments for this measure. A similar pattern of results was found for the total number of Corsi Block sequences correctly recalled where there was a significant treatment by time interaction with post hoc analysis finding a trend for improvement following supplementation with WBE compared to the placebo after 3 months, but not after 6 months. Again, there were no significant differences for the other blueberry treatments for this measure. For the working memory and executive function tasks, there were no significant effects for any of the blueberry treatment compared to placebo at any of the time points. In terms of the markers of cardiovascular health, a main effect of intervention was found with post-hoc analysis revealing significantly lower WBE systolic blood pressure over the 6-month intervention,

however, no such effect was found for the other blueberry treatments. It is interesting, and somewhat unexpected, that no significant differences in cognitive performance were seen after 6 months and the authors posit that this may reflect an element of practice whereby participants improved their strategy to perform these tasks over time with repeated exposure thus reducing task sensitivity to the intervention.

The latest research looking at blueberries for cognitive function in healthy older adults was conducted by Dodd et al. (2019). In this study, the investigators looked at how a blueberry intervention consisting of 579mg of anthocyanins impacted performance in tasks assessing memory and executive function after acute consumption. They also measured blood pressure and took plasma samples to assess how BDNF levels differed in treatment versus control group, to try and underpin the mechanisms of action behind blueberries potential effect on cognitive function. No significant differences were established between blueberry and placebo for cognitive function, however performance was sustained after blueberry intervention at both 2hr and 5 hr time points, whereas performance decreased in the control group. In addition to this, Dodd et al. (2019) also assessed vascular measures to underpin mechanism of actions. For vascular measures, there was a trend towards a reduction in systolic pressure for the blueberry intervention group compared to placebo, however there was no effect of treatment for arterial stiffness, pulse rate or diastolic pressure. BDNF levels decreased in the placebo group compared to blueberry group, where levels at 1hr post intervention, remained similar to baseline levels. Overall, the results suggest that blueberry intervention has the potential of maintaining the level of cognitive function for up to 5 hours post consumption, reducing systolic pressure and maintaining BDNF levels in the blood but this requires further analysis in order to understand whether these effects may be observed after chronic consumption.

In summary, five studies have investigated the effects of pure blueberry intervention in older adults. Results from these studies have been mixed. Of the three studies which considered episodic memory, only two (Miller et al., 2018; Whyte et al., 2018) found significant effects in the different subdomains of delayed recognition and repetition errors. All studies considered executive function, though positive behavioural benefits were only found in two. It could, however, be argued that the effects were found on the more cognitively demanding switch trials of the switching task (Miller et al., 2018) and the Dual Task Adaptive Gait Test (Schrager et al., 2015). Furthermore, the elevated brain activation, which was found in the absence of significant behavioural effects during performance of the less cognitively demanding Stroop task (Bowtell et al., 2017) gives further indication that in order to establish blueberry related executive function benefits within an older age group, the level of task demand should be carefully considered by ensuring sufficient task difficulty for effects to be seen, if any.

Table 2. 5 Key studies investigating the effects of blueberry supplementation on cognition in healthy older adults

Author(s)	Treatment And Amount	Total Anthocyanin (mg)	Total Polyphenol (mg)	Study Design	Study Type	Measurement Time Points	Size (n)	Age Range (Years)	Cognitive Tests (Cognitive Domain Tested in Parenthesis)	Key Findings	Effect Size (Cohen's d)
Schrager et al. (2015)	Frozen BB - 2 cups daily (approx. 300g FE).	N/A	N/A	Placebo controlled, parallel design. Randomisation and blinding not stated.	Chronic	Baseline and week 6	20	61-80	**(1)** Simple Reaction Time (PF) **(2)** Trail Making Test Part B (EF) **(3)** Dual-Task adaptive gait test (DTAG) (EF/PF)	DTAG: Step Errors • BB vs Placebo ($p = .048$)	n/a
Miller et al. (2017)	Freeze dried BB powder, 24g (approx. 150g FE)	460	864	Double-blind, placebo-controlled, parallel design	Chronic	Baseline, day 45 and day 90.	37	60-75	**(1)** Task-switching test (TST) (EF) **(2)** Trail-making test (TMT) (EF) **(3)** California Verbal Learning Test. CVLT-II) (EM) **(4)** Digit span task (DS) (WM) **(5)** Virtual Morris Water Maze (vMWM) (WM) **(6)** Attention Network Task (ANT) (WM)	TST: Switch Cost • BB improvement across visits ($p = .033$) CVLT: Repetition Errors • BB improvement across visits ($p = .031$)	n/a 0.759*
Bowtell et al. (2017)	BB concentrate, 30ml (FE N/A)	387	N/A	Double-blind, placebo-controlled parallel design	Chronic	Baseline and week 12	26	60-75	**(1)** Detection task (PF) **(2)** Groton maze timed chase test (EF) **(3)** Groton maze learning test (EF) **(4)** identification task (EF) **(5)** International shopping list task (EM) **(6)** 1-back and 2-back memory tasks (WM)	2-back task • BB vs Placebo ($p = .05$ trend) fMRI: Brain activity • BB vs Placebo ($p < .001$)	n/a

Whyte et al. (2018)	**(1)** 500 mg Wild BB Powder (WBP500) **(2)** 1000 mg Wild BB Powder (WBP1000) **(3)** 111 mg Wild BB Extract (WBE111)	**(1)** 1.35 **(2)** 2.7 **(3)** 7	**(1)** 35 **(2)** 70 **(3)** 50	Double blinded, placebo-controlled parallel design	Chronic	Baseline, week 12 and week 24	112	65–80	**(1)** RAVLT (EM) **(2)** Object recognition task (EM) **(3)** Corsi Blocks task (EM) **(4)** Serial subtractions tasks (WM) **(5)** The Sternberg memory scanning task (WM) **(6)** Modified Attention Network Task (EF) **(7)** Stroop (EF)	RAVLT: Word Recognition at 12 weeks • WBE111 vs Placebo (p = .038) Corsi Blocks: Total Sequences • WBE111 vs Placebo (p = .069 trend)	0.608** 0.535**
Dodd et al. (2019)	Freeze dried BB powder, 30.1g, (approx. 200g FE)	579	N/A	Double blind, placebo-controlled crossover design	Acute	2hrs & 5 hrs	18	60-75	The global cognitive function measure consisting of: (1) Go-NoGo (EF) (2) Stroop (EF) (3) Digit Switch (EF) (4) Continuous Performance Task (EF) (5) Digit Symbol Substitution Test EF) (6) Random Word Generation (EM) (7) Three-Word Sets Task (EM) (8) N-back (EM) (9) Letter Memory (EM) (10) Location Task (EM) (11) Immediate recall (EM) (12) Delayed Recall (EM) (13) Immediate Recognition (EM) (14) Delayed Recognition (EM)	• No overall effect of treatment on global cognitive function • Pairwise comparison found performance following placebo consumption was significantly worse at 2 hrs compared to 5hrs (p=0.04).	n/a

Note. BB = Blueberry, FE = Fresh Equivalent; EM = Episodic Memory, EF = Executive Function, WM = Working Memory, PF = Psychomotor Function.

* Effects sizes originally reported as partial eta square and converted to Cohen's d for the purpose of this review; ** calculated from estimated marginal means and standard error to give effect size relative to residual individual differences after controlling for the baseline effect

2.3.4 The Effects of Blueberry Polyphenols in Adults With Mild Cognitive Impairment

Similarly, to the other age groupings assessed in this review, there are few studies considering the effects of blueberry supplementation in adults with mild cognitive impairment. To date, 3 studies fulfilled our inclusion and exclusion criteria. Krikorian et al. (2010) investigated for the first time the effects of blueberry on cognition in older adults with MCI. The sample size was a total of 9 participants with a mean age of 72 along with the data from a placebo group of 7 participants gathered from a previous Concord grape juice study (Krikorian et al., 2010). The study employed a randomised, double blind, placebo-controlled trial testing the effects of blueberry for 12 weeks. Dosage of blueberry treatment was calculated according to body weight. More specifically, participants weighing 54-64kg received 444 mL/day and participants in 65-76 kg received 532 mL/day, and participants weighing 77-91 kg received 621 mL/day. The placebo was a grape-flavoured drink that contained no polyphenols. Cognitive measurements from both treatment groups were taken at baseline and 12 weeks and the participants performed a battery consisting of two tasks testing verbal learning and memory which are known to be processed by the hippocampal region. The tasks included Verbal Paired Associate Learning Test (V-PAL) and the California Verbal Learning Test (CVLT).

Analysis of the V-PAL cumulative learning scores showed the cumulative score significantly improved at 12 weeks compared to baseline, as did delayed recall performance during the CVLT. However, no mention is made regarding these comparisons for the placebo either in this paper or the companion study from which the placebo group data was drawn. This raises the possibility that the findings were primarily practice effects. Acknowledging this possibility Krikorian et al. (2010) performed a further comparison with the placebo group on the 12-week time point data which found significantly better V-PAL performance for those receiving

the blueberry intervention, however no such effect was found for the CVLT data. This would indicate that whilst the V-PAL effects would seem to be robust, the effects reported for the CVLT should be considered with caution. Furthermore, although the results yielded significant effects, this was a small study (n=16 which includes data from a placebo group from a different study).

Boespflug et al. (2018) measured working memory performance in a group of MCI participants aged 68-92 (n=16). Additionally, brain activation was assessed using fMRI whilst the participants conducted a cognitive task. The study employed a randomized, double-blind, placebo-controlled parallel study. The cognitive task used was the sequential letter n-back, assessing working memory, as in Bowtell et al. (2017). In this study, there were three different conditions: 0-back, 1-back, and 2-back with the outcome measures being accuracy and reaction time. The treatment was administered as a drink consisting of water and blueberry powder giving a daily dosage of 269 mg of anthocyanins, equivalent to roughly 220g of fresh blueberries. This was administered daily for a total of 4 months, with measurements taken at pre-intervention (baseline) and post-intervention (week 16).

Analysis of working memory performance revealed that for all the 0-back and 2-back conditions there were no significant improvements in reaction time at any of the timepoints. For the 1-back condition, there was a trend towards significance ($p = 0.08$) for accuracy in the blueberry group compared placebo group performance at 16 weeks. However, though a significant difference was found at baseline, with placebo performing significantly faster than blueberry in the 1-back condition, no reaction time differences were found between treatments post-intervention. In terms of fMRI results, there were observed changes in the blueberry treated group, with increased activation in the left pre-central gyrus, left middle

frontal gyrus, and left inferior parietal lobe during the final visit (16 weeks). More specifically, analysis revealed a significant increase of signalling in the left inferior parietal gyrus and left pre-central gyrus during the 2-back condition for blueberry treated group. There were, however, no significant effects of activation under the 0-back and 1-back conditions. In terms of the placebo group, decreased activation was witnessed close to the left post-central gyrus at the final visit compared to baseline.

McNamara et al. (2018) investigated the effects of blueberry supplementation, fish oil, and a combination of the two on the cognition of 94 healthy men and women aged between 62-80 years old who had not been diagnosed with any form of cognitive impairments but did suffer from self-reported cognitive complaints. The sample population was divided into four groups: blueberry powder + placebo oil, fish oil + placebo powder, blueberry powder + fish oil, and placebo powder + placebo oil. The blueberry treatment was equivalent to 25g dry weight of blueberry a day and provided 269 mg of anthocyanins per serving. The intervention lasted a period of 24 weeks with measurements taking place at week 0 (baseline) and week 24, as well as an additional measurement 24 weeks after the intervention period (week 48). A total of 76 participants completed the whole study successfully and a total of 65 took part in the post-intervention measurements. Cognitive assessments used included Dysexecutive Questionnaire (DEX) to assess executive function, Trail Making Tests A and B (TMT-A and TMT-B), Controlled Oral Word Production, and Hopkins Verbal Learning Test (HVLT*)* to assess verbal learning and long-term memory. As well as cognition, there were measurements of red blood cell fatty acid composition, anthocyanin levels in urine, metabolic factors, *APOE* genotyping as well as anthropometrics measurements. In the context of this review, only the cognitive outcomes will be discussed.

Results found that for the DEX, the scores for the blueberry treated group decreased significantly, indicating that fewer negative cognitive symptoms experienced in everyday activities at 24 weeks. This benefit was maintained at the 48 week point, 24 weeks following the cessation of treatment. Furthermore, the blueberry treated group displayed improvements in HVLT recognition memory discrimination performance after 24 weeks, however, this effect was not maintained at 48 weeks. There were no significant blueberry-related improvements for any of the other tasks. Comparing this with the other interventions, the DEX scores also decreased significantly for the fish oil-treated group whilst increases on DEX were seen following placebo. As for the other cognitive tasks, no significant improvements were observed for either the fish oil, the combined fish oil and blueberry group, or the placebo groups. Overall, the researchers concluded that, in a sample of older adults experiencing self-diagnosed cognitive complaints, the blueberry intervention improved cognitive efficiency for everyday life activities, as well as improving resilience against extraneous disturbances during a recognition memory task.

Of the two studies above which considered episodic memory, both found positive effects following blueberry intervention. Interestingly, the recognition memory performance found in healthy adults by Whyte et al. (2018) was also found by McNamara et al. (2018) in adults suffering from mild cognitive complaints, indicating the sensitivity of this measure to blueberry intervention in an aging population. There was little evidence of a working memory effect, with the results of Boespflug et al. (2018) only trending towards significance, however, in a similar fashion to Bowtell et al. (2017) fMRI analysis again showed elevated task related brain activation despite no behavioural effects being found.

Table 2. 6 Key studies investigating the effects of blueberry supplementation on cognition in adults with MCI

Author(s)	Treatment	Total Anthocyanin (mg)	Total Polyphenol (mg)	Study Design	Study Type	Measurement Time Points	Size (n)	Age Range (years)	Cognitive Tests (Cognitive Domain Tested in Parenthesis)	Key Findings	Effect Size (Cohen's d)
Krikorian et al. (2010)	Wild BB juice. By participant weight: **(1)** 54-64 kg = 444 ml **(2)** 65–76 kg = 532 ml **(3)** 77–91 kg = 621 ml (FE N/A)	**(1)** 428 **(2)** 512 **(3)** 598	**(1)** 1056 **(2)** 1266 **(3)** 1478	Double-blind, placebo-controlled parallel design	Chronic	Baseline and at 12 weeks	9	Mean age: 72	**(1)** California Verbal Learning Test (CVLT) (EM) **(2)** Verbal paired associate learning test (V-PAL) (EM)	V-PAL: Cumulative Learning • BB improvement across visits (p = .009) • BB vs Placebo (p = .03) CVLT: Word Recall • BB improvement across visits (p = .04)	1.78* n/a 1.18*
Boespflug et al. (2017)	25 g Freeze-dried BB powder (approx. 150g FE)	269	417	Double-blind, placebo-controlled parallel design	Chronic	Baseline and at 16 Weeks	16	68 –92	**(1)** n-back WM task (WM)	No sig. effects after 16 weeks of supplementation. fMRI: Brain activity • BB relative to baseline (p < .01)	1.82* (left inferior parietal gyrus) and 1.94* (left pre-central gyrus)
McNamara et al. (2018)	25 g Freeze-dried BB powder (approx. 150g FE)	269	417	Double-blind, placebo-controlled parallel design	Chronic	Baseline and at 24 Weeks	19	62-80	**(1)** Dysexecutive Questionnaire (D EX) (EF/WM) **(2)** Trail making test A and Trail making test B (EF) **(3)** Controlled Oral Word Production (EF)	DEX: Cognitive Symptoms • BB vs Placebo (p = .05) HVLT: Memory Discrimination • BB vs Placebo (p = .04)	0.68** 0.68**

4) Hopkins Verbal Learning Test (HVLT) (EM)

Note. BB = Blueberry, FE = Fresh Equivalent; EM = Episodic Memory, EF = Executive Function, WM = Working Memory, PF = Psychomotor Function
*Effect size as reported in original paper; **effect size originally reported as Cohen’s f converted to Cohen’s d for the purposes of this review

2.4 Discussion

Studies investigating the effects of blueberry intervention to date have considered three main age groups, children aged between 7 – 10 years old, middle-aged adults or older adults aged 60 and above. This latter group can be further subdivided into healthy adults and those with MCI. With regards to study type, 5 acute (single administration) interventions and 1 chronic (repeated administration) have been published with children whilst 7 chronic interventions using varying durations of treatment and 1 acute intervention have been published with older adults. The study looking at effects in middle-aged humans utilised an acute intervention. Tasks employed have differed between studies with some considering only one cognitive domain and others a wider range. Furthermore, anthocyanin doses employed have ranged from 1.35 mg to 598 mg in chronic studies and between 127 mg and 579 mg in acute studies. Cognitive results from these studies have been mixed, with results not being seen consistently across the different domains considered, though see below for comments on task sensitivity. Furthermore, as can be seen from Tables 2.3-2.6, there was a spread of effect sizes with Cohen's d ranging between 0.062 and 1.94. Making any strong conclusions regarding expected cognitive outcomes in relation to developmental stages and proposing best practice for future research is therefore not possible given the literature available. The following discussion should therefore be considered in this light.

Within the domain of memory, benefits have primarily been found on episodic measures with significant improvements being found for acute child interventions

on the AVLT in word acquisition, delayed recall, and word recognition (Whyte et al., 2015; Whyte et al., 2016; Barfoot et al., 2018) as well as executive function (Whyte et al., 2020; Barfoot et al., 2021). In middle aged adults improvements were seen for episodic memory with benefits seen for AVLT word recogntion and executive function, with benefits seen during the MANT and Go/No-Go task (Whyte et al., 2021). Interestingly the effect of improved episodic memory performance is also seen in chronic older adult interventions with a number of studies finding positive effects on either the CVLT, HVLT, or AVLT measures of episodic memory (Miller et al., 2017; Whyte et al., 2018; Krikorian et al., 2010; McNamara et al., 2018). This gives some indication that episodic memory is particularly sensitive to anthocyanin blueberry intervention in both children and older adults. It should be noted however that, in a review of the literature considering acute flavonoid interventions of all classes, Bell et al. (2015) reported there was little evidence of a positive episodic memory effect in young adults and further blueberry related research is therefore required to clarify whether episodic memory effects might also be found within this age group.

The positive benefits of executive function (EF) are also present in the literature for all age groups with children showing improved performance following blueberry intervention on the more cognitively demanding response interference trials of the modified flanker and modified attention network tasks (Whyte et al., 2016; Whyte et al., 2017). Similarly, the middle-aged adults displayed maintenance of performance following WBB relative to placebo during the Cued Go/No-Go task and MANT, whereby accuracy scores following placebo intake was significantly reduced (Whyte et al., 2021).

In older adults the results are more equivocal with only two out of the six studies which measured EF reporting blueberry related effects. It should be noted that where there were significant findings, the effects were found on arguably the more cognitively demanding elements of the tasks with results being found on the critical switch trials of the switching task (Miller et al., 2018) and the dual task adaptive gait task involving the simultaneous performance of two tasks at once (Schrager et al., 2015). When considered together, the results of both the acute and chronic studies indicate that blueberry intervention may have an effect on EF; however, task sensitivity is critical with the improvements becoming more evident between treatment and placebo as the cognitive demand of the task increases. This highlights the importance of task sensitivity and demonstrated that some tasks used in the studies presented in this review may not have been sufficiently demanding for differences in performance to be observed between treatment groups.

Tests of working memory (WM) have revealed no evidence of blueberry related benefits in children (Whyte et al., 2015; Whyte et al., 2016). For older adults benefits of WM were found in two of the six studies which considered this domain with the effects being seen either on 1 or 2 n back tasks (Boespflug et al., 2018; Bowtell et al., 2017). However, it should be noted that these effects were trends, and, in both studies, there was no reported statistical correction for the analysis of the multiple n-back versions employed. Taken as a whole, therefore, the literature would suggest there is little benefit to be found for blueberry intervention within this domain.

One explanation for the benefits found in cognitive function could be due to improved memory encoding as a result of elevated levels of BDNF. Pre-clinical studies have shown blueberry's efficacy in increasing the level of brain-derived neurotrophic factor (BDNF) in the hippocampal area of the brain (Williams et al., 2008; Rendeiro et al., 2012). BDNF is a neurotrophin, a protein that plays an important role in cell regeneration, differentiation, survival and death of neurons (Song et al., 2017). Emerging evidence suggests that BDNF plays a significant role in memory, and that BDNF declines as we grow older; this is believed to be one reason why memory loss and cognitive decline is often a frequent effect of ageing (Hattiangady et al., 2004). Moreover, studies have shown that the activation of cAMP-response element-binding protein (CREB), a transcription factor that plays an important role in the formation of long-term memory (Frank & Greenberg, 1994) is positively correlated with an increase in memory after supplementation with blueberry polyphenols in aged rats (Williams et al., 2008) and an increase in spatial memory in young rats (Rendeiro et al., 2012). It is also believed that increased cognition could be due to increased neurogenesis. Studies in animals have shown that neuron proliferation increased after blueberry supplementation (Casadesus et al., 2004). Recent fMRI studies have shown an increase in cerebral blood flow after supplementation with berry polyphenols, particularly in the parietal lobe and occipital lobe (Boespflug et al., 2018; Bowtell et al., 2017). One effect of increased cerebral blood flow is an increase of oxygen and glucose to neurons, which may enhance neuronal activity.

Factors such as time-point, dosage, administration form, and choice of cognitive tasks need to be taken into consideration before recommendations can be made

for future research. From the acute studies looking at time-response effects in children, it seems that there are different responses being produced at different times. For example, results have shown delayed memory performance is best performed 1.15 hrs post consumption (Whyte et al., 2015), whereas improvements in executive function performance is seen at 2hrs (Whyte et al., 2020) and 3 hrs (Whyte et al., 2016; Whyte et al., 2017). The time point differences observed could be due to factors such as absorption rate, digestion, and breakdown of metabolites (Bell et al., 2015) although further testing is necessary in order to understand these mechanisms.

For chronic studies there was one case where cognitive effects for word recognition were observed at the intermediate testing point of 12 weeks but not at the final testing point of 24 weeks (Whyte et al., 2018). This raises questions related to the metabolism and absorption of blueberry polyphenols, after a certain time of ingestion, and at a certain dosage. One possible explanation is that participants may have become habituated to the effect of the blueberry intervention with less cognitive benefit being evident at later stages of treatment. Furthermore, Whyte et al. (2018) consider the possibility of practice effects whereby the performance of each of participant improves over time reducing the early advantage of blueberry intervention. Furthermore, the mechanism of action behind acute interventions may be different to chronic studies due to the quantity and mode of absorption following short versus long intervention periods. Nevertheless, the lack of chronic data, plus the limited range of different cognitive domains within chronic studies tested are not enough for any conclusive points to be made here.

In the papers studied, the anthocyanin content ranged from 1.35 mg (Whyte et al., 2018) to 578 (Dodd et al., 2019). However, higher anthocyanin concentration does not necessarily translate to better cognition compared to lower doses suggesting that a ceiling effect is likely, with higher doses producing no extra benefits. This would seem to correlate with physiological responses to blueberry intervention such as flow mediated dilation (FMD) which can be seen to peak following doses containing 766 mg anthocyanin and tail off at higher doses (Rodriguez-Mateos et al., 2013). It is believed that this improvement in endothelial function is a nitric oxide (NO) mediated response exerted by polyphenolic compounds found in blueberries.

An increase in cerebral blood flow to the brain was found after acute (Dodd, 2012) and chronic (Bowtell et al., 2017; Boespflug et al., 2018) blueberry treatment when compared to placebo. This suggests one mechanism by which blueberries may be exerting a positive effect on cognition. Other studies involving flavonoids and CBF have shown a similar effect, with an increase in CBF observed after an acute intake of citrus juice high in flavanones (Lamport et al., 2016) or cocoa flavanols (Francis et al., 2006). This raises the question whether improved endothelial function might facilitate an increase in peripheral blood flow, and thus cerebral blood flow, which in turn may improve cognitive functioning. This is an area which still requires extensive research and will be explored as part of my experimental studies in this thesis.

In terms of the interventions themselves, there is a large variation in the actual anthocyanin content of the treatments. The flavonoid ratio in equivalent weights

of the blueberry treatments also differed between studies, for example, 30g of freeze-dried blueberry powder contained 253 mg of anthocyanins in Whyte et al. (2016) whereas the equivalent fresh contained 148 mg in Whyte and Williams (2015). This highlights the importance of analysing blueberry powder for polyphenol/anthocyanin content prior to starting an intervention.

In terms of study design, nine studies (Barfoot et al., 2018; Boespflug et al., 2018; Krikorian et al., 2010; McNamara et al., 2018; Miller et al., 2018; Schrager et al., 2015; Bowtell et al., 2017; Whyte et al., 2018; Barfoot et al., 2021) employed a parallel, placebo-controlled design, and six studies (Whyte & Williams, 2015; Whyte et al., 2016; Whyte et al., 2017; Whyte et al., 2021; Dodd et al., 2019) employed a crossover placebo-controlled design. There seems to be no evidence for patterns of effects based on the design as effects were seen in crossover and also parallel designs.

In most cases the blueberry was administered as a freeze-dried powder mixed with water and administered as a drink. Only one study mixed the powder with milk (Whyte & Williams, 2015). Nevertheless, the evidence currently available related to the inhibition of polyphenols by dairy proteins is equivocal. Some studies demonstrate that proteins found in milk have no effect on the bioavailability of polyphenols (Draijer et al., 2016) and some believe that it may affect the bioavailability of some, but not all, polyphenolic compounds (Urpi-Sarda et al., 2010). Ultimately it should be taken into consideration that factors other than dairy proteins may also play a role in the absorption and metabolism of polyphenols, including the gut microbiota and the chemical structure of the

polyphenol (e.g. hydroxyl group have a high affinity for proteins) amongst other dietary factors (Bohn, 2014).

2.5 Conclusion

In conclusion, the cognitive research considering blueberry intervention currently gives an incomplete picture, with no published research as yet having considered infants, teens, or young adults. Findings from the present literature indicate that benefits might be found in most reliably in episodic memory and executive function, with the benefits for working memory at present being more equivocal. More specifically, there is a trend where improvements are seen within the executive function and episodic memory domains for children; for adults there are more memory related improvements and in adults with MCI improvements are found primarily within the episodic memory domain. Therefore, the current literature indicates that blueberry polyphenols have the capacity to improve some aspects of cognition across certain ages, and with further investigation, is a concept which might be applied to specific real-life situations such as learning. This will be explored further in the thesis in order to add to current knowledge surrounding how WBB polyphenols may affect cognitive functioning across the life-course. In addition to this, the chronic effects of WBB intervention will be explored in healthy older adults, to improve our understanding of how daily consumption of WBB, versus a placebo might affect cognitive function within an older cohort.

Chapter Three

Thesis Objective & Research Questions

3.1 Research Gaps

Following on from the findings highlighted in the literature review documented in chapter 2, it is apparent that blueberry polyphenols may have significant benefits for cognitive function. Observations for episodic memory amongst young children and older adults is prominent, but further research, particularly investigating effects in teenagers and middle-aged participants, as well as considering the impact of blueberry polyphenols across a range of cognitive domains is needed. Moreover, evidence so far has demonstrated that polyphenols may have a positive effect on cardiovascular health; however here too, more evidence is required. The current gaps in the literature highlight the need to further investigate the effects across the life course, and also the effects of cognition following chronic WBB consumption, whilst also exploring cardiovascular variables such as flow mediated dilation blood pressure and cerebral blood flow.

3.2 Thesis Objectives

The overall aims of this thesis are to *a)* further explore the effects of blueberries on cognitive function across the lifespan, *b)* assess the specific domains of cognitive function that are sensitive to blueberry polyphenol supplementation and *c)* investigate the potential mechanisms of action underlying the positive effects of blueberry on cognition by assessing changes in both peripheral and central

blood flow, as well as other markers of vascular function. The assessment of vascular health will be conducted with a collaborating team at King's college London in order to understand whether any links between changes in vascular function and cognitive function exist. The results will not be reported in this thesis but instead can be found in the thesis by Wood et al (2022). Nevertheless, a short discussion based on the findings for vascular function will be considered in order to understand and explain the effects seen for cognitive function.

In order to answer these novel research questions, two experimental studies were developed as outlined below.

3.3 Human Intervention Studies

3.3.1 Experiment 1 (The BluLife Study)

An acute study investigating the effects of a WBB intervention (versus placebo) at 2 hrs post consumption, on cognitive function, cardiovascular health, and cerebral blood flow across the life course (age 8-80 years old).

3.3.2 Experiment 2 (The BluFlow Study)

A chronic study investigating the effects of three months repeated daily dosing of WBB (versus placebo) on cognitive function, cardiovascular health, cerebral blood flow and gut microbiota in a group of healthy older adults.

3.4 Methodological Considerations

3.4.1 Intervention Choice

As previously mentioned in chapter 2, there is convincing evidence that polyphenols which exist in blueberries have benefits to human health, particularly cognitive function (Hein et al., 2019) and vascular health (Wood et al., 2019). Wild blueberries of the lowbush variety, are smaller, tend to be less sweet and have a higher skin to flesh ratio. Literature suggests that WBB therefore contains higher anthocyanin levels compared to highbush varieties, as the anthocyanin compounds are concentrated within the skin (Ochmian et al., 2009). Therefore, berry skin consumption is increased as the berries are smaller, compared to larger berries which means higher anthocyanin intake when consuming wild blueberry varieties.

The interventions in these studies will consist of freeze-dried wild blueberry (WBB) powder due to the increased anthocyanin content. In both studies the placebo and WBB interventions were administered as drinks using freeze-dried WBB powder and not as whole fresh fruit. Using a freeze-dried powder instead of fresh fruit has many benefits including easier storage, consistency in term of the polyphenolic content, easier for administering the correct dose and it is more feasible to develop a placebo test drink that is matched to taste and look like a blueberry juice drink. The powders were stored in a freezer set at -20 degrees Celsius as previous studies have demonstrated the anthocyanin levels remain stable at this temperature (Lohachoompol et al., 2004). The freeze-dried wild blueberry powder used in these investigations was provided by the Wild Blueberry Association of North America (WBANA). The freeze-dried blueberry powder used in the two studies were from different harvest batches and contain different levels of micronutrients. In the BluLife study, the treatment drink contained 133.2mg of

anthocyanins per serving, which was equivalent to 106 g of fresh weight blueberries. This dose was chosen as it represented a realistic portion which could be easily consumed by people of all ages and is just over the recommended serving size for the 5-a-day guidelines (NHS, 2018). In the BluFlow study, the anthocyanin content in the WBB powder was approximately 264mg per serving, which was equivalent to 178g of fresh blueberries. This dose was chosen based on previous studies which demonstrated improvements for cognitive function (Hein et al., 2019) using this dose. Full breakdown of chemical composition and formulations will be provided in the study specific chapters (BluLife Study, chapter 5), (BluFlow Study, chapter 6).

3.4.2 Study Design Considerations

The first study (BluLife) employed a randomised, double blind, placebo-controlled crossover design investigating the acute effects of a wild blueberry intervention on cognitive function and cardiovascular health in participants aged 8-80 years old. This design allows the participant to complete each experimental condition (active and placebo treatment), which ultimately reduces variability relating to individual differences. Acute testing time point was chosen as 2hr post-consumption of treatment as studies have documented that this time point is when the metabolite levels peaks in the human body and may therefore lead to increased absorption (Feliciano et al., 2016).

The second study (BluFlow) employed a double blind, parallel design, consisting of a group treated with a WBB intervention and a group treated with a placebo.

Participants consumed their allocated treatment daily with cognitive function and cardiovascular health assessed at baseline and after 84 days of treatment. For both studies, measurements of each outcome variable were conducted at baseline, before any administration of treatments, to ensure that random variations in measurements such as cognitive performance were controlled. Lastly, to avoid potential experimenter bias or expectations, testing was fully double-blinded for both studies until all statistical analyses were finalised.

3.4.3 Participants

For the first study (the BluLife study), participants in the following age groups were selected: 8-10, 14-18, 22-28, 40-50, and 65-80. This is because these ages provide a good snapshot of the cognitive developments expected across the life course. The youngest age category in this study is the 8-10 year olds. This age group were chosen as six studies have been published looking at the effects of WBB on cognitive function in children aged 7-10. It is documented that children from the age of 7 begin to develop cognitive flexibility and executive function capacities (Anderson, 2002) which allows them to complete tasks which have been previously used in nutritional intervention cognitive studies. The maximum age category is 65-80 year olds and, due to dementia risk being higher as age increases, it was decided that maximum age of 80 years old would allow us to identify participants who are eligible, cognitively and vascularly healthy and free from medication. All participants, irrespective of age group, were screened prior to the study to check their health, to ensure they were non-smokers, were within a

healthy BMI category (between the range of 18.5–24.9) and to ensure they are free of medications, as certain medications may have an effect on the efficacy and absorption of the WBB polyphenol intervention, as well modulating vascular responses such as blood pressure.

For the BluFlow study, the age category chosen was healthy older adults aged 65-80. This population was chosen based on previous findings showing that age-related cognitive differences and decline is detectable in this age range (Miller et al., 2011). Cognitive decline is expected as age increases, regardless of whether older adults are diagnosed with dementia or not. This decline in cognition is normal and often combined with a reduction in physical ageing such as a reduction in musculoskeletal strength and gait such as walking speed and balance (Miller et al., 2011; Salzman et al., 2010; Prince, 1997). More specifically to cognitive function, it is believed that a reduction in synaptic plasticity may lead to slower psychomotor speed, as well as working memory and episodic memory being examples of domains which are particularly prone to age-related changes (Bherer, 2015).

3.4.4 Cognitive Domains Targeted

The tasks chosen for the battery included Auditory Verbal Learning Test (AVLT), Corsi Blocks Tapping Task, Serial 3s and 7s, Task-switching test (TST) (Figure 3.1). A full description of each task is outlined in chapter 4. The tasks selected for this battery were chosen for numerous reasons. Firstly, they were chosen because of a known hypothesized relationship between flavonoids and cognitive function.

Secondly, they are suitable for assessment in all ages. Lastly, a range of tasks were chosen which offered a broad coverage of different cognitive domains. AVLT measures immediate and delayed episodic and has been used extensively in studies investigation the effects of nutritional interventions. Evidence from literature suggests sensitivity of this task following blueberry consumption (Whyte *&* Williams 2015; Whyte, Schafer and Williams, 2016; Barfoot et al., 2018, Whyte et al., 2018). The Corsi Blocks tapping task which assesses visuospatial working memory, has previously demonstrated a trend for better performance following blueberry consumption relative to placebo (Whyte et al., 2018). Serial Subtractions Task which measures working memory have also shown sensitivity following an anthocyanin-rich blueberry and grape intervention (Phillip et al. 2019). Lastly, effects following blueberry consumption relative to placebo have been found for the Task Switching Test, which assesses executive function (Miller et al., 2017; Whyte et al., 2019). The Task Switching Test is the most cognitively demanding task in the battery, as it requires the participant to remember the instructions for two different tasks at the same time, whilst continuously switching between doing the two tasks.

As well as these cognitive measures, mood was measured via the Positive and Negative Affect Schedule (PANAS) questionnaire which was administered before and after the cognitive battery. The reason for adding a mood questionnaire pre and post task in the studies featured in this thesis is to compare how mood changes during testing, which may help us understand whether any negative or positive mood effects are related to the cognitive tasks rather than the intervention. Randomized controlled trials with blueberry have shown significant

increases in positive mood following blueberry consumption. For instance, Khalid et al., (2017) found significant increase in positive mood scores after acute consumption in children aged 7-10 and young adults aged 18-21. More recently, Fisk et al. (2020) found significant decrease in low mood symptoms after chronic WBB consumptions in a group of adolescents aged 12–17-years-old.

The total duration of the cognitive battery was calculated to last an average of 45 minutes, this was to increase cognitive fatigue. The tasks were also assessed for practicality in terms of cognitive measurement during cerebral blood flow assessment, and laptop compatibility for optimum task performance, for example, using a touch-screen mode instead of a computer mouse.

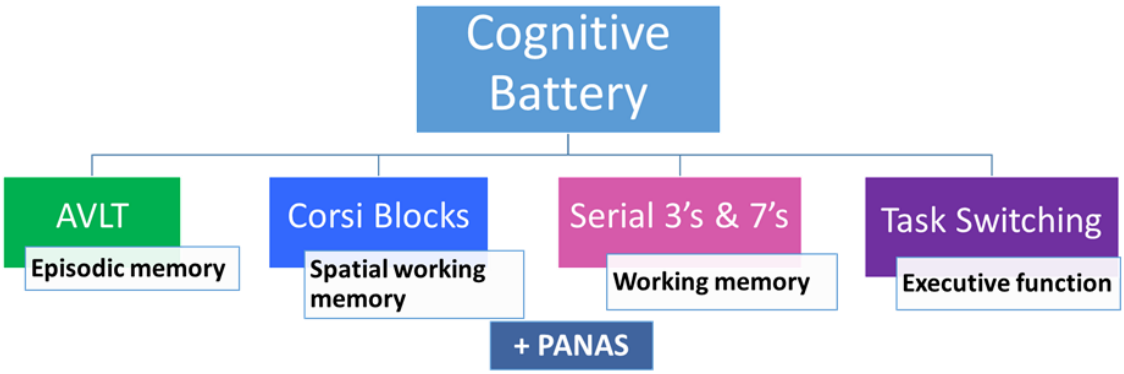


Figure 3. 1 Cognitive tasks and their dependent variables

3.4.5 Practice Effects

Repeated testing is often required when assessing the effectiveness of nutritional products. As a result, this could lead to practise effects. Practise effects refer to the improvement in performance in cognitive tasks following repeat testing, regardless of intervention consumption (Bartels et al., 2010). Naturally, the more times a participant conducts the task, the better at the task they become. This may lead to false representation of nutritional products being invested (Bell et al., 2018). To avoid practice effects, there were sufficient number of matched versions of all the tasks, which were presented in a randomised, counter-balanced order on each test day. Furthermore, each version was matched for level of difficulty. In addition to this the participants were required to complete a familiarization session whereby they completed a single fixed version of the tasks to ensure that they fully understood the tasks and instructions prior to the intervention testing.

3.4.6 Vascular Measures

Although not a feature of the data described in my thesis, a number of vascular measures were also taken (described in detail in Wood (2022) thesis). Flow-mediated dilation (FMD) has been widely used in RCTs looking at the effects of polyphenols on cognitive health. It is a quick, non-invasive procedure that can be used to detect changes in endothelial function in humans across the lifespan. Office blood pressure is a convenient and practical measure of peripheral blood pressure, although it is subject to variability based on the participant's state of mind (stressed or nervous participants may not give a correct indication of blood pressure). Therefore, in addition to office blood pressure measures, a 24hr blood

pressure (ABPM) device was also used to assess the blood pressure of participants across a 24hr period for the BluFlow study only.

3.4.7 Cerebral Blood Flow (CBF)

Cerebral blood flow parameters were assessed due to interest in whether there could be any correlation between cognition and cardiovascular health, and whether any observed changes in cognition is improved, due to improvements in cardiovascular health, and whether that is mediated by increases in cerebral blood flow. In both studies, a transcranial doppler ultrasound (TCD) was used for the measurement of cerebral blood flow parameters in adults and children. This technique uses non-imaging ultrasound and is a non-direct measurement of CBF, as it assesses blood flow velocity in the middle cerebral artery (MCA). Currently, transcranial Doppler ultrasound is used clinically to assess blood flow velocities in children with sickle cell disease (Verlhac, 2011) and also to diagnose cerebral vasospasm in adults, as well as assessing risk factors for vascular disorders including ischemic stroke (Purkayastha & Sorond, 2012).

The basic principles of TCD are based on the 'Doppler Effect'. Figure 3.2 outlines the mechanism of the Doppler Effect during TCD measurements.

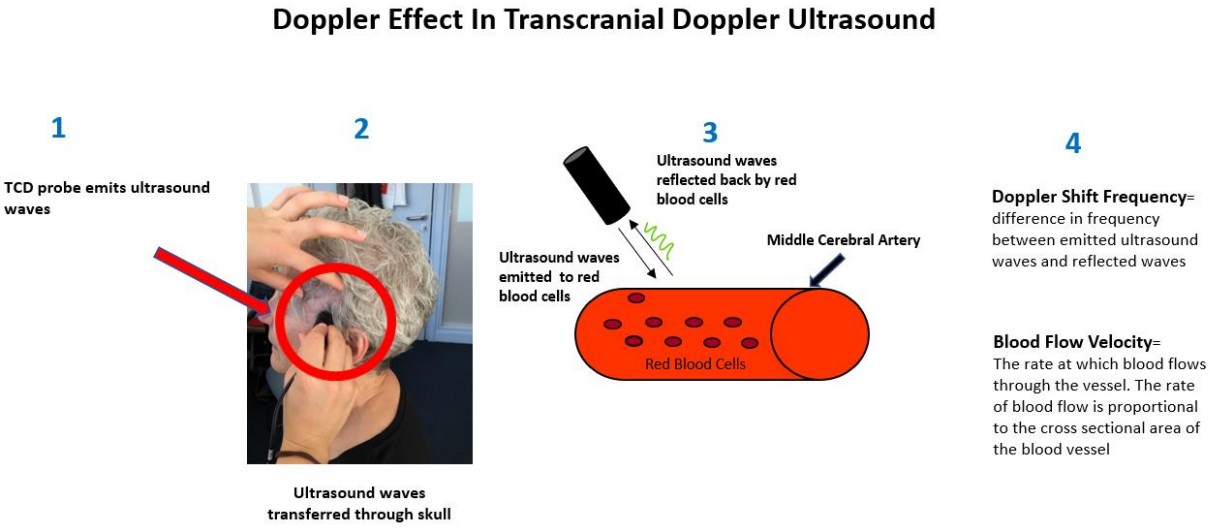


Figure 3. 2 Figure to show the basic principle of TCD sonography. Information sourced from D'Andrea et al. (2016) and Purkayastha & Sorond (2012)

TCD has also been used in previous study involving cocoa (Massee et al. 2015; Marsh et al., 2017 Sorond et al. 2008) and resveratrol (Evans et al., 2017) but with mixed results. To my knowledge, no studies as yet have investigated the effects of an anthocyanin-rich wild blueberry intervention on cerebral blood flow velocity using TCD. Therefore, TCD will be used in the BluLife Study and BluFlow Study to understand further whether TCD offers a good measurement of representing changes in blood flow mediated by the consumption of flavonoids. Taking into consideration the study population which includes human subjects of all age groups (8-80 years old), TCD was chosen as it offers many practical advantages. TCD is a safe, non-invasive, relatively inexpensive, provides rapid real-time results, and is portable, making it the most convenient choice for observing the effect of a nutritional intervention. A series of training sessions on measurement techniques and analysis were provided by a clinical vascular scientist at King's College Hospital, London. It was decided that resting state and active state of

cerebral blood measurement were to be taken, to understand how CBF changes when the participant is engaged in a cognitive activity versus when the participant is resting. The active state was measured during the practise trial of the task-switching test (TST). This was chosen because the TST is the most cognitively demanding task in the battery involving increased activity in the prefrontal cortex and the parietal cortex (Loose et al., 2017). In addition to this, the TST practise trial duration was sufficient for 10 TCD readings to be taken, compared to other practise trials in other tasks which were shorter in length. A practise trial was necessary in order to ensure the headband and probe was fully set-up and in place prior to assessment during the assessed task.

3.4.8 Low Flavonoid Diet

Participants in the BluLife study were required to follow a low-polyphenol diet in the 24 hrs prior to each study day; this is standard procedures practised by our laboratory both at University of Reading and King's College London. A guide sheet (Appendix A) was given to each participant highlighting examples of low polyphenol foods that were allowed, as well as the types of food which were prohibited. In addition, examples of meal plan ideas were given to participants verbally. In the BluFlow study, the participants did not follow a low polyphenol diet 24hrs before study days. This is because I was interested in observing the effects of treatment over and beyond the participants' habitual diet. In both studies, participants were required to attend the laboratory on each test day after having an overnight fast. They were provided with a standardised low-polyphenol breakfast upon arrival consisting of two slices of white bread, toasted, with a thin

layer of Philadelphia low fat cream cheese or vegan alternative. A small pot (125g) of Danone Activia 0% Fat Vanilla Yoghurt and a glass of water were also provided. Full product descriptions can be found in Appendix B. Moreover, each participant in both studies were asked to complete a 24hr dietary recall, to check for compliancy in the BluLife Study, and to identify whether any high polyphenol foods were consumed the night before for the BluFlow Study.

3.4.9 Urine and Plasma Metabolites level

Various studies have conducted the assessment of polyphenols in urine and plasma to understand the relationship between polyphenol bioavailability and cognitive (Barfoot et al., 2021) and vascular outcomes (Rodriguez-Mateos et al. 2013; Rodriguez-Mateos et al. 2019). Assessing metabolites pre and post consumption may also give us an indication if, and if so which, metabolites peak at the time of observed improvement to cognition and/or vascular health. Therefore, in order to further understand the absorption and bioavailability of polyphenols in humans, collections of 24hr urine samples were taken in all participants and plasma samples were taken from adults only in both studies, for polyphenol metabolite assessment following WBB versus placebo consumption.

3.4.10 Data Analysis and Choice of Statistical Technique

Data collected during screening and practice sessions were discarded and not analysed, only data from formal test days was analysed. A linear mixed model (LMM) approach was used for analysis of all cognitive and vascular outcomes to ensure consistency in approach. LMM was chosen as it allows for the control of multiple factors including fixed factors such as baseline performance in a cognitive

task, or treatment, and random factors such as participant ID to control for individual differences. Another advantage of using a LMM approach is that it does not require balanced data which means that missing samples can be included, thereby increasing the power of the analysis. Treatment was included as fixed factors in the model, whilst baseline performance was included as a covariate. The covariance structure can be selected, including unstructured matrix, which is applied to assumed unpredictable data or the ARH1 matrix which would be applied to assumed heterogeneous data or if there is a lack of degrees of freedom for example. In terms of reporting the LMM results, the significance ($p \leq 0.05$) or non-significance ($p \geq 0.051$) of the dependent variable, which is treatment in this case, is reported. Post-hoc analysis using a Bonferroni correction was applied to all pairwise comparisons where there were significant treatment-related effects ($p<0.05$). Bonferroni was deemed the best method as it may reduce type 1 error and therefore reduce the chances of observing a false positive outcome.

Chapter Four

Materials and Methodology

4.1 Ethical Approval & Training

Ethical approval was received in accordance with King's College of London Research Ethics Committee guidelines. Training and certification was received for the principles of Good Clinical Practice (GCP) (National Institute for Health Research), Human Tissue Act (HTA), gaining informed consent from participants, level 2 food safety (Chartered Institute of Environmental Health) and Emergency First Aid at Work. In addition to this, training in venepuncture was completed in preparation for phlebotomy.

4.2 Recruitment

Recruitment was conducted using a variety of methods. Both the BluLife and BluFlow study was advertised via newspapers (London Metro, and Evening Standard) and a family-orientated magazine (*Families*, SouthWest London). As well as this, schools and universities were contacted with posters/leaflets displayed in a variety of university facilities, public libraries (Waterloo, Lambeth, Chelsea, Victoria) community centres (Waterloo, Lambeth) and supermarkets. Finally, participants were also recruited via direct email correspondence with individuals or social organisations/groups (University of the Third Age (U3A). Social media platforms (Twitter, Facebook) were also used to publicise the study. Examples of adverts can be seen in appendix C.

4.3 Case Report Form (CRF)

A case report form (CRF) was administered to each participant for data recording at each study visit including the screening session which documented the variables that were collected at each test session. Each CRF was labelled with a participant ID in order for anonymity to be retained in line with good clinical practice (GCP) and ethical principles. These forms were carefully dated and recorded by a researcher involved in the study. Demographic information was recorded and stored securely online, whilst the paper-based CRFs were stored in a locked office. The CRF can be seen in appendix D.

4.4 Informed Consent

Prior to attending the screening session, an email containing a participant information sheet was sent to each prospective participant. If still happy to take part, and if participants fulfilled the initial study requirements, they were asked to give written consent by signing and dating both the information sheet and consent form. The researcher also signed each form, and a copy of the signed documents were given to the participants for their records. The participant information sheet can be seen in appendix E and consent form can be seen in appendix E.

4.5 Screening

Participants were invited for a screening session. At screening the participants were informed fully about what the study entails, they were asked questions about their health and medical history via the screening CRF and their blood pressure was measured (see section 4.8.2 for full description of blood pressure

assessment). General inclusion criteria for the studies in this thesis included healthy, non-smoking, willing to maintain their normal eating/drinking habits and exercise habits to avoid changes in body weight over the duration of the study. Certain conditions such as cardiovascular disease, diabetes, hypertension and mild cognitive impairment resulted in exclusion from the study. Anthropometric information was collected including height, measured in centimetres using a stadiometer before BMI was measured using a body composition analyzer (TANITA body composition analyser, Type BC-418 MA) which also assessed weight (see section 4.14 for full description). In addition to this, participants were asked to practise the cognitive battery twice, each time using different version of the tasks which had been matched for level of difficulty. This practise enabled the participants to become familiar with the cognitive tasks and to reduce incidence of practise effects during the test days themselves.

4.6 Cognitive Battery

With the assistance of a post-doctoral researcher, a cognitive battery was developed to include tasks assessing a range of cognitive domains including episodic memory, visuo-spatial memory, working memory, and executive functioning. The purpose of this was to fulfil the research gaps outlined in chapter 3, which was to comprehensively understand whether WBB polyphenols had any effects on a range of different cognitive domains, and whether WBB had any effects on the cognitive functioning and vascular health across the life-course following acute consumption and on healthy older adults following chronic consumption.

4.6.1 Cognitive Battery Duration and Equipment

Total duration of the cognitive battery was approximately 45 minutes. All tasks were programmed and delivered via E-Prime 2.0 or 3.0. Participants conducted the tasks on a laptop with a touch screen adaptation for use during the Corsi Blocks task. Laptop models were Toshiba Portege X20W-D-0Q and Toshiba Portege X20W-E-13J both with a screen size of 12.5 inches and screen resolution of 1920 x 1080. Participants were asked to wear in-ear headphones throughout the cognitive battery, in order to be presented with the audio stimuli during AVLT, as well as to minimise noise and distractions from external sources.

4.6.2 Cognitive Task Description

4.6.2.1 Rey's Auditory Verbal Learning Task (RAVLT)

The first task implemented in the battery was the RAVLT, a word-list learning task where the words were presented auditorily. The RAVLT version used in this study is an adaptation of the version by Lezak et al. (1982) and has been widely used in our laboratory. For each matched version of the task there were two different word lists each containing 15 words (list A and List B). The participant listened to list A for a total of 5 times and was given 1 minute per trial to recall as many words from the list as they could remember. Words were presented at a rate of 1 second per minute. In total the participant was recalling list A for a number of 5 times (R1-R5). Subsequently, the participant heard the 15 words from list B (also referred to as the interference list) and was given 1 minute to recall words from this list only (R6). On completion of their recall of the interference list, the participant was then

asked to recall words from list A only without any reminders of the word list beforehand (R7). After a delay of approximately 30 minutes, during which time the remaining cognitive tasks were completed, participants again verbally recalled List A (R8). Finally, the participants performed a visual recognition task whereby participants were presented with 50 list A words, which appeared on the computer screen one by one. The list also contained 15 list B foils and 20 previously unseen words. The aim, therefore, of the visual recognition task was for the participant to identify list A words only by pressing the correct corresponding keys (B which had a sticker with the letter 'Y' on to indicate 'yes', and the N key for 'no') on the laptop keyboard. Different word lists, matched for concreteness and familiarity, were used at each test session (Whyte et al. 2016). A number of different outcome variables were measured from this task: performance on R1 indicates immediate memory, the total number of words (total acquisition) correctly recalled from R1-R5 gives a measure of total word acquisition, proactive interference which is calculated from subtracting performance on R1 minus performance on R6 which corresponds to the list B trial, retroactive interference calculated from subtracting performance on R5 from performance on R7. For both proactive and retroactive interference, the lower the score is, the least interference has been experienced. Performance on R8 gives an indication of delayed memory and finally delayed recognition, as measured by the performance on the recognition task, calculated according to the number of correctly identified list A words. A full list of words included for each task version can be seen in appendix G.

4.6.2.2 Corsi Blocks Tapping Task

An electronic version of the Corsi-blocks tapping task was included in the battery, used before by Whyte et al. (2018). This task measures short-term visuo-spatial working memory (Kessels et al., 2000; Berch et al., 1998; Busch et al., 2005) and involves remembering the sequence of a series of blocks lighting up on the screen. Each block is highlighted for a total of 1000ms and the sequences are randomized to vary between 2-9 blocks in length. Once the full sequence is completed, the participant is prompted by the word 'go' on the screen to recall the sequence by pressing the corresponding blocks on the touch-screen (Figure 4.1). The participant was immediately given feedback on their performance whereby if they tapped the sequence correctly a 'Correct! Well done' appeared and if they tapped the sequence incorrectly a 'Sorry-you got it wrong' message appeared. Dependent variables measured in this task included the number of sequences correctly recalled and the number of blocks correctly tapped. The task consisted of a total of 32 sequences presented to the participants in a random order. The laptop used in both studies had a touch-screen design meaning the participant were able to directly tap on the screen.

Figure 4. 1 Example of Corsi Blocks scenario. Blocks are highlighted one at a time

4.6.2.3 Serial 3s and 7s

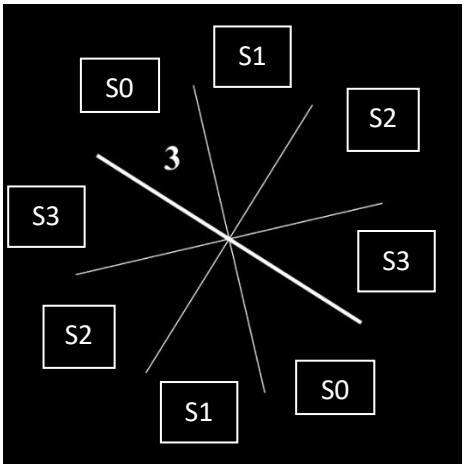
This task assesses working memory and has been modified from a previous version (Scholey et al., 2001; Scholey et al., 2010; Scholey et al., 2013). It involved participants subtracting the number '3' from a randomly generated starting number between 800 and 999, and then repeatedly subtracting '3' from the new number they have generated. For example, if the starting number was '850', the participant would be expected to correctly type '847', followed by '844' and subsequently '841'. Participants were asked to type their responses using the number pad on the laptop keyboard. The task lasted for a duration of 2 minutes and the participants were required to type as many answers as possible. The responses were calculated based on the previous number, therefore if the participants had inputted an incorrect response previously this does not affect their score. To help them familiarise with the process, the participant completed a 20 second practise trial prior to the 2-minute test. The dependent variable was

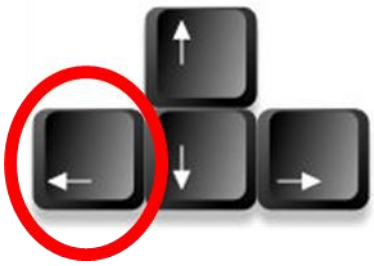
accuracy, calculated from the total number of correct responses given during the two-minute trial. The same process is repeated during serials 7s, which required the participant to subtract 7 from a stating number for a total of 2 minutes.

4.6.2.4 Task Switching Test (TST)

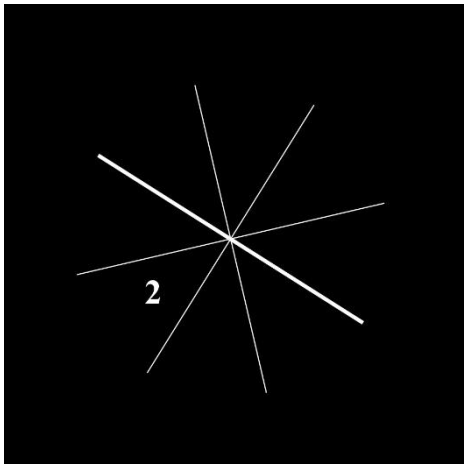
This task assesses executive functioning and is based on a version utilised by Miller et al. (2018). Here, participants must change between conducting two different tasks with different sets of instructions. A display of a circle divided into 8 segments was shown (Figure 4.2). These segments were divided by a bold line, leaving 4 segments at the top and 4 segments at the bottom. A number between 1-9 appeared sequentially in each segment in a clockwise direction. The numbers appeared for a total of 3000ms or until the participant made a response. When the number appeared in a segment above the bold line, the participant must press either the left or the right arrow key depending on whether the number is odd or even. In this case, the correct response would be the left arrow key for odd numbers and right arrow key for even numbers (Figure 4.2). When the number appeared in a segment below the bold line the participant had to press either the left or right arrow key depending on whether the number is higher or lower than the number 5. In this case, the number 5 did not appear as a randomly generated number, only numbers 1-4 or 6-9 appeared. The correct response is the left arrow key for numbers higher than 5 and the right arrow key for numbers lower than 5. Task type refers to either the high/low paradigm or the odd/even paradigm. In terms of switch trials, S0 represented the first trial per task type, which is followed by S1, S2 and S3. In total, there were 4 trials per task type. The task-switching

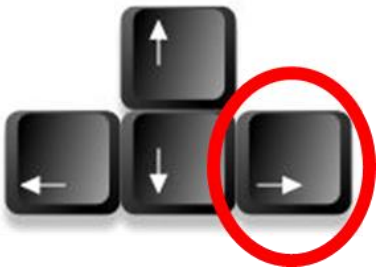
paradigm therefore assessed how the performance of task is affected when the participants are required to switch between these two different sets of instructions. Dependent variables included accuracy, calculated by the mean number of correct responses overall, the mean number of correct responses per task type, and switch trial accuracy which looked at the accuracy scores when participants switched to a new task (switch 0 and corresponding switches of the same task S1, S2 and S3). In addition to this, overall reaction time, and time taken (ms) for the participant to respond to the first trial per task type (ms) were also observed.





Number above bold line is an odd number. Correct response is to press the left arrow key





Number below bold line lower than 5 number. Correct response is to press the right arrow key

Figure 4. 2 Example of switching task scenario and description of correct responses

4.6.3 Mood Assessment

4.6.3.1 Positive and Negative Affect Schedule (PANAS)

PANAS-Now (Watson et al. 1988) assessed the mood of participants and aimed to capture the current state of mood. The PANAS involved participants responding to 20 mood adjectives presented sequentially (see appendix H for full list of mood adjectives), participants simply marked on a 5-point scale to indicate how much of that adjective they were feeling at that moment. Ten adjectives referred to positive words such as 'interested' and 'proud'. Ten adjectives referred to negative words such as 'upset' and 'nervous'. A rating of '1' conveys the feeling of 'not at all' and a rating of '5' refers to 'extremely. The positive affect score was calculated by adding scores corresponding to the 10 positive adjectives (out of 5 per word) to form an overall positive affect score (out of 50). Similarly, negative affect score was calculated by adding the scores corresponding to the negative adjectives (out of 5 per word) to form an overall positive affect score (out of 50).

As mentioned in chapter 3, a mood questionnaire was assessed pre- and post- task in the studies featured in this thesis. This is to compare how mood changes during testing, which may help understand whether any negative or positive mood effects are related to the cognitive tasks rather than the intervention. Figure 4.3 below outlines the order of tasks.

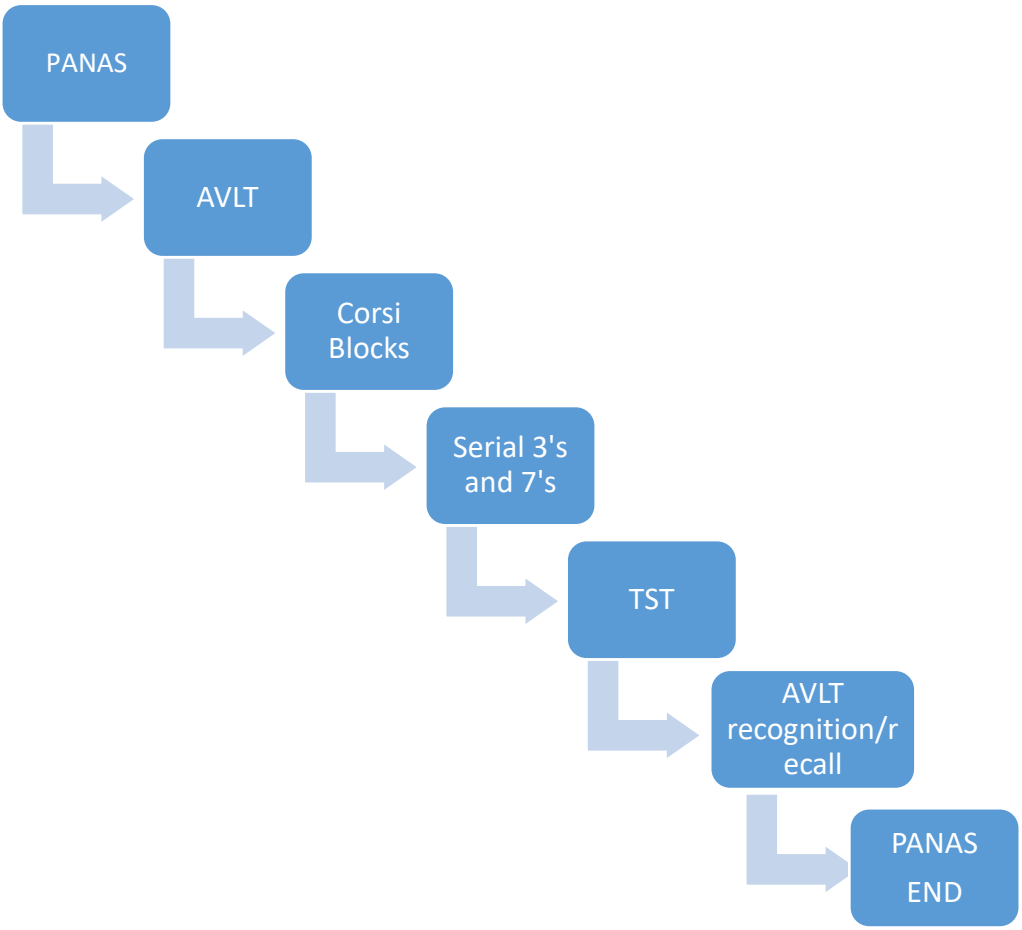


Figure 4. 3 Flowchart demonstrating the order in which the cognitive tasks were conducted

4.7 Counterbalancing

A total of 6 versions of the battery matched for difficulty were developed, two versions for the screening/practise sessions, two for the baseline test sessions on each study day, and two for the post-intervention timepoint on each study day. These versions were presented to each participant in a randomised format.

4.8 Vascular Measures

Vascular measures were assessed to understand how WBB affected vascular function. The information reported here is given for context only and are not the primary focus of this thesis. The vascular results are presented more comprehensively in a separate thesis (Wood, 2022).

4.8.1 Flow Mediated Dilation (FMD)

The assessment of the effects of WBB on FMD was conducted, using a non-invasive procedure, which looks at the elasticity of the brachial artery after hyperaemia is induced. It uses high-resolution Doppler imaging ultrasound to observe a pulsatile flow, indicated by red colouring and venous flow indicated by blue colouring. The procedure begins by placing ECG patches on the volunteers using the 3-electrode system RA (right arm), LA (left arm), LL (left leg). This was followed by the application of ultrasound gel on the inside of the inner upper arm. Next, a sphygmomanometric cuff was placed on the forearm just below the elbow. Before pressure was applied, the brachial artery was imaged in order for the blood flow and diameter of the artery to be measured before stress was applied. Imaging was conducted using a 12MHz transducer (Vivid I, GE Healthcare, Buckinghamshire, UK). Next, shear stress was applied to the brachial artery, to induce temporary hyperaemia, by inflation of the sphygmomanometric cuff. The cuff remained inflated at 180mmHg for 5 minutes. After the 5 minutes, the cuff was deflated and images were captured at the following time points; at 20, 40, 60 and 80-seconds, to assess dilation of the endothelium post deflation. After the procedure, the images were saved to be analysed by a trained assessor using an automatic edge-

detection software (Brachial Analyser, Medical Imaging Applications, Iowa City, USA). The outcome variable here is the change in brachial artery diameter (post deflation) relative to pre-deflation.

4.8.2 Peripheral Office Blood Pressure

The participants were asked to rest in a supine position with their head supported by a pillow for a duration of 10 minutes in order for their blood pressure and heart rate to stabilise. Systolic blood pressure, diastolic blood pressure, and heart rate were recorded using an automated digital sphygmomanometer (Omron M6, Kyoto, Japan). Before the procedure participants were asked whether they needed to empty their bladder and to remain silent and still with legs uncrossed during measurement to avoid variability caused by movement. Each reading was conducted with the cuff placed on the upper right arm at heart level, with the arm resting on the bed adjacent to their body, this was the same for each session in order for consistency to be maintained. In addition to this, the room temperature was closely monitored and recorded in order to observe whether atmospheric conditions and variations in seasons and temperatures may affect blood pressure, as previously recorded by literature (Modesti, 2013). Three readings were taken spaced a minute apart and an average was calculated using the final two readings for statistical analysis, which formed the outcome variable.

4.9 Cerebral Blood Flow (CBF)

A transcranial doppler ultrasound device was used to assess cerebral blood flow following treatment consumption. The brand of the TCD non-imaging ultrasound device was EZ-Dop (DWL, ScanMed Medical Instruments, Germany). The procedure involved an ultrasound transducer which was placed on the trans-temporal window together with a small amount of ultrasound gel. Blood flow was measured via the middle cerebral artery, which forms part of the main internal carotid arteries and is characterised by distinct peaks (Figure 4.4). CBF measurements were taken from the right middle cerebral artery (MCA) on the right side of the head. The probe was held in place over the trans-temporal window by TCD headband (DiaMon® Smart Medical, Germany), for a comfortable hands-free measurement, allowing for consistent recording, free of any movement (Figure 4.5). Resting and active blood flow measurements were collected at baseline and post-intervention. More specifically, for the BluLife study measurements took place at baseline and 2 hours post consumption, and for BluFlow this was at baseline and at 3 months following daily treatment consumption. For resting blood flow, a total of 10 recordings whilst the participant was in a seated position either on a bed at 120 ° angle or on a chair. They were asked to remain silent and rest with their legs uncrossed. For active blood CBF, measurements were taken whilst the participant conducted the practise trials for the TST. Outcome variables in this case included (i) mean Blood Flow Velocity (BFV) (cm/s) which is the rate of speed at which the blood flows through the artery, any changes in BFV is a direct representation of changes occurring in CBF, and (ii) Pulsatility Index of the MCA which is a measure of resistance in pulsatile flow calculated via (vmax-vmin)/ v mean. The

temperature of the testing room was closely monitored and recorded for any fluctuations in temperature, as this is known to affect blood flow (Mrozek et al., 2012).

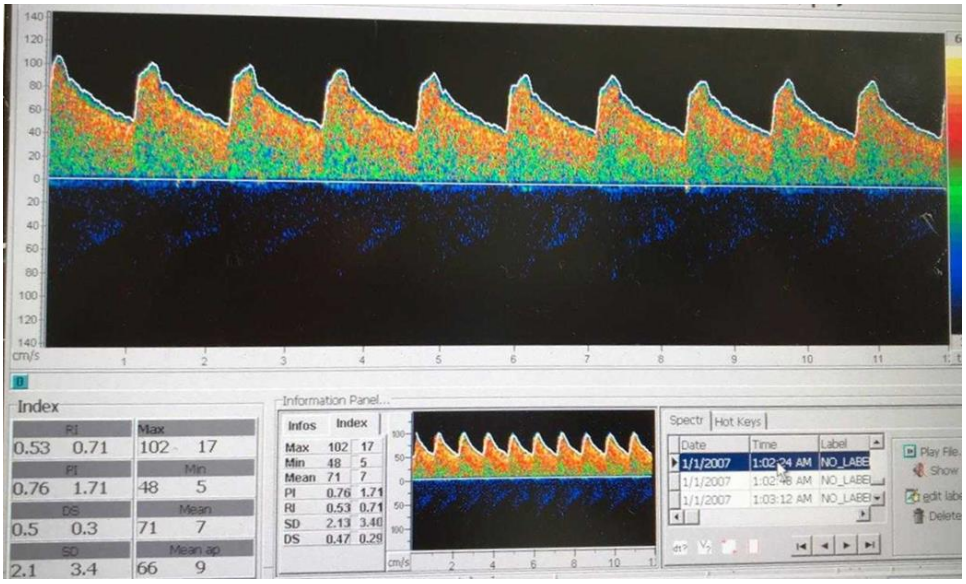


Figure 4. 4 Spectral image demonstrating expected waveform of the middle cerebral artery

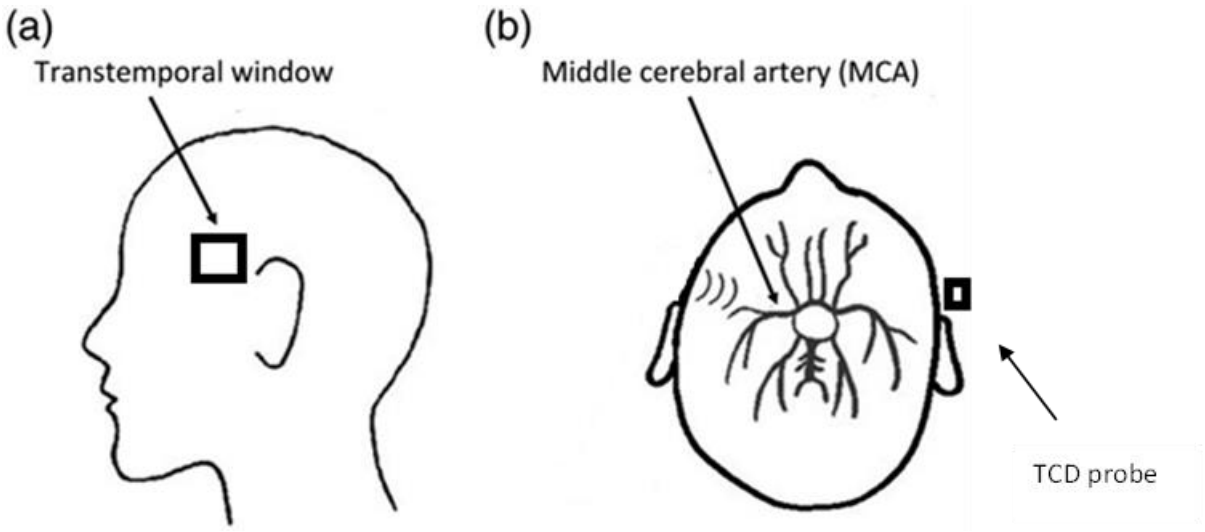


Figure 4. 5 (A) The location of the transtemporal window on the left side of a human subject. (B) A transcranial Doppler (TCD) probe placed against the trans-temporal window on the right-hand side. Image adapted from Fantini et al. (2016)

4.10 Phlebotomy

Blood samples were collected in all subjects aged 18 years or older, via venepuncture using collection needles (21g Green BD Vacutainer Safety Lok). Six different tubes were used for blood collection (Table 4.1) in order to analyse a range of plasma biomarkers: total blood lipids, HDL cholesterol, LDL cholesterol, triglycerides, blood glucose level, and markers of liver and kidney function (full list can be seen in appendix I). Additionally, blood samples were analysed for polyphenol content in order to estimate the bioavailability of polyphenols in the body after blueberry consumption. One EDTA K3 (3ml) blood tube was sent without processing to an external laboratory where they were analysed for full blood count at Affinity Biomarker labs (White City Campus, London).

Table 4. 1 Plasma Biomarkers And Vacutainers Used For Collection Of Blood Samples

Vacutainer	Colour Top	Quantity (ml)	Outcome
EDTA K3	Purple	3	Full blood count
Serum separator	Yellow	8.5	Liver Function and blood Lipids
Fluoride oxalate	Grey	3	Blood glucose
Heparin	Green	6	Plasma polyphenols
EDTA	Purple	10	Plasma polyphenols
Cell preparation tubes (CPT)	Blue	8	Nutrigenomic analyses- Peripheral blood monocytes (PBMC).

4.11.2 Methodology for Processing of Blood Samples

One EDTA (10 mL) was centrifuged for 10 minutes, set at a temperature of 4°C and speed setting at 1800 rcf. On completion a volume of 600 µl of the centrifuged sample was pipetted into 4 Eppendorf's pre-prepared with 12 µl of formic acid 2%, (Fisher Scientific, Loughborough, UK), and also into 3 empty aliquots. These were then stored in a -80°C freezer. The smaller EDTA vacutainer (4ml) was not prepared in our lab and instead was sent to a central lab (Affinity Biomarker labs, (White City Campus, London) for analysis. Preparation of heparin vacutainers involved pipetting 1ml of the plasma sample and then centrifuging for 10 minutes, also at a setting of 4°C and 1800 rcf. A volume of 600 µl were pipetted in to 4 aliquots, 2 of them containing 12 µl of formic acid, and samples stored in a -80°C freezer. The serum vacutainer was centrifuged for 10 minutes at room temperature, and at 1800 rcf. Once centrifuged, 1ml was centrifuged into 2 aliquots, each, and one of the aliquots was also sent to the central lab for analysis. The other aliquot was stored in an -80°C freezer. The fluoride oxalate vacutainer was centrifuged at room temperature for 10 minutes, also at a setting of 1800 rcf. Identical to the serum vacutainer, 1ml was centrifuged into 2 aliquots, each, with one of the aliquots sent to the central lab for analysis. Plasma samples for glass cell preparation tubes (CPT) were also collected in order to isolate peripheral blood monocytes for the purpose of nutrigenomic analysis. However analysis and results of this will not be reported in this thesis. In order to isolate the peripheral blood monocytes, several steps were conducted. Firstly, the CPT were carefully inverted 8 times, and then centrifuged at 1500 rcf, for 30 minutes at room

temperature. Then, the top half of the plasma was discarded, and the white cell layer collected and transferred to a 50ml tube. Phosphate-buffered saline (PBS) solution was added until the total volume was 15ml. This tube was then inverted 5 times in order to mix the cells. A secondary round of centrifuging was conducted for 15 minutes at 1400 rpm at room temperature. After this, the supernatant was carefully discarded, leaving only the cell pellet. 1 ml of PBS was added, lightly mixed on a vortexer, and the mix was transferred to an Eppendorf for a third round of centrifuging, for 15 minutes at 2000 rpm at room temperature. Finally, the supernatant was aspirated leaving the cell pellet, which was stored in a -80°C freezer.

4.11.3 Urine Collection

Participants were asked to collect 24hr urine samples to look at urinary polyphenol metabolites after blueberry and placebo consumption. Participants were given a 3-litre urine collection container. To ensure preservation of samples, the containers were prepared with 3g of L-ascorbic acid 99% (Sigma-Aldrich) before each study visit. The containers were issued to the participant in an insulated cooler bag, along with an ice pack to ensure the samples were kept cold. When the samples were left at home with the ice packs, the participants were instructed to store it in a cool environment away from any heat sources such as radiators.

4.11.4 Processing of Urine Samples

Once the urine containers were returned, a falcon tube was used to collect 15ml of urine and centrifuged for 15 minutes at 1500 rcf and at a temperature setting

of 4°C. The supernatant was then pipetted (1200 µl) and 24 µl formic acid was added into three separate aliquots, and 1500 µl with no formic acid was pipetted into two aliquots. The samples were the stored into a -80°C freezer until further analysis.

4.12 Polyphenol Analysis

The process of analysis of polyphenol metabolites in the plasma and urine samples followed a method validated by colleagues Domínguez-Fernández & Xu et al. (2021). The validation method used a total of 110 standards for urine and 119 standards for plasma.

4.12.1 Quality Check samples

This process was conducted by colleague Yifan Xu. In brief, three different types of quality checks were completed for the sequence run and this included QC solvent, QC pool-spike and QC pool-non spike. For QC solvent, a HPLC vial filled with mobile phase A (1.5 mL) was used. QC pool comprises of the pooled plasma samples and urine sample of all participants (10 µl per sample). Subsequently, the QC pooled samples underwent Sample Preparation and Microelution Solid-Phase Extraction (µ-SPE). Lastly, the QC plasma and urine samples were spiked for use as reference samples and inserted into vials for multiple injections.

4.12.2 Sample Preparation and Microelution Solid-Phase Extraction (µ-SPE)

Firstly, the urine and plasma samples were removed out of the -80°C freezer and placed on ice until visibly defrosted. The centrifuge was then turned on and set to

a temperature of 4°C.Once thawed, the urine samples were diluted with HPLC-grade water (1:5 ratio) and centrifuged for a total of 15 minutes at 15000g rcf. Samples were then pipetted into microcentrifuge Eppendorf tubes containing 353µL of phosphoric acid (4%) and mixed on a vortexer. A 96-well plate was then prepared with 35µl HPLC water, 5µl isotope standard mix in preparation for solid phase extraction. 600 µL of each sample were loaded into the 96-well plate and 5 µl of taxifolin were pipetted into the each well. After this, 200 µL of HPLC water was used to wash the wells, and subsequently 200 µL of acetic acid (0.2%) was also used for washing the well. Once washed, 30 µL of elution solution was added to each sample-containing well, and pressure was applied using semi-automated positive pressure (Positive Pressure 96 Processor, Waters, Eschborn, Germany). This was repeated a further 2 times, each time with 30 µL of elution solution being added. An adhesive seal was added immediately upon completion to minimise the amount of evaporation and mixed on a plate mixer for 30 seconds.

4.12.3 Ultra-High-Performance Liquid Chromatography-Mass Spectrometry (UPLC-MS)

The polyphenol metabolites in the urine and plasma samples were detected using UPLC-MS, on a Shimadzu triple quadrupole (LCMS8060, Shimadzu, Kyoto, Japan). The column used in this experiment was a Raptor Biphenly, 2.1 x 50 mm, 1.8 µm (Restek, 9309252) with compatible Raptor Biphenyl Guard Cartridges 5 X 2.1 mm. UPLC-MS machine configuration was performed by my colleague Yifan Xu (standard operating procedure can be seen in appendix K). Data analysis and processing was conducted by myself and colleague Eleanor Wood. Quantification

of polyphenolic metabolites was conducted using the Lab Solutions Insight (Shimadzu, Kyoto, Japan) using standard curves. Target peaks were detected using a reference retention time. The concentrations of each metabolite were calculated via integration, which is the action of measuring and calculating the area under the signal curve. The signal curve is then normalized with a default method from the software. Once integrated, values were calibrated in an excel workbook using pre-set formulas developed by colleague Yifan Xu.

4.12.4 HPLC-DAD Analysis of Anthocyanins in Treatment Powders

The analysis of anthocyanin content in the treatment powder was conducted by colleague Zhicheng Zhang using high performance liquid chromatography with diode-array detection (HPLC-DAD). In brief, two grams of each powder was combined with 50ml of acidified methanol (0.1 % HCL in MeOH). This was then vortexed, sonicated and centrifuged to form a supernatant. Following this, a concentrator (Eppendorf 5301, Eppendorf UK ltd, Stevenage, UK) was used to evaporate the supernatant samples to dry at room temperature. The concentrated sample was then mixed and dissolved with 20% acidified methanol (0.1 % HCL in MeOH). The identification process used anthocyanin standards. Before the samples were transferred to the HPLC vials, they were filtered through 0.22 µm filters. The HPLC-DAD analysis followed similar method described by Pertuzatti et al. (2016). An Agilent 1100 series HPLC system (Agilent Technologies, Cheshire, UK) equipped with a diode array detector and a Poroshell 120 EC-C18 column (100 × 2.1 mm, 2.7 µm particle size; Agilent Technologies, Cheshire, UK)

was used to separate the anthocyanin metabolites. Here, the temperature was set at 40 °C and the injection volume was set at 5 µL.

The mobile phase A and B were acidified water (1% formic acid, v/v) and acidified acetonitrile (1% formic acid, v/v). The reported gradients were: 0–5 min, 5% B; 5–35 min, 5–17% B; 35–50 min, 17–27% B; 50–60 min, 27–90% B; 60–65 min, 90% B; 65-70 min, 90-5%B; 70-80 min, 5% B, with a flow rate of 0.2 mL/min. The eluate was monitored at 520 nm for all samples. Authentic standards were used to obtain the calibration curves.

4.13 Health & Lifestyle Questionnaires

Food Frequency Questionnaires (FFQ) and food diaries were administered to each participant in order to assess their overall habitual nutrient and energy intake, whilst physical activity was measured using a physical activity questionnaire. These questionnaires were administered to all participants at baseline. Analysis and findings of the health & lifestyle questionnaire data including the FFQ data and IPAQ data was beyond the scope of this thesis and not included in this thesis. The findings are discussed in Wood (2022) and will be featured in publications related to this study.

4.13.1 The EPIC-Norfolk Food Frequency Questionnaire

Participants completed the EPIC-Norfolk FFQ (version 6-CAMB/PQ/6/1205) to monitor their habitual intake of different food items and allow calculation of their nutrient and energy intakes to be assessed. This FFQ consisted of two parts. Part 1, which comprised of 130 food items ranging from fruit and vegetables,

meat, dairy, confectionaries, cereals and non-alcoholic and alcoholic drinks. The participants indicate using a tick (✓) how often they consumed that particular food item, on average, in the past year. There are 6 responses categories, which ranged from 'Never/less than a month' to '6+ per day'. Part 2 consisted of questions relating to salt intake, brand of cereals consumed, type and quantity of milk consumed, cooking methods and supplements. A copy of the full EPIC-Norfolk FFQ can be seen in appendix L.

4.13.1.1 Analysis of EPIC FFQ

Analysis of FFQ's were conducted by research students and comprised of two stages. The first stage involves transcribing results from paper copies an online data entry template downloaded from https://www.epic-norfolk.org.uk/for-researchers/ffq/. For this the installation of Microsoft Access was required. Once the software is installed, data entry consists of recording responses from the questionnaires into the database using drop down options or by clicking on options directly. This was then exported as a CSV file for the next step to be used with the FFQ EPIC Tool for Analysis (FETA). FETA allows the conversion of the FFQ data into specific data such as nutrient, or food groups using pivot tables. Polyphenol intake through diet was calculated using Phenol-Explorer and calculated according to food group or compound group.

4.13.2 Physical Activity Questionnaire

The International Physical Activity Questionnaire (IPAQ) (October 2002) was administered at baseline to each participant in both studies to determine how active participants were on average over a 7-day timeframe. The IPAQ takes into consideration not just exercise but also commuting habits (walk, cycle car etc) and other physically demanding activities such as housework and gardening. A copy of the IPAQ can be seen in Appendix M.

4.13.2.2 Analysis of IPAQ

The questionnaire responses were tallied up into a composite score referred to as a metabolic equivalent task (MET) score to indicate which category of physical activity the participant belongs to. The MET score is calculated by adding the total minutes of physical activity conducted per week. Categories include 1 (low and inactive), 2 (moderately active), 3 (highly active). Table 4.2 outlines the scoring system.

Table 4. 2 Categories of 'High', 'Moderate' 'Low' Levels of physical activities and the minimum requirements needed to achieve these

High	**Moderate**	**Low**
At least 3 days of vigorous exercise a week to equal a minimum score 1500 MET a week	A minimum of 3 days of vigorous exercise, at least 20 minutes each time	If the number of days of exercise, or the MET score is not sufficient enough to reach the 'Moderate' or 'High' category.
At least 5 days of exercise (any type), a score of a minimum of 3000 MET a week	A minimum of 5 days of moderate exercise, at least 30 minutes each time	
	A minimum of 5 days of any exercise type, a score of 600 MET a week	

4.13.3 Twenty-Four Hour Dietary Recalls

At the start of each study visit, each participant was given a log sheet to record everything they had consumed (food and drink) in the previous 24hrs. This includes recording information such as serving size, food item, brand and time of consumption. An example of the recall sheet is given in appendix N.

4.14 Body Anthropometrics

A TANITA body composition analyzer (Type BC-418 MA) was used to calculate overall body weight, body mass index (BMI), fat mass (kg), fat free mass, basal metabolic rate. This was measured for each participant at their screening session. In addition, in the BluLife study this was conducted at the screening session and at the end of the 3-month intervention period (pre and post intervention). The outcome variable of interest was BMI.

4.15 Data Analysis

4.15.1 Outlier Procedures

For the cognitive data, the outlier removal process involved conducting a Z-score analysis of all data points per participant for each task. Any data points which had a corresponding Z-scores identified as being equal to or greater than 3.29 was removed. For TST reaction time, all data points under 200 ms were automatically excluded from the dataset as they were perceived as outliers.

4.15.2 Statistical Analysis

Results (except where stated) were analysed using the Statistical Package for Social Sciences (SPSS) (Version 27.0, IBM, UK). Cognition, mood, CBF, FMD, blood pressure and metabolites data were analysed using linear mixed modelling (LMM). A linear model was performed for every dependent variable of each cognitive task:

1) AVLT: Total acquisition, immediate recall, proactive interference, retroactive interference, delayed recall, word recognition
2) Corsi Blocks: correct sequence, correct blocks
3) Serial subtractions: Serial 3s accuracy, serial 7s accuracy
4) TST: Accuracy, RT
5) Mood: (PANAS positive score pre-tasks, positive score post tasks, negative score pre-tasks, negative score post tasks).

A linear mixed model was also performed for every dependent variable of the vascular health outcomes (flow-mediated dilation, blood pressure) and cerebral blood flow measures (blood flow velocity, pulsatility index). Lastly, a linear mixed model was performed for each polyphenol metabolites in plasma, and urine.

In order to model repeated measures, an unstructured covariance matrix was implemented and baseline performance was included as a covariate for all analyses.

For TST analysis; switch trial (1,2,3,4,5) and task type (high/low, odd/even), as well as interactions with treatment (Treatment x Switch trial, Treatment x Task Type, Treatment x Switch Trial x Accuracy) were also included in the model as fixed factors to understand how cognitive load differs between different treatment

groups. For all significant treatment-related effects on each outcome variable, post-hoc pairwise comparisons using a Bonferroni correction to correct type 1 error, was applied (p<0.05).

Chapter Five:

The BluLife Study- A Randomised, Double-blind, Placebo controlled, Crossover Trial Investigating the Acute Effects of Wild Blueberry (poly)phenols on Vascular Function and Cognitive Performance in Healthy Individuals Across the Life-Course

Experimental design was formulated by myself, Eleanor Wood and our supervisory team of Ana Rodriguez-Mateos and Claire Williams. Cognitive testing setup and analysis was conducted by myself, with some aspects of cognitive analysis (AVLT scoring) conducted by project students. FMD procedure and analysis was conducted by Eleanor Wood and is reported in detail in (Wood, 2022). TCD procedure and analysis, phlebotomy and all biochemical analyses of urinary and plasma metabolites was conducted jointly by myself and Eleanor Wood.

5.1 Introduction

As discussed in Chapter 1, the importance of nutrition for human health is paramount. Having a healthy balanced diet throughout the life-course may help prevent chronic diseases such as cardiovascular disease, and a nutritious diet through life may also decrease the incidence and slow down progression of dementia. The importance of a healthy diet from early years onwards is vital. Not only does balanced nutrition affect children's growth and physical development, but it may also have an effect on their cognitive development which will ultimately have an impact on their learning in educational settings (Norris et al, 2022). In addition to this, studies suggest that childhood nutrition may have a long-term effect on their health as adult (Haas, 2007). Recent research investigating the effects of early nutrition predicted that a healthy childhood diet may result in up to 25% reduction in the probability of adult obesity (Wang et al., 2021). Studies have also suggested that foods consumed by

young children during early years may affect their eating behaviour as an adult (Birch et al., 2007), which suggests that having a healthy diet as a child may lead to a healthy diet in adulthood.

Moreover, observational studies have demonstrated that malnutrition in infancy may negatively affect intelligence quotient (IQ) in adulthood (Waber et al., 2013). Despite this, a recent report by the Health Survey for England 2019 suggested that the 5 a day recommendation was met by only 18% of children aged 5-15 and 28% of adults (Health Survey for England, 2019). Nevertheless, recent campaigns surrounding healthy eating is being taken more seriously, especially those regarding children's eating (Sustain, 2021; Fare Share, 2021) and obesity in adults (Change4Life, 2021; Food Active, 2021). The driving force behind the encouragement of correct nutrition is being driven by the ever-growing research demonstrating the importance of foods for health and wellbeing, especially within educational settings. As covered in chapter 1, clinical trials are starting to demonstrate that foods high in polyphenols may have the ability to improve attention, focus, and mood, all of which are important in a classroom setting, but also within the professional workplace environment.

As discussed previously in chapter 2, studies have demonstrated that anthocyanin-rich blueberries have the potential to offer an array of cognitive benefits to humans of different ages. Studies investigating the effects of WBB in young children aged 7-10 found positive benefits for delayed recall (Whyte and Williams, 2015; Whyte, Schafer and Williams 2016), episodic memory (total acquisition) (Barfoot et al., 2018) executive functioning (Whyte, Schafer and Williams 2016; Barfoot et al., 2021), and reaction time (Whyte et al. 2020). One study investigating the cognitive effects of WBB in middle-aged adults found maintenance of

performance during an executive functioning task, whereas performance significantly reduced for placebo group (Whyte et al., 2021).

Studies investigating the effects of WBB in healthy older adults found positive benefits for episodic memory (word recognition) (Whyte et al., 2018) and executive functioning (Miller et al., 2017). No studies to date have considered the effects of WBB in teenagers and young adults, nor have the impact of WBB in all age groups been compared in a single study.

This gap in research led to the development of this first study; the BluLife study, a life-course study designed to measure the acute effects of WBB polyphenols in 5 different age groups which were chosen to reflect a span of different ages and developmental stages in life. The BluLife study was designed to not only assess age groups representative of the life course, but also to test a range of tasks that measure a range of cognitive domains, to see if different ages respond to WBB supplementation differently in terms of cognitive domains affected.

As outlined in Chapter 2, the potential mechanisms of action underlying changes in cognitive function following WBB consumption are still being investigated. One potential mechanism of action is through the improvements in vascular health, as it is hypothesised that improvement in arterial health could lead to improvements in blood flow to the brain, and as a consequence, better cognitive function. To better understand the mechanism behind WBB polyphenol's actions, measures of cardiovascular health including flow mediated dilation (FMD), and blood pressure (BP) will be assessed to observe whether any correlations with improved cognitive performance exists. To date, several studies have found improvements in FMD after polyphenol supplementation in healthy young adults (Rodriguez-Mateos et al., 2013) and in adults with metabolic disorders (Curtis et al., 2019). An increasing number of studies are being conducted looking at polyphenols' effects on vascular health (Wood et al.,

2019). Promising effects have thus far been shown for flow mediated dilation, with 9 studies showing improvements in endothelial function post WBB intervention, both acutely and chronically (Wood et al., 2019).

For blood pressure, results have been mixed, with only a few studies showing significant reduction in blood pressure after intervention with blueberry polyphenols. After conducting a parallel RCT investigating the effects of blueberry polyphenols in a group of middle-aged adults suffering from metabolic disorders, Basu et al. (2010) found significant reduction of BP after 8 weeks of intervention for the blueberry group compared to the placebo group. Similarly, Johnson et al. (2015) also found positive effects of blueberry polyphenols on blood pressure after daily consumption of a freeze-dried blueberry powder for 8 weeks, in a group of pre-menopausal women who were pre-hypertensive or diagnosed with stage 1 hypertension. However, it is worth noting that both these studies were conducted in adults suffering from metabolic disorders, and to this day studies which exhibit positive effects for blood pressure following WBB in healthy adults is limited. To my knowledge, only one study has shown a reduction in systolic 24hr Ambulatory Blood Pressure Monitoring (ABPM) in a group of healthy young males after daily blueberry consumption for 4 weeks (Rodriguez-Mateos et al., 2019). This highlights the need for further investigation into the effects of WBB consumption on blood pressure in healthy humans.

As documented in chapter 2, two studies to date have shown increased cerebral blood flow following chronic WBB supplementation (Bowtell et al., 2017; Boespflug et al., 2018). In the BluLife study, cerebral blood flow measures will be taken in order to understand how CBF differs across the life-course and to also observe whether any correlations exist between changes in cognitive function and cardiovascular health following WBB consumption. To my

knowledge, the BluLife Study will be the first study to investigate the acute effects of WBB polyphenols on cerebral blood flow using TCD across the life course, and also the first study to look at the effects WBB intervention on FMD in children and teenagers. The vascular results are presented more comprehensively in a separate thesis (Wood, 2022); however, the results found for flow-mediated dilation and blood pressure will be briefly outlined here to help elucidate potential mechanisms of action behind any cognitive improvements observed in the BluLife study.

It is believed that well-being correlates with cognitive functioning (Khalid et al., 2016; Bierman et al., 2005) and depressive symptoms are associated with a reduction in executive functioning (Khalid et al., 2017; Gohier et al., 2009). Various epidemiological studies have suggested that long-term intake of fruit and vegetables is associated with improved psychological wellbeing and reduced incidence of depression (Chang et al., 2016; Mihrshahi et al., 2015; Godos et al., 2018). As discussed in chapter 3, two randomized control intervention trials with WBB have shown significant increases mood following acute (Khalid et al., 2017) and chronic WBB consumption (Fisk et al., 2020). However further research is required to understand whether acute WBB consumption may have an effect on mood in middle-aged adults and older adults.

Lastly, a handful of studies have looked at the levels of polyphenolic compounds following berry consumption but only one study has investigated the bioavailability of WBB polyphenols in children (Barfoot et al., 2021). In order to further understand the absorption and bioavailability of polyphenols in humans, collections of 24hr urine samples will be taken in all participants and plasma samples will be taken from adults for polyphenol metabolites assessment following WBB versus placebo consumption.

5.1.1 Research Aims:

1. To test the effects of wild blueberry (WBB) polyphenols on cognitive function, assessed using a cognitive battery consisting of 4 different tasks assessing different cognitive domains including the AVLT, Corsi blocks tapping task, Serial 3s and 7s and the Task-Switching Test.
2. To determine the acute effect of blueberry (poly)phenols on cerebral blood flow across the life course
3. To measure the acute effects of blueberry (poly)phenols vs placebo on mood across different age groups
4. To determine the acute effects of blueberry (poly)phenols vs placebo on flow-mediated dilation (FMD) and office blood pressure across the life course. This will be presented in a separate thesis (Wood, 2022)
5. To assess the acute effects of blueberry (poly)phenols vs placebo on plasma and urinary polyphenols across the life course

5.2 Methods and Materials

Methodological procedures specific to this intervention study are reported in this chapter, all other methodological detail was previously described in Chapter 4.

5.2.1 Ethics

This study was granted ethical approval by the BDM Research Ethics Subcommittee at King's College London. Study Reference: HR-18/19-9091.

5.2.2 Clinical Trials Study Registration

This study was registered on clinical trials.gov and allocated the following Clinical Trials.gov ID: NCT03592966.

5.2.3 Study Design

A crossover, placebo controlled RCT was conducted. Participants who expressed an interest in the study were invited for a screening and cognitive familiarisation session. The cognitive and physiological data collected during this visit were taken purely for determining whether the participant was eligible to take part, and therefore this data was not included in subsequent analyses. If eligible and if participants were still happy to take part, they were invited to take part in the study 2-7 days after screening. In total, four visits were scheduled: visit 1, urine drop-off 1, visit 2 and urine drop-off 2 (24hr before Study Visit 1 and Study Visit 2) (Figure 5.1). Randomisation was conducted using Research Randomizer https://www.randomizer.org/.

The BluLife Study

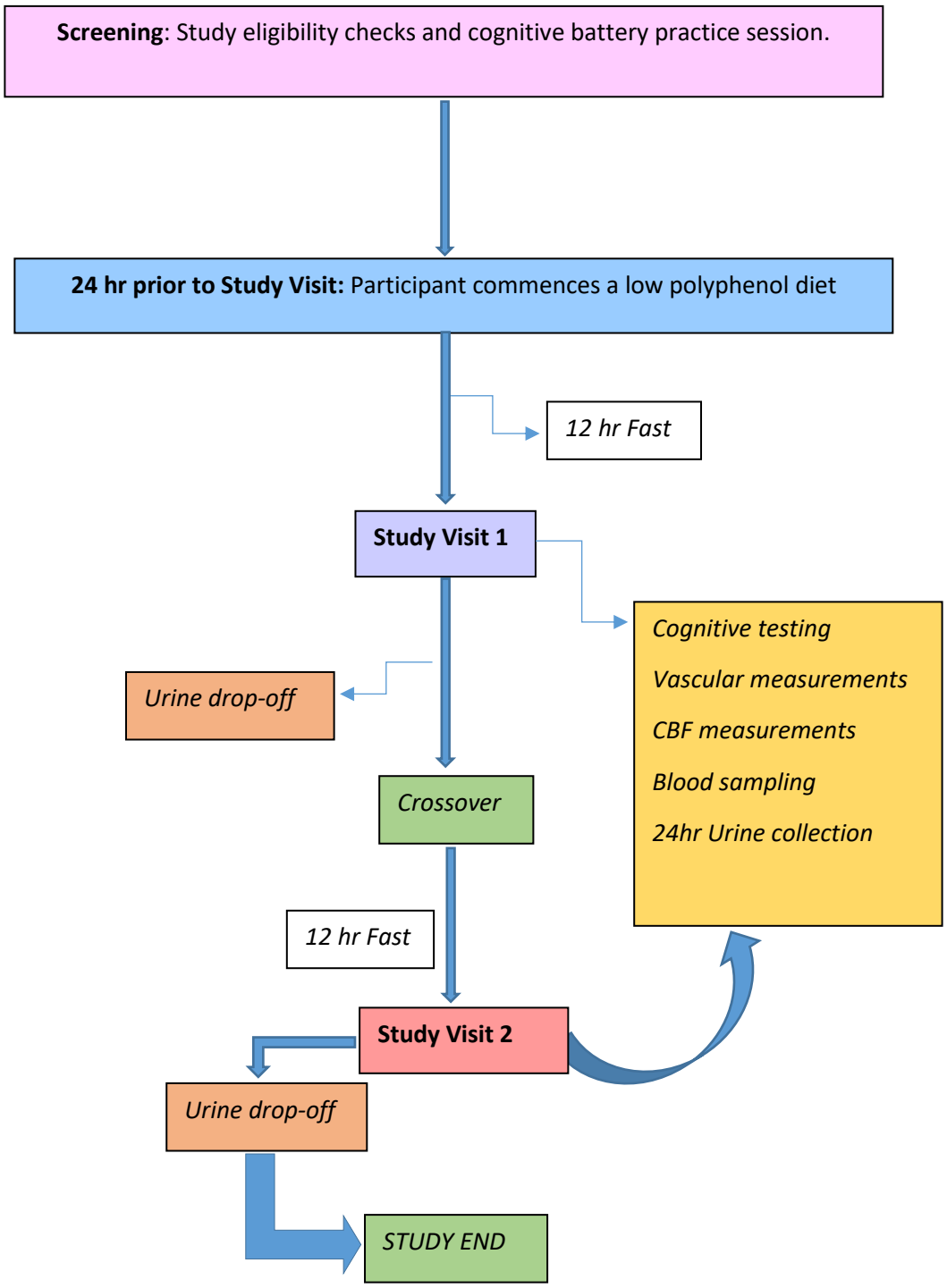


Figure 5. 1 Flow diagram demonstrating the study process and order of study days

5.2.4 Study Population

The study aimed to recruit 20 participants in the following age groups: 8-10, 14-18, 22-28, 40-50, 65-80, totalling 100 participants. The sample size was based on power calculations from both cognitive function and FMD outcomes from previous studies. The number of participants required to yield a power of 0.95 was 20 per intervention. This was based on a previous study by Whyte et al. (2017) who conducted a crossover RCT looking at the effects of WBB on the cognitive function in children aged between 7-9 and with an observed medium effect size of d= 0.44. This number agreed with the required number for FMD where it was calculated that for a crossover study with 2 intervention arms which had a power of 0.8 and significance of 0.05, 20 subjects were required in order for a difference of 0.6% to be detected following acute WBB and placebo supplementation (Rodriguez-Mateos et al., 2013).

Participants recruited were healthy human subjects from five different age categories. The age categories consisted of ages between 8-10, 14-18, 22-28, 40-50 and 65-80 years old. These age categories were chosen, as it was believed that they adequately represented different developmental stages of the life course. Additionally, as previously mentioned there have been a handful of studies looking at the cognitive effects of blueberry in children aged between 8-10, and adults aged between 65-80, however, no studies have investigated the effects of blueberries on 14-18, 22-28, 40-50 correct at the time of protocol development in September 2017. Therefore, these age groups were included in order to identify the impact of WBB across the full life-course. The overall study population was 96 with following numbers per age group: 8-10 (n=12), 14-18 (n=20), 22-28 (n=23), 40-50 (n=21), 65-80 (n=20). Due to COVID-19 pandemic lockdown, the full 20 was not achieved for the 8-10 year olds. The

extra number of participants recruited into the 22-28 (n=3) and 40-50 (n=1) age groups were to take into account incomplete study days due to factors such as drop-outs, disrupted study days e.g., fire alarms and incompletion of cognitive tasks due to technological issues. The participants were screened according to the inclusion and exclusion criteria outlined in chapter 4, section 4.5.

5.2.5 Treatments

The freeze-dried whole-fruit blueberry powder was manufactured by the Wild Blueberry Association of North America (WBANA), Maine, USA. The placebo powder was manufactured by North Carolina State University, Raleigh, NC 27695, USA. The serving of the WBB powder was 13.2g, therefore each treatment drink was calculated to contain 133.05 mg of anthocyanins per serving, equivalent to 119 g of fresh weight blueberries. The anthocyanin composition of the WBB treatment powder was analysed in our lab by Zhicheng Zhang as per the protocol outlined in chapter 4 section 4.12.4. In total, the WBB powder was composed of 37.82% malvidins, 29.02% delphinidins, 18.38% petunidins, 12.18% cyanidins and 2.80% peonidins (Figure 5.2).

The placebo powder was formulated to contain the same macro- and micronutrients, but with no polyphenols. Ingredients in the placebo included 'Flavor, FD&C red 40, FD & C blue 1, FD&C blue 2, fructose, glucose, citric acid (vitamin C), cellulose (plant fibre), soluble fibre, pectin, silicon dioxide' (developed by North Carolina State University, USA). Table 5.1 outlines the main ingredients present in the WBB and placebo powders.

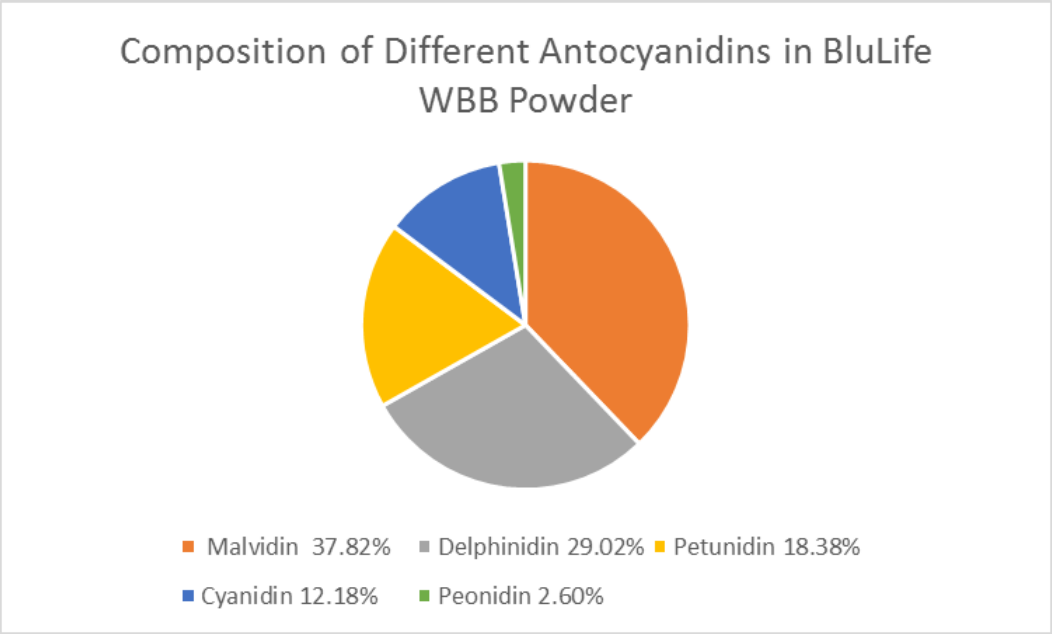


Figure 5. 2 The overall proportion of the anthocyanidins in the BluLife WBB powder

Table 5. 1 BluLife intervention and placebo powder composition

Ingredients	**Freeze-dried Wild Blueberry Powder (13.2 g)**	**Placebo (13.2 g)**
Anthocyanins (mg)	133.2	0
Glucose (g)	4.49	4.29
Fructose (g)	4.75	4.69
Fibre (g)	2.11	2.5
Vitamin C (mg)	44.2	45.0
Citric acid (g)	*	0.13
Silica (g)	0	0.13
Flavour (g)	0	0.10
Red colouring (%)	0	0.02
Blue colouring 1 (%)	0	0.003
Blue colouring 2 (%)	0	0.01

*not available

5.2.5.1 Treatment Blinding

The powders were shipped to the principal investigator and once received by the researchers at the clinical site the blueberry powders were weighed and placed into identical opaque sealed sachets with numbering to distinguish between the treatments. An independent party not involved with the research study blind-coded the blueberry intervention and placebo drink. This was blinded to any researchers who have contact with participants in order to ensure double-blinding throughout the study procedure until statistical analysis was complete.

5.2.5.2 Drink Administration

The blueberry intervention contained 13.2g of powder which was mixed with 250ml of water. Both drinks were served in an opaque, lidded bottle and opaque straw and refrigerated prior to consumption. Drinks were referred to as either A or B therefore the researchers and participants remained blind to the interventions. Both powders were stored in a -20°C freezer.

5.2.6 Test Procedure

As described in chapter 3, participants were required to follow a low polyphenol diet in the 24 hrs prior to each study day. A guide sheet was given to each participant highlighting examples of low polyphenol foods that were allowed, as well as the types of food which were prohibited. In addition, examples of meal plan ideas were given to participants verbally.

All testing was conducted at the Metabolic Research Unit, Department of Nutritional Sciences, King's College London. Participants were asked to attend the laboratories in an overnight fasted state and breakfast was served upon arrival. Breakfast was a standardised low-polyphenol breakfast consisting of two slices of white bread, toasted, with a thin layer of

Philadelphia low fat cream cheese or vegan alternative. A small pot (125g) of Danone Activia 0% Fat Vanilla Yoghurt and a glass of water were also provided.

Whilst participants waited to be served their breakfast, they were asked to fill out the 24hr diet recall which required participants to fill out everything they had to eat and drink in the 24hrs prior to their study visit. This was to measure compliance, ensuring that no polyphenol-rich foods were consumed the day before each study day. When participants completed their breakfast, they were taken to study room and asked to lie down for 10 minutes on a clinic chair in a supine position. This was to ensure they were fully relaxed prior to getting a blood pressure measurement. Three readings were taken, with the intention of discarding the first reading and taking a mean of the last two readings. After this, participants remained in the supine position and FMD measurement took place. Cerebral blood flow measures were then collected (resting CBF) and followed by participants commencing the cognitive battery. Around halfway through the battery, before the participant conducted the practise round of the Task Switching Test (TST), the participant was asked to alert the researcher by manually ringing a bell or pressing a call button. This was because a second reading of cerebral blood flow was taken whilst the participants was conducting the practise task (active CBF). Once finished, all participants over the age of 18 years had a blood sample taken. After this, the participant consumed either a blueberry or control drink within a 10-minute timeframe (Rodriguez-Mateos et al., 2013). After consumption of their allocated intervention, they were asked to wait in the research unit waiting room for 1.5 hrs during which they were allowed to watch tv, read or do their personal work. No food was allowed during the breaks but the participants were permitted to drink as much water as they desired. They were also reminded to collect any urine in the jug each time they visited the bathroom. During the break participants were given two food frequency questionnaires and a physical activity

questionnaire to complete. Once the break was over participants were asked to come back into the study room for their post-intervention measurements which were completed in the same order. Once the test session was fully completed, the participant was asked whether they think they received the WBB treatment or the placebo treatment and also whether they were still wanted to continue with study visit 2. Lastly, participants were reminded to continue collecting urine for the remainder of the day and to bring this back the following day. Study visit 2 was scheduled, following the exact same format as study visit 1 (see Figure 5.3 for order of measurements).

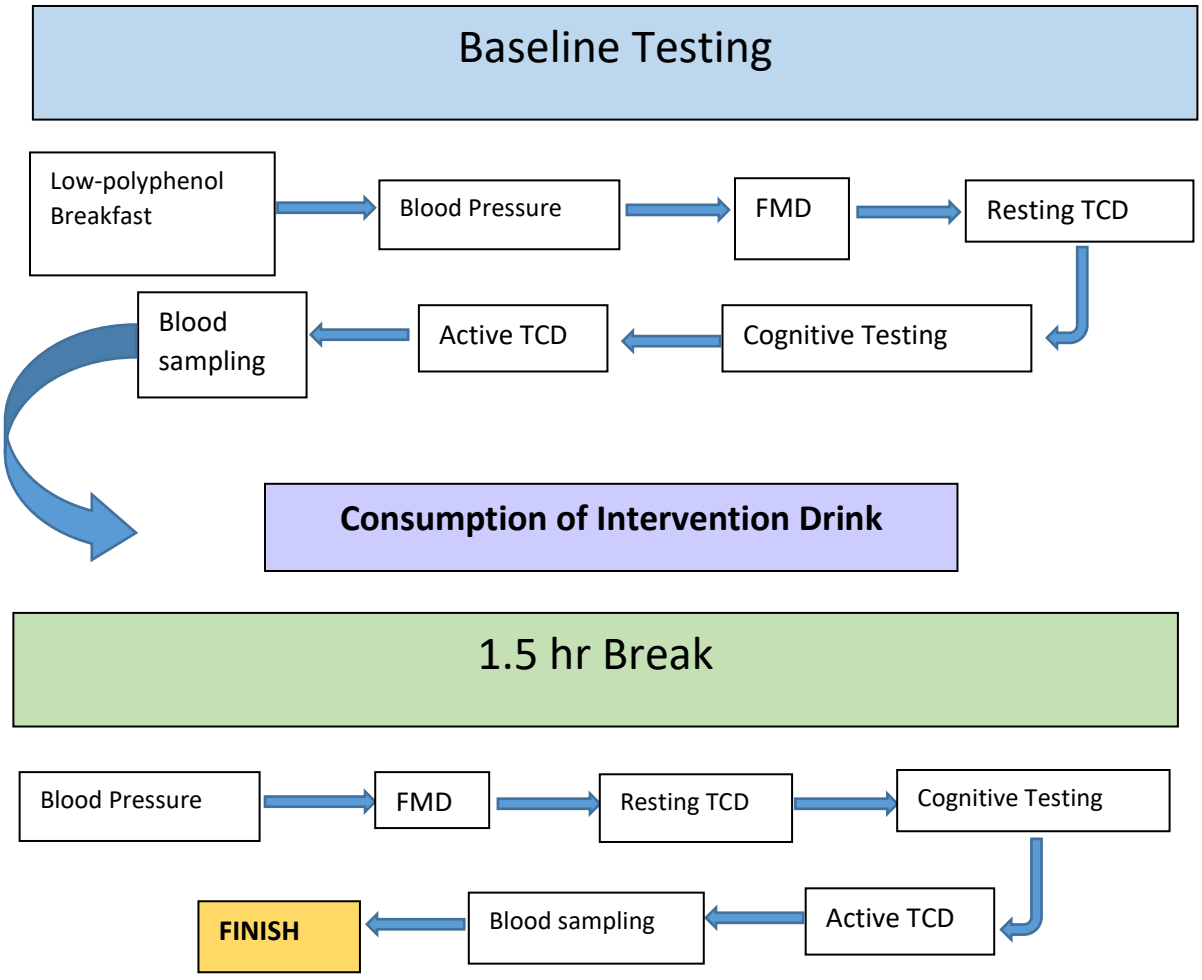


Figure 5. 3 Flow diagram to show the order of measurements at the study days

5.2.7 Statistical Analysis

Results (except where stated) were analysed using the Statistical Package for Social Sciences (SPSS) (Version 27.0, IBM, UK). Cognition, mood, CBF, FMD, blood pressure and metabolites data were analysed using linear mixed modelling (LMM). A linear model was performed for every dependent variable of each cognitive task:

1) AVLT: Total acquisition, immediate recall, proactive interference, retroactive interference, delayed recall, word recognition
2) Corsi Blocks: correct sequence, correct blocks
3) Serial subtractions: Serial 3s accuracy, serial 7s accuracy
4) TST: Accuracy, RT
5) Mood: PANAS positive score pre-tasks, positive score post tasks, negative score pre-tasks, negative score post tasks).

A linear mixed model was also performed for every dependent variable of the vascular health outcomes (flow-mediated dilation, blood pressure) and cerebral blood flow measures (blood flow velocity, pulsatility index). Lastly, a linear mixed model was performed for each polyphenol metabolites in plasma, and urine.

In order to model repeated measures, an unstructured covariance matrix was implemented and baseline performance was included as a covariate for all analyses. In addition to this, treatment (placebo, WBB), Age Group (8-10, 14-18, 22-28, 40-50, 65-80) and treatment x age group were included as Fixed Factors in LMMs to compare the effects of treatment across different age groups. For TST analysis, switch trial (1,2,3,4,5) and task type (high/low, odd/even), as well as interactions with treatment (Treatment x Switch trial, Treatment x Task Type, Treatment x Switch Trial x Accuracy) were also included in the model as fixed factors to

understand how cognitive load differs between different treatment groups. For all significant treatment-related effects on each outcome variable, post-hoc pairwise comparisons using a Bonferroni correction to correct type 1 error, was applied (p<0.05) and age-related post-hoc analysis was explored to understand how cognitive function differs across ages irrespective of treatment.

5.3 Results

5.3.1 Baseline Demographics

Table 5. 2 Baseline characteristics for both placebo and wild-blueberry treatment groups. Data are means (SD) or numbers (%)

	8-10 (n=12)	**14-18 (n=20)**	**22-28 (n=22)**	**40-50 (n=20)**	**65-80 (n=20)**
Gender (m/f)	8/4	9/11	11/11	10/10	9/11
Ethnic Origin					
White British	8%	10%	32%	15%	70%
White Other	25%	40%	27%	45%	5%
White Irish	0%	5%	5%	0%	0%
Black Caribbean	8%	0%	5%	10%	0%
Black African	8%	10%	0%	5%	0%
Mixed White & Black	17%	0%	5%	0%	0%
Chinese	8%	5%	18%	10%	15%
Pakistani	0%	5%	5%	5%	0%
Indian	0%	5%	0%	5%	0%
Asian	17%	5%	0%	5%	0%
Other mixed Background	8%	15%	5%	0%	10%
Body Composition					

BMI (kg/m^2)	16.90 (4.65)	20.87 (2.85)	22.91 (2.80)	24.61 (3.35)	23.21 (2.61)
Body Fat %	21.80 (7.12)	24.27 (7.35)	21.30 (7.93)	25.68 (9.61)	25.33 (6.52)

5.3.2 Cognitive Function Results

Table 5. 3 Pre-consumption and post-consumption scores per age group for placebo and WBB for AVLT, Corsi Blocks, Serials, and TST. LMM fixed effect statistics for treatment demonstrated significantly better performance following wild blueberry treatment for AVLT Total Acquisition and all three Corsi Blocks outcome variables. Significantly improved performance was observed following placebo for AVLT proactive interference

Dependent Variable	*Baseline Placebo Mean (SD)*	*Post-consumption Placebo Mean (SD)*	*Baseline WBB Mean (SD)*	*Post-consumption WBB Mean (SD)*	*LMM Treatment-related Statistics*
AVLT					
Total Acquisition					
8-10	37.00 (9.12)	29.50 (11.95)	32.45 (15.35)	31.64 (13.85)	p=0.042
14-18	46.05 (8.81)	45.95 (8.43)	45.37 (6.55)	45.78 (5.42)	p=0.823
22-28	56.05 (8.43)	56.05 (9.66)	55.55 (8.89)	53.55 (9.14)	P=0.223
40-50	45.00 (10.58)	42.53 (11.98)	48.67 (9.95)	48.00 (9.38)	p=0.123
65-80	44.16 (9.12)	40.26 (11.95)	43.65 (7.46)	44.94 (7.09)	p=0.016
Total Mean	**46.69 (1.11)**	**44.38 (1.34)**	**46.58 (1.27)**	**46.19 (1.20)**	**F(1,67.98)=6.29, p=0.015***
Immediate Recall					
8-10	4.33 (1.23)	4.40 (1.08)	5.00 (2.00)	4.90 (1.37)	p=0.993
14-18	6.00 (1.21)	5.95 (1.43)	5.30 (1.49)	6.47 (1.87)	p=0.075
22-28	7.04 (2.44)	7.78 (2.34)	7.39 (2.33)	7.13 (2.38)	p=0.063
40-50	5.32 (1.25)	5.39 (1.50)	5.80 (1.47)	6.07 (1.44)	p=0.279
65-80	5.63 (1.01)	5.30 (1.26)	5.74 (1.52)	5.84 (1.54)	p=0.189
Total Mean	**5.83 (1.77)**	**5.99 (1.98)**	**6.00 (1.98)**	**6.26 (1.93)**	***F*(1,80.76)=1.13, *p*=0.290**
Proactive Interference					
8-10	-1.17 (1.75)	-0.83 (1.99)	0.55 (2.51)	1.00 (2.10)	p=0.036
14-18	-0.35 (2.03)	0.10 (2.25)	-1.00 (2.32)	0.95 (1.51)	p=0.152
22-28	-0.78 (1.91)	0.13 (2.20)	-0.09 (2.35)	-0.96 (1.94)	p=0.051
40-50	-0.63 (1.77)	-0.44 (2.38)	-0.60 (1.77)	0.07 (1.53)	p=0.476
65-80	0.00 (1.56)	-0.90 (1.17)	-0.37 (2.81)	0.42 (1.68)	p=0.042
Total Mean	**-0.55 (1.82)**	**-0.33 (2.05)**	**-0.36 (2.38)**	**0.18 (1.87)**	***F*(1,94.32)=4.85, *p*=0.030***
Retroactive Interference					
8-10	2.33 (2.64)	1.25 (1.66)	1.30 (1.42)	2.27 (2.15)	p=0.269
14-18	2.55 (2.26)	2.25 (2.00)	2.90 (2.69)	2.00 (2.11)	p=0.656
22-28	1.26 (1.82)	1.74 (2.32)	1.78 (2.00)	2.00 (1.73)	p=0.705
40-50	2.67 (2.59)	3.44 (2.90)	3.33 (3.29)	3.40 (2.32)	p=0.742
65-80	2.95 (2.32)	3.60 (1.98)	3.26 (1.91)	4.32 (2.31)	p=0.511
Total Mean	**2.30 (2.33)**	**2.52 (2.36)**	**2.79 (2.53)**	**2.78 (2.27)**	***F*(1,76.46)=1.01, *p*=0.318**

Delayed Recall					
8-10	8.20 (2.70)	4.67 (3.74)	7.22 (3.67)	5.87 (2.80)	*p=0.404*
14-18	7.00 (4.38)	6.45 (4.10)	8.44 (4.29)	7.22 (4.18)	*p=0.676*
22-28	11.74 (2.94)	10.00 (3.73)	11.00 (3.33)	9.59 (3.79)	*p=0.926*
40-50	8.22 (3.00)	6.18 (3.97)	7.80 (3.82)	7.31 (3.07)	*p=0.223*
65-80	7.05 (3.52)	4.75 (3.18)	6.42 (2.69)	5.41 (2.76)	*p=0.465*
Total Mean	**8.60 (3.66)**	**6.75 (4.20)**	**8.44 (3.88)**	**7.37 (3.75)**	***F*(1,78.80)=2.18, *p*=0.144**
Word Recognition-List A (out of 15)					
8-10	10.42 (3.32)	8.17 (3.76)	8.08 (3.45)	7.83 (2.82)	*p=0.997*
14-18	12.40 (3.52)	11.05 (2.58)	12.58 (2.17)	11.35 (2.78)	*p=0.707*
22-28	13.30 (4.25)	13.96 (4.34)	13.24 (1.55)	13.05 (1.53)	*p=0.248*
40-50	12.10 (2.34)	11.20 (2.55)	11.72 (2.91)	11.76 (2.19)	*p=0.453*
65-80	12.55 (1.64)	12.00 (1.62)	12.42 (1.71)	12.42 (1.39)	*p=0.424*
Total Mean	**12.34 (3.22)**	**11.62 (3.50)**	**11.92 (2.79)**	**11.60 (2.65)**	***F*(1,58.91)=0.13, *p*=0.722**
Word Recognition-List B & distractor words (out of 35)					
8-10	31.00 (4.47)	30.33 (5.35)	25.50 (6.13)	27.00 (7.77)	*p=0.954*
14-18	34.85 (8.32)	32.95 (2.63)	33.21 (2.37)	32.95 (2.31)	*p=0.623*
22-28	35.26 (8.01)	35.91 (10.98)	33.33 (3.12)	32.82 (3.70)	*p=0.194*
40-50	32.75 (3.52)	30.65 (5.33)	32.50 (3.17)	32.24 (3.44)	*p=0.280*
65-80	29.45 (4.76)	29.05 (4.57)	31.05 (4.20)	28.63 (5.77)	*p=0.467*
Total Mean	**32.88 (6.61)**	**32.03 (7.03)**	**31.60 (4.49)**	**31.07 (5.10)**	***F*(1,48.18)=0.37, *p*=0.848**
Corsi Blocks					
Correct Sequence					
8-10	13.67 (5.26)	11.17 (5.70)	12.42 (5.27)	11.08 (5.62)	*p=0.885*
14-18	17.45 (4.89)	19.65 (7.32)	20.00 (6.76)	21.50 (6.02)	*p=0.670*
22-28	19.50 (7.55)	22.23 (7.09)	22.77 (5.94)	25.70 (6.11)	*p=0.155*
40-50	18.55 (7.61)	16.25 (7.45)	15.65 (5.98)	19.71 (5.57)	*p=0.021*
65-80	14.75 (5.60)	13.84 (5.55)	12.67 (5.82)	16.11 (6.66)	*p=0.160*
Total Mean	**17.11 (6.62)**	**17.25 (7.65)**	**17.35 (7.22)**	**19.48 (7.58)**	***F*(1,82.93)=6.15, *p*=0.015***
Correct Blocks					
8-10	24.17 (5.61)	19.58 (8.32)	20.25 (6.11)	18.75 (7.45)	*p=0.923*
14-18	24.68 (6.55)	26.00 (7.07)	26.40 (6.86)	28.44 (6.11)	*p=0.283*
22-28	27.05 (7.71)	28.26 (6.96)	26.91 (11.88)	29.33 (6.89)	*p=0.627*
40-50	26.90 (7.11)	23.55 (7.88)	23.76 (7.63)	29.18 (5.79)	*p=0.007*

65-80	24.45 (5.71)	22.68 (6.99)	21.33 (7.23)	24.53 (7.31)	*p=0.398*
Total Mean	**25.60 (6.67)**	**24.54 (7.74)**	**24.17 (8.71)**	**26.61 (7.51)**	***F*(1,89.43)=4.64, *p*=0.034***
Serials Subtraction Task					
Serial 3's Accuracy					
8-10	9.92 (3.78)	9.00 (4.35)	9.50 (6.08)	21.00 (28.14)	*P=0.003*
14-18	28.15 (7.86)	27.85 (9.48)	30.90 (13.30)	29.11 (10.58)	*p=0.570*
22-28	43.55 (14.03)	44.04 (13.28)	39.23 (14.93)	43.00 (12.65)	*p=0.569*
40-50	25.56 (9.88)	27.06 (11.60)	24.83 (12.11)	27.00 (12.05)	*p=0.567*
65-80	21.40 (10.75)	21.53 (11.17)	19.71 (8.64)	22.63 (9.29)	*p=0.682*
Total Mean	**27.48 (14.64)**	**28.40 (15.22)**	**26.71 (14.89)**	**30.03 (16.11)**	***F*(1,74.05)=0.351, *p*=0.555**
Serial 7's Accuracy					
8-10	5.33 (2.65)	5.10 (2.56)	5.60 (3.75)	6.00 (4.31)	*P=0.812*
14-18	14.95 (7.69)	16.10 (7.95)	16.00 (7.99)	17.37 (8.45)	*p=0.756*
22-28	25.14 (8.00)	26.57 (8.95)	23.55 (8.66)	26.57 (9.17)	*p=0.412*
40-50	17.05 (8.68)	16.21 (8.75)	15.72 (8.47)	17.29 (9.80)	*p=0.335*
65-80	14.78 (9.03)	15.79 (9.12)	13.68 (8.67)	14.24 (8.89)	*p=0.861*
Total Mean	**16.93 (9.66)**	**17.49 (10.25)**	**16.15 (9.52)**	**17.74 (10.59)**	***F*(1,88.75)=0.831, *p*=0.365**
TST					
Overall TST Accuracy					
8-10	0.64 (0.19)	0.68 (0.16)	0.59 (0.23)	0.59 (0.24)	*p<0.001*
14-18	0.87 (0.14)	0.87 (0.17)	0.88 (0.14)	0.90 (0.11)	*p<0.001*
22-28	0.95 (0.05)	0.95 (0.06)	0.95 (0.06)	0.95 (0.05)	*p=0.734*
40-50	0.94 (0.08)	0.95 (0.06)	0.94 (0.08)	0.94 (0.06)	*p=0.435*
65-80	0.95 (0.09)	0.93 (0.10)	0.95 (0.08)	0.95 (0.08)	*p=0.090*
Total Mean	**0.89 (0.15)**	**0.89 (0.14)**	**0.88 (0.17)**	**0.89 (0.01)**	***F*(1,758.64)=1.35, *p*=0.247**
Overall TST Reaction Time (ms)					
8-10	1115.91 (254.74)	1123.55 (235.68)	1070.71 (293.54)	1038.01 (252.08)	*p<0.001*
14-18	957.62 (256.93)	882.78 (237.97)	931.82 (255.40)	908.41 (270.82)	*p<0.001*
22-28	802.22 (177.99)	766.29 (163.70)	817.76 (173.71)	742.31 (154.94)	*p=0.734*
40-50	901.69 (286.61)	834.83 (256.87)	890.27 (321.65)	830.44 (261.10)	*p=0.435*
65-80	935.86 (266.10)	915.00 (265.20)	969.13 (288.47)	927.68 (266.72)	*p=0.090*
Total Mean	**923.48 (265.07)**	**880.46 (254.82)**	**923.06 (277.63)**	**873.22 (259.11)**	***(F(4,679)=6.56, p<0.001#***

#Significant at < 0.01, *Significant at < 0.05

5.3.1 Auditory- Verbal Learning Task (AVLT)

Figure 5.4 (A) shows a significant difference between treatment on mean total acquisition (F(1,67.98)=6.29, p=0.015). Cognitive benefits were observed following WBB consumption relative to placebo whereby WBB attenuated a decline in performance as seen in placebo. Overall, participants across the life-course recalled a mean of 46.19 words across trials 1-5 following post-WBB in comparison to post-placebo group who recalled a mean of 44.38 words. In addition to this, there was also a significant Treatment x Age Interaction (F(4,68.05)=2.58, p=0.045; Figure 5.4 (B) with post-hoc analysis revealing significantly better performance following WBB for the 8–10-year-olds relative to placebo at 2 h (mean total acquisition = 31.64 vs 29.50 respectively, p=0.042). A significant improvement was also observed for the 65–80-year-olds following WBB consumption relative to placebo at at 2hr (mean total acquisition= 44.94 vs 40.26 respectively, p=0.016). For immediate recall, there was no significant main effect of treatment, however a trend towards significance for the treatment x age group interaction was observed (F(4,79.69)=2.21, p=0.076). This trend was driven by 14-18 year olds (p=0.075, trend for improved performance following WBB realtive to placebo) and 22-28 year olds (p=0.075, trend for improved performance following placebo relative to WBB). In addition to this treatment-related effect there was also a significant main effect of age group (F(4,84.69)=4.98, p=0.001), with best performance on immediate recall for the 22-28 year olds, followed by the 14–18-year-olds, 40-50 year olds, 65-80 year olds and lastly the 8-10 year olds (Table 5.3).

For delayed recall there was no significant main effect of treatment or treatment x age interaction, but there was a significant effect of age group (F(4,81.01)=3.35, p=0.014). Overall,

the 22-28 year olds recalled the most words at the delay recall point, followed by the 14-18 year olds, 40-50 year olds, 8-10 year olds year olds and lastly 65-80 year olds (Table 5.3).

Interestingly, although proactive interference performance at the 2h time point was worse when compared to baseline for both treatments, (higher score equals worse performance), a significant main effect of treatment was observed for proactive interference whereby placebo attenuated a decline in performance seen in the WBB ($F(1,94.32)=4.85$, $p=0.030$; Figure 5.4 (C).

A significant treatment x age group analysis was also observed ($F(4,94.56)=3.14$, $p=0.018$), with post-hoc analysis revealing that the 8-10 year olds and 65-80 year olds experienced significantly less interference following placebo consumption compared to WBB ($p=0.036$ and $p=0.042$, respectively). In contrast to this, pairwise comparisons showed that the 22-28 year olds experienced significantly less interference following WBB consumption compared to placebo ($p=0.051$; Figure 5.4 (D). This suggests that the 8-10 year olds and 65-80 memorised the original word list more strongly (List A) whereas the 22-28 year olds encoded list B more strongly.

For retroactive interference, no significant main effect of treatment, or treatment x age group interaction, was observed. However, there was a significant effect of age group ($F(4,80.12)=5.05$, $p<0.001$). Overall, the 8–10-year-olds experienced the least interference, followed by the 22-28 year olds, 14-18 year olds, 40-50 year olds and lastly the 65-80 year olds experiencing the most interference (Table 5.4). This indicates that the younger age categories were less affected by List B affecting their ability to correctly recall List A words, compared to older age groups, which were more affected the ability to recall list A words

upon hearing the distractor list. No treatment-related effects were seen for word recognition.

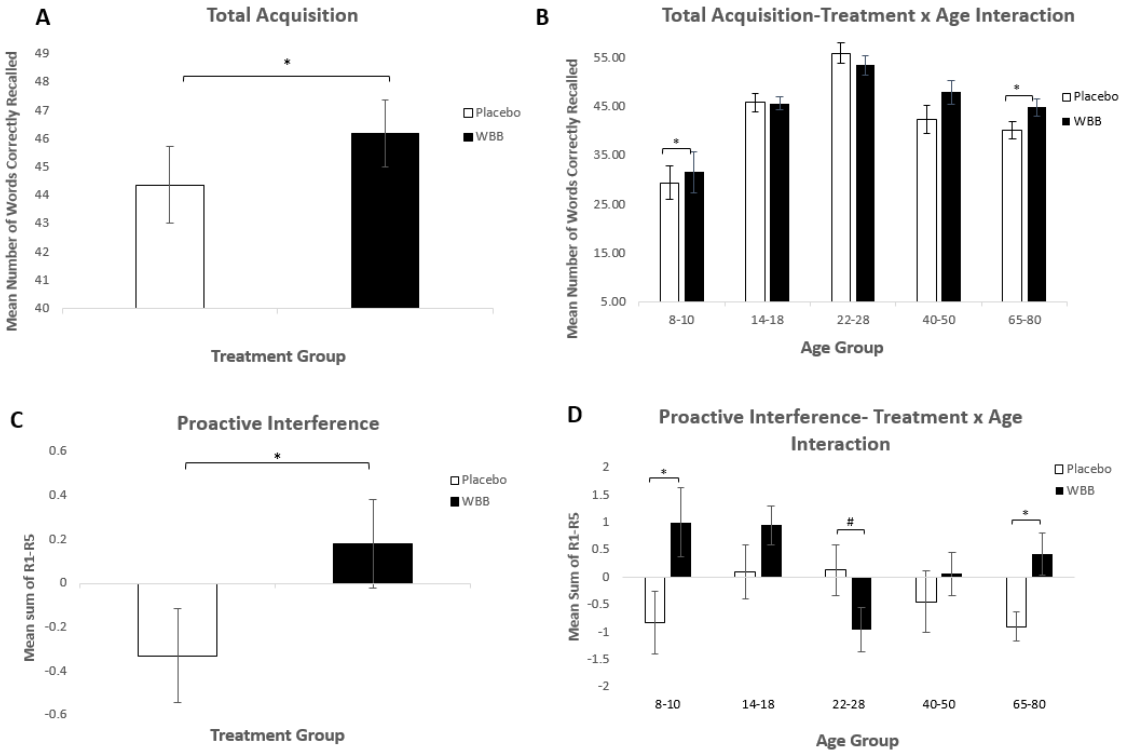


Figure 5. 4 **A)** Mean of words correctly remembered across (R1 to R5) (± SEM) at 2hr following placebo and WBB consumption. Performance was significantly better following WBB consumption compared to placebo (p=0.015). **B)** Mean of correct responses across (R1 to R5) trials per age group (± SEM) at 2hr following placebo and WBB consumption. A significant treatment x age group interaction (p=0.045) revealed significantly better performance for WBB consumption in comparison to placebo for the 8-10 year olds (p=0.019) and 65-80 year olds (p=0.018). **C)** Mean proactive interference (R1 minus R6) (± SEM) at 2hr following placebo and WBB consumption. Significantly better performance following placebo consumption in comparison to WBB (*p*=0.030). **D)** Mean proactive interference (R1 minus R6) for each age group (± SEM) at 2hr following placebo and WBB consumption. A significant treatment x age group interaction (p=0.030) revealed significantly better performance for placebo consumption in comparison to WBB for the 8–10-year-olds (p=0.036) and 65-80 year olds (p=0.042). Significant improvement in performance was observed for the 22-28 year olds following WBB versus placebo intake (p=0.051).

Table 5. 4 Mean scores (SD) for the number of words correctly recalled per age group, irrespective of treatment, at baseline and post-consumption of treatment.

Age Group	**Mean Score**									
	Total Acquisition (R1-R5)		Immediate Recall (R1)		Proactive Interference (R1-R6)		Retroactive Interference (R5-R7)		Delay Recall (R8)	
	Baseline	Post	Baseline	Post	Baseline	Post	Baseline	Post	Baseline	Post
8-10	34.83 (12.41)	30.52 (12.64)	4.64 (1.62)	4.65 (1.23)	-0.35 (2.27)	0.04 (2.21)	1.86 (2.20)	1.74 (1.94)	7.74 (3.14)	5.24 (3.29)
14-18	45.72 (7.70)	45.87 (7.07)	5.65 (1.39)	6.21 (1.66)	-0.68 (2.18)	0.51 (1.95)	2.73 (2.46)	2.13 (2.03)	7.68 (4.34)	6.82 (4.10)
22-28	55.80 (8.57)	54.80 (9.38)	7.22 (2.37)	7.46 (2.35)	-0.43 (2.15)	-0.41 (2.13)	1.52 (1.91)	1.87 (2.03)	11.37 (3.13)	9.80 (3.72)
40-50	46.72 (10.29)	45.09 (11.02)	5.53 (1.35)	5.70 (1.49)	-0.62 (1.74)	-0.21 (2.03)	2.97 (3.00)	3.42 (2.61)	8.03 (3.35)	6.67 (3.59)
65-80	43.92 (7.34)	42.47 (7.79)	5.68 (1.28)	5.56 (1.41)	-0.18 (2.25)	-0.26 (1.57)	3.61 (2.20)	3.95 (2.15)	6.74 (3.11)	5.05 (2.97)

5.3.2 Corsi Blocks Tapping Task

Figure 5.5 A shows a significant main effect of treatment for the number of sequences correctly tapped was observed following WBB consumption (F(1,82.93)=6.15, p=0.015), whereby participants correctly tapped more sequence post WBB (mean=19.48) consumption relative to placebo (mean=17.25).There was no significant treatment x age group interaction, however there was a significant effect of age group observed (F(4,87.93)=10.30, <0.001) with the 22-28 year olds correctly memorising the highest number of sequences (mean=23.88), followed by the 14-18 year olds (mean=20.53), 40-50 year olds (mean=17.84), 65-80 year olds (mean=14.97) and then the 8-10 year olds (mean=11.13) (Table 5.5).

Similarly, a significant main effect of treatment was observed for the number of blocks correctly tapped (F(1,89.43)=4.64, p=0.034), irrespective of sequence, whereby participants tapped more correct blocks following WBB consumption (mean= 26.61) relative to placebo (mean=24.54) (Figure 5.5 B). A significant effect of age group was also observed (F(4,90.65)=6.87, p<0.001), with the 22-28 year olds correctly memorising the most blocks (mean=28.77), followed by the 14-18 year olds (mean=27.16), 40-50 year olds (26.14), 65-80 year olds (mean=23.61) and then the 8-10 year olds (mean=19.17) (see Table 5.5)

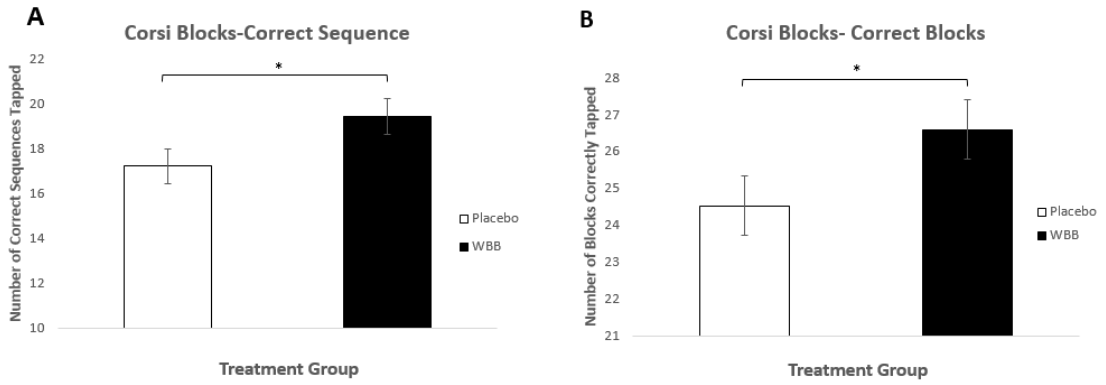


Figure 5. 5 **A)** Mean of number of sequences correctly remembered (± SEM) at 2hr following placebo and bluebery consumption. Performance was significantly better following WBB consumption compared to placebo irrespective of age (p=0.015). **B)** Mean number of blocks correctly memorised (± SEM) at 2hr following placebo and bluebery consumption. Overall score was significantly higher following WBB consumption compared to placebo (p=0.034).

Table 5. 5 Mean scores (SD) at 2hr post consumption of treatment for the number of sequences and blocks correctly tapped per age group, irrespective of treatment.

Age Group	**Mean Score**	
	Sequence	**Blocks**
8-10	11.13 (5.54)	19.17 (7.73)
14-18	20.53 (6.71)	27.16 (6.66)
22-28	23.88 (6.79)	28.77 (6.87)
40-50	17.84 (6.79)	26.14 (7.47)
65-80	14.97 (6.15)	23.61 (7.12)

5.3.3 Task-Switching Test

No significant main effect of treatment was observed for TST accuracy, however, a significant Treatment x Age Interaction ($F(4, 760)=16.11$, $p<0.001$) was found (Figure 5.5 A). Individual age group post-hoc analysis revealed 8–10-year-olds performed significantly better after placebo intake (mean accuracy=0.827) compared to WBB (mean accuracy=0.758) (p=0.001) and the 14–18-year-olds showed the opposite pattern performing significantly better following WBB intake (mean accuracy=0.908) relative to placebo (mean accuracy=0.875)

(p<0.001). In addition to this, a trend for improvement following WBB was observed for the 65–80-year-olds (mean accuracy=0.917) compared to placebo (mean accuracy=0.901) (p=0.090). No significant differences between treatments were seen for the 22-28 (WBB mean accuracy=0.914, placebo mean accuracy=0.911) and 40-50 year olds (WBB mean accuracy=0.0.909, placebo mean accuracy=0.915). Moreover, a significant effect of age group was also observed (F(4,95.49)=13.00, p<0.001), with the 22-28 year olds displaying the highest score for accuracy (mean=0.950), followed by the 40-50 year olds (mean=0.946), 65-80 year olds (mean=0.944), 14-18 year olds (mean=0.885) and the 8-10 year olds (mean=0.637) (Table 5.5). Lastly, analysis revealed no significant difference between WBB and placebo for treatment x task type, meaning that performance was not different during the high/low or odd/even trial types following WBB or placebo treatment (F(1,764.73)=0.001, p=0.972). Similarly, no significant treatment x Switch Trial interaction was observed (F(3,763.74)=0.114, p=0.952).

As with accuracy, no overall effect of treatment was observed for TST RT. Nevertheless, there was a significant Treatment x Age Interaction (F(4,679)=6.56, p<0.001). Individual age group analysis revealed significantly quicker performance amongst the 8–10-year-olds following WBB consumption (mean RT=1038.01 ms) relative to placebo (mean RT=1123.55 ms) (p<0.001) and significantly slower performance in the 14-18 year olds following WBB consumption (mean RT= 908.41 ms) relative to placebo (mean RT=882.78) (p=0.008) (Figure 5.5 B). An effect for age group was also observed (F(4, 86.51)=6.35, p<0.001). Overall, the 22-28 year olds displayed fastest performance (mean=754.63ms), followed by the 40-50 year olds (mean=832.82ms), 14-18 year olds (mean=895.63 ms), the 65-80 year olds (mean=920.97ms) and lastly the 8-10 year olds (1080ms) (Table 5.6). As with accuracy, analysis revealed no significant difference between WBB and placebo for treatment x task

type, meaning that reaction time was not different during the high/low or odd/even trial types following WBB or placebo treatment ($F(1,701.38)=0.002$, $p=0.961$). Similarly, no significant treatment x Switch Trial interaction was observed ($F(3,701.60)=0.814$, $p=0.486$).

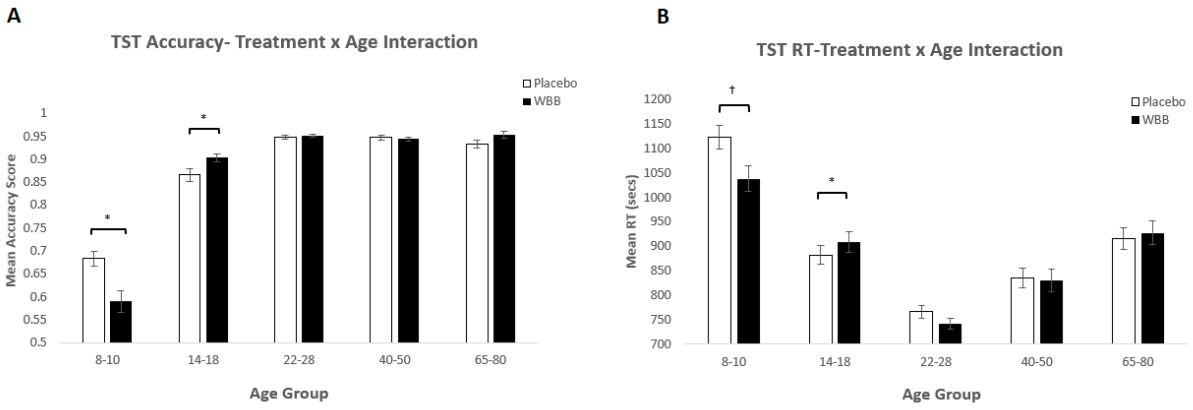


Figure 5. 6 **A)** Mean TST accuracy per age group (± SEM) at 2hr following placebo and blueberry consumption. There was significantly better performance following WBB consumption in comparison to placebo for the 8-10 year olds ($p<0.001$) and 14-18 year olds ($p<0.001$). **B)** Mean TST reaction time per age group (± SEM) at 2hr following placebo and blueberry consumption. There was significantly quicker performance following WBB consumption in comparison to placebo for the 8-10 year olds (†$p<0.001$) and 14-18 year olds (*$p<0.008$).

Table 5. 6

Mean scores (SD) at 2hr post consumption of treatment for TST accuracy and RT per age group, irrespective of treatment.

Age Group	**Mean Score**	
	TST Accuracy	**TST RT (ms)**
8-10	0.64 (0.21)	1080 (247.21)
14-18	0.89 (0.14)	895.63 (254.90)
22-28	0.95 (0.05)	754.63 (159.73)

40-50	0.95 (0.06)	832.82 (258.38)
65-80	0.94 (0.09)	920.97 (265.50)

5.3.4 Serials 3s and 7s

No significant main effects of treatment or treatment x age interaction was observed for serial 3s or serial 7s accuracy. A significant effect of age group was observed for serial 7s performance ($F(4,92.82)=2.62$, $p=0.040$) with the 22-28 year olds performing better overall (mean score= 26.57) followed by the 14-18 (mean score=16.72) & 40-50 year olds (mean score=16.72), 65-80 year olds (mean score=15.06) and lastly the 8-10 year old scoring the lowest (mean score= 5.57) (Table 5.7). No effects of age were seen on the easier serial 3s task.

Table 5. 7 Mean scores (SD) per age group at 2hr post consumption of treatment for number of correct responses during a 2 minute serials task, irrespective of treatment.

Age Group	**Mean Score**	
	Serial 3s	**Serial 7s**
8-10	10.58 (5.71)	5.57 (3.53)
14-18	28.45 (9.90)	16.72 (8.12)
22-28	43.52 (12.83)	26.57 (8.96)
40-50	27.03 (11.65)	16.72 (9.14)
65-80	22.08 (10.15)	15.06 (8.92)

5.4 Mood Analysis

For the mood analysis, a significant main effect of treatment was observed for negative affect (NA) when measured at the start of the cognitive battery ($F(1,81.83)=5.82$, $p=0.018$) whereby the negative mood score was significantly different between WBB and placebo. Here, the negative mood scores were lower following WBB consumption (mean NA score=13.63) when compared to placebo consumption (mean NA score=13.99) (Figure 5.7 A). A significant Treatment x Age interaction ($F(4,81.82)=2.72$ $p=0.035$) was also observed, whereby post-hoc pairwise comparisons revealed a significant difference between treatment for 8-10 year olds with WBB treatment leading to a lower rating of negative affect (mean NA score=16.25) relative to placebo (Mean NA score=17.50) (p=0.004). No other age groups showed any significant difference (Figure 5.7 B).

To compare with positive affect pre-tasks, no significant effect of treatments was observed ($F(1,81.15)=0.491$, $p=0.485$). Overall, participants scored a mean of 29.98 following WBB consumption, and a mean of 29.73 following placebo consumption (Figure 5.7 C). The treatment x age group interaction for positive affect was not significant (Figure 5.7 D). No significant main effects of treatment were observed for positive post-task either, or negative affect post-task. Generally, the combined positive post-task score following WBB consumption was lower than the mean pre-tasks score (mean=27.36) which was higher

compared to the positive post-task score obtained for placebo (mean=26.77). Similarly, no significant effect of treatment was observed for negative affect post task scores. The mean negative affect posts tasks scores for WBB were 13.6, whereas for placebo it was 13.99.

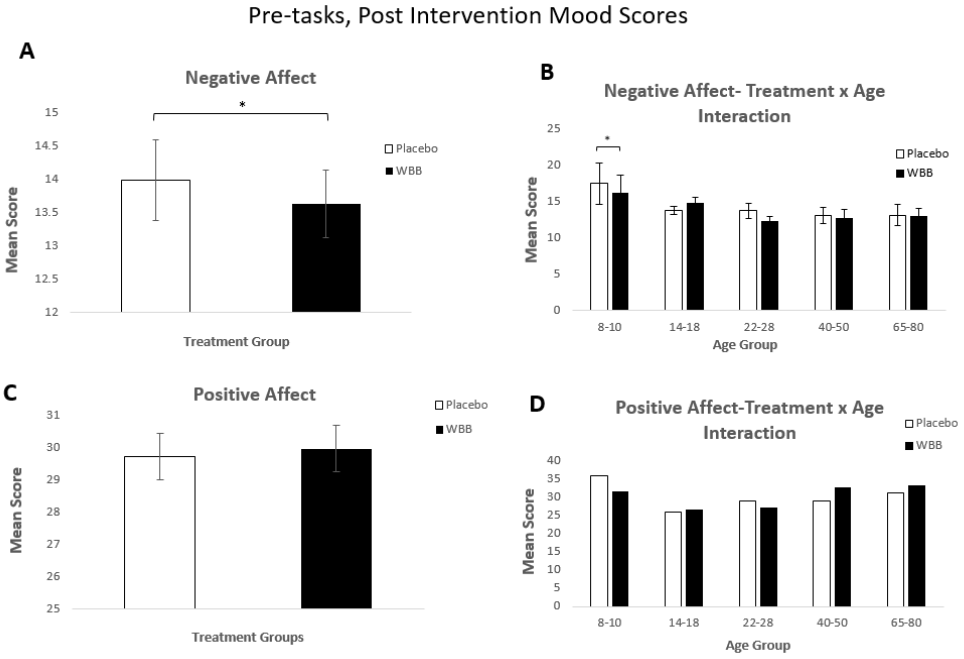


Figure 5. 7 **A)** Mean negative affect score (± SEM) at 2hr following placebo and WBB consumption before commencement of cognitive tasks. Negative affect scores were significantly less following WBB consumption compared to placebo (p=0.018). **B)** Mean negative affect score (± SEM) at 2hr following placebo and WBB consumption across the age groups before commencement of cognitive tasks. Negative affect scores were significantly less following WBB consumption compared to placebo for the 8-10 year olds (p=0.004). **C)** Mean positive affect score (± SEM) at 2hr following placebo and WBB consumption before commencement of cognitive tasks. **D)** Mean positive affect score (± SEM) at 2hr following placebo and WBB consumption across the age groups before commencement of cognitive tasks.

5.5 Cerebral Blood Flow Results

5.5.1 Mean Blood Flow Velocity

For resting TCD, no significant main effect of treatment was observed (F(1,79.91)=0.214, p=0.645) (Table 5.8) and there was no effect of age group or treatment x age group interaction (F(4,79.58)=0.303, p=0.875). Similarly for active TCD, there was no main effect of treatment (F(1,67.60)=2.39, p=0.127) or treatment x age group analysis (F(1,79.91)=0.214, p=0.645), however a significant effect of age group was observed (F(4,67.44)=1.39, p=0.248). Generally, the 8-10 year olds had the highest blood flow velocity (81.89 cm/s), followed by the 14-18 year olds (71.32 cm/s), 22-28 year olds (61.52 cm/s), 40-50 year olds (58.18 cm/s) and 65-80 year olds (56.70cm/s). The difference between the 8-10 year olds and 40-50 year olds was significant (p=0.030). The difference between the 8-10 year olds and 65-80 year olds was near significance (p=0.098) however the variability was too large for significance to be obtained (95% CI: -13.10 to 0.57).

5.5.2 Mean Pulsatility Index

There was no significant main effect of treatment for pulsatility index whilst the participant was at rest (F(4,79.20)=24.99, p=0.153) and there was no significant treatment x age group interaction (F(4,68.45)=1.01, p=0.407) (Table 5.8). However, a significant main effect of age group was observed (F(4,79.20)=24.99, p=0.001). Generally, the 65-80 year olds had the

highest resting PI (1.03 cm/s), followed by the 8-10 year olds (mean= 0.95), 14-18 year olds (mean=0.91), 22-28 year olds (mean= 0.91) and 40-50 year olds (mean=0.86).

Similarly, during the active state, there was no significant main effect of treatment for pulsatility index ($F(1,75.05)=0.25$, $p=0.617$), and there was no significant treatment x age group interaction (($F(4,74.78)=0.57$, $p=0.685$). Nevertheless, a significant main effect of age group was observed ($F(4,79.20)=5.00$, $p=0.001$). Generally, the 65–80-year-olds exhibited the highest pulsatility index during the active state (mean=1.04), which was significantly different to the 40–50-year-olds (mean=0.87) ($p<0.001$), 22-28 year olds (mean=0.92) ($p=0.20$), 14-18 year olds (mean=0.92) ($p=0.033$), and 8-10 year olds (mean=0.87) ($p=0.011$).

Table 5. 8

Mean resting and active blood flow velocity pre and post consumption of WBB and placebo.

Dependent Variable (Cerebral Blood Flow)	*Baseline Placebo Mean (SD)*	*Baseline WBB Mean (SD)*	*Post-consumption Placebo Mean (SD)*	*Post-consumption WBB Mean (SD)*	*Treatment Effect*
Resting					
Blood Flow Velocity	66.72 (14.06)	67.38 (13.56)	64.98 (13.13)	64.98 (13.13)	$F(1,79.91)=0.214$, $p=0.645$
Pulsatility Index	0.96 (0.15)	0.96 (0.16)	0.93 (0.15)	0.92 (0.14)	$F(1,68.43)=2.091$, $p=0.153$
Active					
Blood Flow Velocity (cm/s)	66.50 (14.52)	66.45 (12.86)	63.86 (11.99)	64.70 (13.03)	$F(1,67.60)=2.385$, $p=0.127$
Pulsatility Index	1.06 (0.88)	0.96 (0.15)	0.93 (0.14)	0.92 (0.14)	$F(1,75.05)=0.249$, $p=0.619$

5.6 Polyphenol Analysis Results

5.6.1 Plasma Polyphenols Analysis

A total of 70 polyphenolic metabolites were quantified in plasma including derivatives of benzoic acid, phenylacetic acid, propionic acid, benzaldehyde, pyrogallol, benzene diols and triols, hippuric acid, cinnamic acid, flavanol, and flavonol (full list of raw means pre and post consumption can be seen in appendix O). A significant main effect of treatment was observed for 13 compounds whereby levels were significantly higher following WBB consumption relative to placebo. These metabolites included four derivatives of benzoic acid, one propionic acid derivative, one pyrogallol derivative, six cinnamic acid derivatives and one flavanol derivative (Table 5.8). In addition to this, a trend towards significance ($p<0.1$) was observed for 4 cinnamic acid derivatives whereby the levels were higher following WBB consumption relative to placebo (Table 5.8). Significant increases (at $p<0.05$) were also observed for one metabolite following placebo intake versus WBB including one benzoic acid derivative (Table 5.9).

Interestingly, a number of treatment x age group interactions were apparent, and significant differences were observed for 7 metabolites including for 2 Pyrogallol derivatives: 2-Methylpyrogallol-O-Sulfate ($F(2,50.55)=3.82$, $p=0.29$) with pairwise comparisons revealing a higher concentration in plasma following WBB treatment compared to placebo for the 22-28 year olds. Likewise, for Pyrogallol-2'-O-Sulfate ($F(2,21.54)=11.72$, $p<0.001$) where pairwise-comparisons found higher concentrations post placebo consumption relative to WBB for the 65-80 year olds. Two benzoic acid derivatives were also significant: 2,4-dihydroxybenzoic acid

(*F*(2,45.77)=7.10, p=0.002) whereby a significantly higher concentration was observed following placebo for the 22-28 year olds relative to WBB, however a significant increase in metabolite concentration was observed following WBB treatment for the 40-50 year olds relative to placebo. For isovanillic acid 3-O-sulfate (*F*(2,44.74)=3.92, p=0.027), pairwise comparisons revealed a significant increase following WBB consumption relative to placebo, for the 22-28 year olds and 40-50 year olds. Treatment x age group analysis for was significant for one cinnamic acid derivative: Isoferulic Acid 3-O-Sulfate (*F*(1,28459989.08)=4.95, p=0.026), with pairwise comparison revealing a higher concentration following WBB compared to placebo for the 22-28 year olds. Likewise, for one propionic acid derivative: 3-(2-hydroxyphenyl) propionic acid with pairwise comparisons revealing significantly higher concentration following WBB consumption relative to placebo for the 65-80 year olds (*F*(2,33.89)=4.91, p=0.013). And lastly one flavonol derivative: Quercetin 3-sulfate (p=0.013) where pairwise comparisons revealed significantly higher concentration following WBB consumption relative to placebo for the 22-28 year olds (*F*(2,20.44)=5.38, p=0.013).

Finally, as shown in Figure 5.8 A, total polyphenols analysis revealed a non-significant increase in total metabolites following WBB consumption compared to placebo (*F*(1,60.40)=1.55, p=0.219). A significant age x treatment interaction was also apparent (*F*(2,60.09)=0.33, p=0.718), with the 22-28 year olds exhibiting the highest total polyphenol metabolites following WBB consumption relative to placebo at 2hr (Figure 5.8 B), no significant differences between treatments were observed for the other age groups.

Table 5. 9 Mean ± SD plasma concentrations for pre- and post-intervention metabolites that showed significant effects

	Plasma Concentration (nM)				
(Poly)phenol Metabolite	**Placebo Mean (±SD)**		**WBB Mean (±SD)**		**Treatment Effect**
	0hr	2hr	0hr	2hr	
Benzoic Acid Derivatives					
*2-Hydroxy-4-methoxybenzoic acid [b]**	166±198	164±191	140±198	136±190	(*F*(1,27.79)=6.67, p=0.015)
*Protocatechuic acid sulfate mix [a]**	43±130	13±13	15±12	85±72	(*F* (1,3.56)=10.97, *p*=0.035)
*Isovanillic acid 3-O-sulfate [a]**	363±389	240±302	293±238	289±305	(*F*(1,46.15)=16.66, p<0.001)
*Syringic acid [a]**	162±127	153±91	147±118	175±99	(*F*(1,44.61)=4.89, p=0.032)
*4-Methylgallic-3-O-sulfate [a]**	276±1094	83±739	152±365	121±225	(*F*(1,29.03)=53.74, p<0.001)
Propionic acid derivatives					
*3-(2-hydroxyphenyl) propionic acid[a]**	283±287	261±274	250±293	522±704	(*F*(1,34.21)=4.62, p=0.039)
Pyrogallol derivatives					
*1-Methylpyrogallol-O-sulfate [a]**	1167±269	607±829	501±1576	406±626	(*F*(1,13.37)=9.55, p=0.008).
Cinnamic acid derivatives					
Cinnamic acid [a#]	253±108	248±111	235±98	254±113	(*F*(1,24.43)=4.07, p=0.055)
*Caffeic acid-3-glucuronide [a]**	12±11	10±8	10±9	14±10	(*F*(1,40.23)=6.52, p=0.015)
*Dihydro Caffeic Acid 3-O-Sulfate Sodium Salt [a]**	791±920	718±794	748±833	724±770	(*F*(1,27.64)=9.22, p=0.005)
*Ferulic Acid 4-O-Sulfate Disodium Salt [a]**	435±1638	72±142	155±36	463±294	(*F*(1,31.88)=61.81, p<0.001)
Dihydro Isoferulic acid 3-O-Sulfate [a#]	251±424	227±558	285±279	283±511	(*F*(1,56.53)=2.94, p=0.092)
Dihydroferulic acid 4-O-β-d-glucuronide [a#]	245±253	198±202	198±190	234±216	(*F*(1,19.38)=3.18,p=0.090)
*Isoferulic Acid 3-O-Sulfate [a]**	605±462	745±449	658±404	591±465	(*F*(1,28395102.71)=4.96,p=0.02
*Dihydro isoferulic acid 3-O-β-d-glucuronide [a]**	84±76	76±65	72±69	85±68	(*F*(1,37.54)=9.25, p=0.004)
Ferulic Acid 4-O- β -D-Glucuronide[a#]	608±782	644±795	634±802	614±727	(*F*(1,34.21)=3.90, p=0.056)
*Chlorogenic acid [a]**	4±5	4±1	3±1	50±43	(*F*(1,22.17)=11.46, p=0.003)
Flavanol derivatives					
*(-)-Epicatechin-3-O-sulfate [a]**	498±566	544±559	482±586	777±777	(*F*(1,4.39)=9.35, p=0.033)

Data is represented as (nM) ±SD
*Significance at the p<0.05 level for Treatment
[#]Trend towards significance at p< 0.10 for Treatment
[a]Higher metabolite concentration post WBB consumption
[b]Higher metabolite concentration post placebo consumption

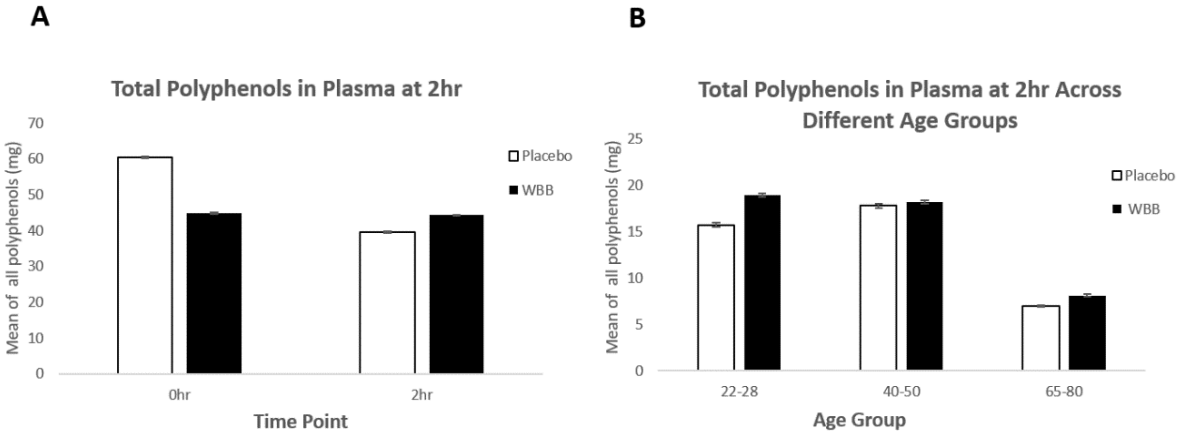


Figure 5. 8 **A)** Mean (± SEM) total polyphenol measured in plasma at 0hr and 2hr following placebo and WBB intake. **B)** Mean (± SEM) total polyphenol in plasma at 2hrs post consumption for placebo and WBB participants across different age groups.

5.6.2 Urinary Polyphenol Concentration

A total 76 polyphenolic metabolites were quantified in urine following consumption of placebo and WBB treatment. Full list of means per treatment group can be seen in appendix O. Following the consumption of WBB and placebo, a significant increase at $p<0.05$ was found for 26 compounds following WBB consumption relative to placebo. These metabolites included 7 benzoic derivatives, one phenylacetic acid derivative, one propionic acid derivative, three benzene diols and triols, two hippuric acid derivatives, eleven cinnamic acid derivative, and one flavonol derivative (Table 5.10). As a reminder, urine collection took place once following placebo consumption and once following WBB consumption therefore baseline values will not appear here.

Trend for significance following WBB consumption relative to placebo was found for 4 metabolites which included 2 propionic acid derivatives, one cinnamic acid derivative, and one benzoic acid derivative. There was no significant effect of treatment observed for any metabolites following placebo consumption, however a trend for significance following placebo consumption was observed for three metabolites which included a benzoic acid derivative, a phenylacetic acid derivative and a flavonol derivative (Table 5.10).

Interestingly, treatment x age interactions were seen for 15 metabolites. This included four benzoic acid derivatives including 2,4-dihydroxybenzoic acid (F (4,88.53)=3.690, p=0.008), where pairwise comparisons revealed higher concentration following placebo intake compared to WBB in the 65-80 year olds. For 2-Hydroxy-4-methoxybenzoic acid (F (4,87.37)=3.725, p=0.008), pairwise comparisons revealed higher metabolite concentration following placebo intake compared to WBB for the 8-10 year olds and higher metabolite concentration following WBB intake versus placebo for the 65-80 year olds.

For protocatechuic acid sulfate mix, (F (4,86.23)=2.893, p=0.027), pairwise comparisons revealed significant difference between placebo and WBB metabolites concentration for the 65-80 year olds, with higher levels observed following WBB intake. Similarly, pairwise comparisons for 4-Methylgallic-3-O-sulfate concentration found higher levels following WBB intake versus placebo for the 65-80 year olds (F (4,89.84)=3.084, p=0.020). There was also a significant treatment x age group analysis for two phenylacetic derivatives: 3,4-dihydroxyphenylacetic acid (F (4,66.14)=2.850, p=0.030) with pairwise comparison revealing significantly higher metabolite concentration post-consumption of WBB relative to placebo for the 8-10 year olds and 65-80 year olds.

In contrast to this, pairwise comparisons for DL-p-Hydroxyphenylacetic acid concentration found higher levels following placebo intake versus WBB for the 65-80 year olds (F (4,87.65)=3.015, p=0.022). A significant treatment x age group interaction was observed for one benzaldehyde derivative: 4-Hydroxybenzaldehyde, (F (4,73.83)=2.635, p=0.041) with pairwise comparison revealing significantly higher metabolite concentration post-consumption of WBB relative to placebo for the 8-10 year olds and 65-80 year olds. A significant treatment x age group interaction was observed for three benzene diols and triols derivates: 4-Methylcatechol (F (4,89.02)=25.856, p<0.001) with significant pairwise showing increased levels following WBB consumption for the 22-28 year olds and 65-80 year olds. Similarly, for 4-Methylcatechol-O-Sulfate, (F (4,88.17)=4.89, p=0.001) with pairwise comparisons revealing significantly higher levels following WBB relative to placebo for the 65-80 year olds and for Catechol-O-1-Glucuronide (F (4,88.84)=3.665, p=0.008) there was significant increase following WBB consumption relative to placebo for the 14-18 year olds and 65-80 year olds. A significant interaction was observed for one hippuric acid derivative, (F (4,87.72)=4.914, p=0.001) whereby increased levels of hippuric acid metabolites were observed for the 8-10 year olds, 14-18 year olds, 22-28 year olds, and 65-80 year olds following WBB consumption relative to placebo.

Significant age x treatment interactions were observed for three cinnamic acid derivatives including dihydro ferulic Acid 4-O-Sulfate (F(4,82.72)=3.500, p=0.011) where pairwise comparisons revealed significantly higher metabolite concentration following WBB consumption relative to placebo for the 14-18 year olds and significantly higher metabolite concentration following placebo consumption relative to WBB for the 65-80 year olds. For isoferulic acid (F(4,88.17)=5.770, p<0.001), pairwise comparisons revealed increased levels

following WBB consumption relative to placebo across all ages; 8-10 year olds, 14-18 year olds, 22-28 year olds, 40-50 year olds and 65-80 year olds. The same observations were found for 3-Feruloylquinic acid ($F(4,87.97)=18.38$, $p<0.001$) with increased levels of metabolites following WBB consumption relative to placebo across all ages; 8-10 year olds, 14-18 year olds, 22-28 year olds, 40-50 year olds, and 65-80 year olds. Lastly, one flavonol derivative was found to have a significant treatment x age group comparison: Quercetin-7-O-ß-D-glucuronide ($F(4,89.97)=8.421$, $p<0.001$) where pairwise comparisons revealed significantly higher concentration following WBB consumption relative to placebo for the 65-80 year olds.

Total polyphenols analysis revealed a significant increase in total metabolites detected in urine between treatments ($F(1,88.76)=65.17$, $p<0.001$) with higher excretion following WBB consumption compared to placebo (Figure 5.9 A) . A significant treatment x age interaction was also observed ($F(4,88.57)=2.83$, $p=0.029$), showing higher levels of metabolites excreted in urine following WBB treatment relative to placebo. Post-hoc analyses showed this was significant for the 8-10 year olds and 14-18 year olds 22-28 year olds and 65-80 year olds, whilst only a trend towards significance was observed for the 40-50 year olds (Figure 5.9 B).

Table 5. 10 Raw mean ± SD urinary concentrations for urinary metabolites that showed significant effects

	Total 24 h Urinary Excretion (µg)		
(Poly)phenol Metabolite	**Placebo Mean (±SD)**	**WBB Mean (±SD)**	**Treatment Effect**
Benzoic Acid Derivatives			
3-Hydroxybenzoic Acid [a]*	3976±6211	4065±9516	(*F* (1,32.23)=4.48, p=0.042)
2,4-Dihydroxybenzoic Acid [b#]	21199±51076	17806±24751	(*F* (1,89.79)=2.96, p=0.089)
2,3,4-Trihydroxybenzoic Acid [a#]	2682±1710	2635±1634	(*F* (1,71.30)=3.221. p=0.077)
Protocatechuic Acid Sulfate Mix [a]*	1167±1195	1478±1392	(*F* (1,88.12)=14.29, p<0.001)
Isovanillic Acid 3-O-Sulfate [a]*	3714±3774	5326±4805	(*F* (1,88.28)=12.41, p<0.001)
Syringic Acid [a]*	1736±1296	2246±1604	(*F* (1,84.57)=18.13, *p* <0.001)
Protocatechuic Acid-2-Sulfate Mix [a]*	134±141	209±211	(*F* (1,73.04)=17.47, p<0.001)
Protocatechuic Acid 3-O-ß-D-Glucuronide [a]*	68±48	78±54	(*F* (1,82.05)=6.18, p=0.015)
Protocatechuic Acid [a]*	590±1187	690±808	*F* (1,25.39)=5.27, *p*=0.028)
Phenylacetic acid derivatives			
3,4-Dihydroxyphenylacetic Acid [a]*	6271±5784	8312±6789	(*F* (1,68.09)=25.31 p<0.001)
DL-P-Hydroxyphenyllactic Acid[b#]	3291±5178	3019±3886	(*F* (1,89.23)=2.982, p=0.088)
Propionic acid derivatives			
(R)-(+)-2-(4-Hydroxyphenoxy)-Propionic Acid [a#]	8009±5288	8969±6202	(*F* (1,86.23)=3.57, p=0.062)
3-(2,3-Dihydroxyphenyl)Propionic Acid [a]*	2743±1595	2802±1586	(*F* (1,88.18)=10.07, p=0.002)
3-(4-Hydroxy-3-Methoxyphenyl)Propionic Acid [a#]	75793±121429	101154±165478	(*F* (1,89.57)=3.32, p=0.072)
Benzene diols and triols			
4-Methylcatechol-O-Sulfate [a]*	15566±16782	20335±23219	*(F* (1,89.10)=20.68, p<0.001)
4-Methylcatechol [a]*	11644±10848	18737±19659	(*F* (1, 90.37)=73.78, p<0.001)
Catechol-O-1-Glucuronide [a]*	1049±1198	1604±1585	(*F* (1,90.45)=21.04, p<0.001)
Hippuric acid derivatives			
3-Hydroxyhippuric Acid [a]*	122781±151961	151630±143564	(*F* (1,82.51)=9.96, p=0.002)

Hippuric Acid [a]*	2092649±1427211	3304277±2335141	(*F* (1,88.47)=63.98, p<0.001)
Cinnamic acid derivatives			
Caffeic Acid-4-Glucuronide [a#]	480±394	582±386	(*F* (1,90.08)=3.16, p=0.079)
Caffeic Acid-3-Glucuronide [a]*	258±276	361±319	(*F* (1,90.17)=8.88, p=0.004)
Dihydro Caffeic Acid 3-O-Sulfate Sodium Salt [a]*	7362±5654	8003±5437	(*F* (1,76.03)=3.99, p=0.049)
Ferulic Acid 4-O-Sulfate Disodium Salt [a]*	50775±42788	59822±41453	(*F* (1,88.63)=5.76, p=0.018)
Ferulic Acid 4-O- B -D-Glucuronide [a]*	18718±17640	28232±22338	(*F* (1,89.32)=14.37, p<0.001)
Isoferulic Acid [a]*	104601±205022	598448±616133	(*F* (1,88.15)=73.84, p<0.001)
P-Coumaric [a]*	923±867	1444±1651	(*F* (1,88.34)=14.99, p<0.001)
Sinapic Acid [a]*	1210±817	1346±970	(*F* (1,89.57)=11.09, p=0.001)
Chlorogenic Acid [a]*	1303±1842	2237±2006	(*F* (1,65.50)=19.76, p<0.001)
3-Feruloylquinic Acid [a]*	2080±1988	5561±5275	(*F* (1,88.04)=130.67, p<0.001)
Caffeic Acid-2-Sulfate Mix [a]*	3067±3633	4322±4186	(*F* (1,72.47)=7.50, p=0.008)
Caffeic Acid [a]*	1255±746	1622±967	(*F* (1,89.47)=21.72, p<0.001)
Flavonol derivatives			
Quercetin-7-O-ß-D-Glucuronide [a]*	123±75	122±86	(*F* (1,90.67)=7.48, p=0.008)
Quercetin 3-Sulfate Potassium Salt [b#]	635±1041	447±575	(*F* (1,89.50) =3.56, p=0.062)

Data is represented as (nM) ±SD
*Significance at the p<0.05 level for Treatment
[#]Trend towards significance at p< 0.10 for Treatment
[a]Higher metabolite concentration post WBB consumption
[b]Higher metabolite concentration post placebo consumption

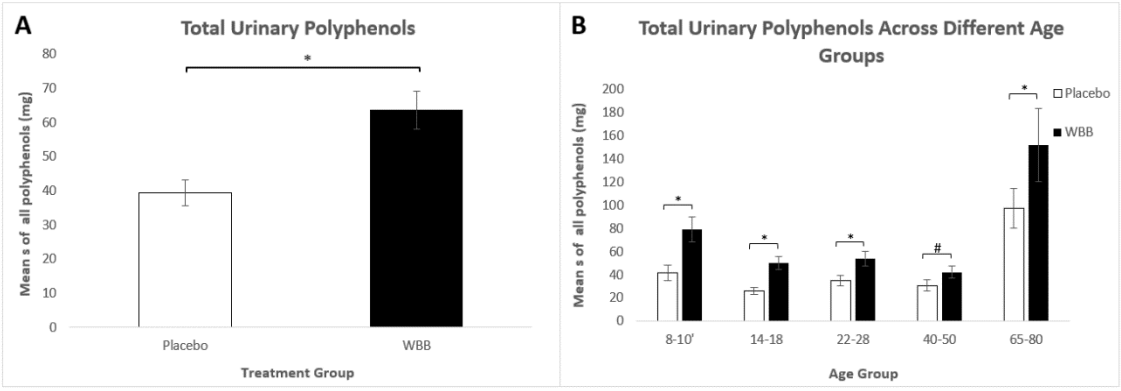


Figure 5. 9 **A)** Mean (± SEM) total polyphenol measured in urine following placebo and WBB intake. **B)** Mean (± SEM) total polyphenol in urine at post consumption of placebo and WBB for participants across different age groups. Significantly higher metabolite concentration was observed post consumption of WBB relative to placebo for the 8-10, 14-18, 22-28 and 65-80 year olds. A trend towards significance was observed for the 40-50 year olds

* $p<0.05$

# $p<0.1$

5.8 Discussion

The BluLife study aimed to elucidate the short-term effect of a single WBB treatment on cognitive function, cardiovascular health, and cerebral blood flow across the lifespan. Importantly, this study examined how WBB polyphenols acutely affected cognitive domains including episodic memory, visuospatial memory, working memory, and executive functioning; and examined how WBB treatment influences FMD, blood pressure and cerebral blood flow to elucidate if changes in blood flow may underlie the cognitive effects. Plasma and urinary metabolites were also examined following treatment to understand how levels of metabolites in plasma and urine may vary following the consumption of WBB, which may give us an insight into the bioavailability of polyphenolic compounds.

In summary, this found that WBB provided benefits for episodic memory and working memory, 2 hrs post-intervention. The findings from AVLT task revealed significant benefits to total acquisition performance ($F(1,67.98)=6.29$, $p=0.015$), whereby WBB consumption maintained cognitive performance during this recall but performance was seen to decline following placebo consumption. This is in line with previous research by Barfoot et al. (2019) who found an effect of treatment for total acquisition amongst children aged 7-10 after acute consumption of WBB compared to placebo. In terms of episodic memory domain and older adults, Whyte et al. (2018) observed a significant treatment by time interaction with post hoc analysis finding improvement after supplementation with a wild blueberry extract compared to the placebo following 3 months consumption. Similarly, Miller et al. (2017) found significant effects of WBB for episodic memory in terms of significant reduction of repetition errors made during the California Verbal Learning Test (CVLT) following 3 months consumption of WBB.

Interestingly, a significant effect of placebo was observed for proactive interference, with participants performing better after placebo intake compared to WBB for this measure. Post-hoc analysis revealed that the significantly improved performance for placebo-treated participants was seen in the 8-10 and 65-80 year olds who also performed the best at total acquisition but following WBB consumption. On the contrary, the significant improvement for performance following WBB compared to placebo was observed for the 22–28-year-olds, who scored the lowest for total acquisition after consuming WBB. This suggests that those who performed best on total acquisition encoded list A more strongly and therefore encoded list B less effectively. On the other hand, those who were less affected by proactive interference may have encoded list A less strongly and therefore had the capacity to encode list B more effectively. Finally, no significant effects of treatment were observed for retroactive interference, indicating that WBB in this case did not alter the effect of encoding new words on the recall of previously encoded information, and in support of this finding, no studies to date have yet seen effects of an anthocyanin-rich intervention on retroactive interference. This is also in line with the observations for proactive interference, whereby following WBB consumption, participants were less affected by the interference list, list B, on their ability to recall list A.

The findings on improvement in proactive interference following placebo consumption in young children is in line with previous research by Whyte & Williams (2015) who also found significantly better scores for proactive interference for placebo relative to WBB following acute WBB consumption. This study had similar population size to the young children sample size in the BluLife study (n=14) and also implemented a crossover RCT design. For older adults, no studies thus far have provided evidence regarding WBB and proactive interference. This

study therefore suggests that older adults in this case encoded List A, and did not let list B interfere with their ability to recall List A.

Interestingly, no significant effects were observed for better immediate recall following WBB treatment, with scores remaining unaffected by treatment type across all age groups. To my knowledge, no studies have yet shown any effects for immediate recall following an anthocyanin-rich intervention. Previous studies have found significant improvement in delayed recall of a word list amongst younger participants (Whyte and Williams, 2015; Whyte, Schafer and Williams 2016), however this effect was not replicated in this study. Similarly, in this study no treatment-related effects were seen for either list A word recognition or list B word recognition. Previous studies have found significant improvements for word recognition in older adults after 3 months consumption of WBB (Whyte et al., 2018), however no study has yet found this effect utilising an acute dosing paradigm.

Nevertheless, as expected, a significant effect of age was observed for all AVLT outcomes other than proactive interference. Generally, the 22–28-year-olds performed the best and the 8-10 year olds performed least well (Table 5.3). This gives us an indication of the cognitive function across the life course, with the cognitive peak expected amongst young adults, whom have been in formal education for longer compared to teenagers or children, combined with greater cognitive flexibility compared to middle aged and older adults. Although not conclusive, some studies have indicated that cognitive function may start to decline in middle-aged adults (Ferreira et al., 2015). For older adults, aged 60 and above, cognitive decline is documented to be steeper (Whitley et al., 2016) as well as this age group being at an increased risk of developing dementia due to changes in neuropathology which often is a natural process of ageing (Peters, 2006). In the BluLife study, these observations were replicated and

episodic memory was most influenced amongst the 8-10 year olds and 65-80 year olds, indicating that WBB polyphenols are able to modulate episodic memory performance in humans at an age where cognition is either most compromised or in humans who have not yet achieved, or have surpassed, their peak cognitive functioning.

Turning to working memory, a significant effect of treatment was observed for the number of sequences correctly recalled during the Corsi Blocks tapping task, with a significant improvement in performance observed following WBB intake compared to placebo. To my knowledge this is the first study to show a significant effect on Corsi Blocks performance following an anthocyanin-rich intervention although Whyte et al. (2018) did find a trend for improved performance on Corsi Blocks correct sequences following a 3-month long supplementation with WBB, compared to placebo. As expected, the number of correct blocks which were tapped was also significant following WBB, but this is to be expected given the correlation between these two outcome measures.

The Serial 3s and 7s subtractions task showed no significant effects of treatment. To my knowledge, no other studies thus far have used serials subtraction task to test the acute effect of WBB. Nevertheless, Whyte et al. (2018) who tested the chronic effects of WBB also failed to observe any treatment-related effects during serial 3s and 7s. Reasons for this may be due to subtraction task not being sensitive enough to WBB intervention for a significant improvement in working memory to be observed under these conditions.

For executive functioning, no main effect of treatment was observed for TST accuracy; however, a significant treatment x age interaction was demonstrated, with individual age group analysis revealing improved accuracy scores following WBB consumption for the 14-18 year olds and significant improvements in performance observed following placebo for the 8-

10 year olds. For TST RT, a significant main effect of treatment was found overall and individual age group analysis revealed significant quicker performance for the 8-10 year olds, and significant slower performance for the 14-18 year olds following WBB intake relative to placebo. This speed-accuracy trade-off could help explain accuracy score, whereby the 8-10 year olds were conducting the task too quickly and therefore were more likely to make mistakes, which indicates that increased speed had a cost to accuracy, whereas the 14-18 year olds were significantly slower than the placebo but perhaps taking their time meant that their performance was more accurate. Nevertheless, the improvements in psychomotor speed during the Attention Network Task (ANT) which assesses executive functioning, has been previously observed by Whyte et al. (2020) who found significant reaction time benefits following WBB consumption versus placebo in a group of 7-10 year olds. Similarly, previous research by Barfoot et al. (2019) found that reaction time during the Modified Attention Network Task (MANT), assessing executive functioning, was significantly reduced following acute WBB consumption but not placebo. To back this up, although Barfoot et al. (2021) observed no significant improvements to reaction time following chronic WBB consumption in children, they did observe a significant increase in RT following placebo during a cognitively demanding trial of the MANT task, suggesting that in this case, chronic WBB supplementation had a protective effect on reaction time regardless of cognitive difficulty. Reasons for these observations may be that blueberry polyphenols may influence the psychomotor speed domain, resulting in decreased reaction time, particularly in younger children, compared to teenagers or adults.

In this study it was demonstrated that WBB consumption may acutely improve psychomotor speed amongst young children aged 8-10. These improvements in attention and reaction time may be a useful enhancement when it comes to boosting learning in children in the classroom

setting. Furthermore, the improvement observed for accuracy scores during TST for the 14-18 year olds is promising. It is during these ages that the teenagers undergo important exams that dictate the choices they can choose as an adult, such as obtaining a university place. Previous studies have suggested that executive functioning and working memory may be linked to educational achievement and performance, particularly in the fields of mathematics and reading. For instance, an early study by De Jong (1998) revealed worse performance during a working memory assessment in children with reading disabilities, versus children not experiencing reading abilities. Similarly, investigations conducted by St Clair-Thompson et al. (2006) found associations between lower English and mathematics scores with worse performance in working memory tasks in children. The findings of the BluLife study therefore are promising as it suggests that WBB consumption may boost executive functioning 2 hrs post consumption in teenagers as well as finding overall treatment effect for Corsi Blocks which assesses working memory.

Some aspects of mood were improved following WBB consumption relative to placebo, which is in line with previous work by Khalid et al. (2017) and Fisk et al. (2020) using similar interventions, intervention lengths and tasks. Although a significant improvement in positive affect at either pre or post task following WBB intake relative to placebo was not observed, an overall reduction in negative affect after WBB consumption just before commencing the cognitive battery was seen. This suggest that participants felt less negative about the prospect of completing the tasks after consuming WBB compared to after consuming placebo. This finding is promising, as feelings of elevated mood or reduced bad mood, may be important for learning, and subsequently, performance during cognitive tasks. This is because people who experience positive emotions, or a reduction in negative emotions, may be more likely to feel motivated, and possess a greater sense of achievement, which as a result may

positively affect their learning and focus (Valiente et al., 2012). This is crucial for students in an educational setting but may be something that could also be translated to adults within a professional workplace environment.

Considering the vascular measures, treatment with WBB significantly improved FMD amongst all age groups except the 8-10 year olds (the results are discussed in more detail in thesis by Wood, 2022). Reasons for a lack of observation amongst the 8-10 year olds may be that the FMD assessment may not be sufficiently sensitive to detect vascular reactivity in young children. The largest FMD increase (%) observed was in the 22-28 year old group, which is in line with previous studies which have also demonstrated benefits in healthy young adult males of this age group following consumption of WBB polyphenols (Rodriguez-Mateos et al., 2013; Rodriguez-Mateos et al., 2014; Rodriguez-Mateos et al., 2019; all reviewed in Wood et al., 2019). To my knowledge this is the first study to demonstrate significant FMD effects following acute WBB consumption in teenagers, middle-aged adults and older adults following a WBB intervention. This observation is promising as it demonstrates that small lifestyle changes, such as the consumption of blueberries, may positively enhance our vascular systems.

No concurrent effects were seen for CBF parameters in this instance. Here, no main effect of treatment was observed for either mean resting or mean active blood flow velocity, or indeed for mean resting or mean active pulsatility index. Nevertheless, differences in these measures were observed between the different ages as active CBF velocity for the 8-10 year olds was higher compared to the other 4 age groups and significantly so when compared to the 40-50 year olds. This is to be expected as CBF velocity is known to progressively decrease as we age (Tarumi e al, 2018; Lu et al., 2011).

Previous studies have suggested that an increased pulsatility index together with a decreased mean blood flow velocity may indicate cardiovascular risk (Pase et al., 2012) and Alzheimer's disease risk (Roher et al., 2011). Indeed, in this study a higher pulsatility index for the 65-80 year olds was observed, which was significantly different to all other age groups. The results are preliminary and therefore not conclusive; however, an increased pulsatility index and decreased mean blood flow velocity may be a common occurrence amongst older adults, as the cardiovascular health decreases and risk of dementia increases amongst older population (Rivera-Rivera et al., 2016).

There are few studies looking at the effects of WBB polyphenols on cerebral blood flow. To my knowledge, excluding the BluLife study, only one study has previously been conducted to look at the effects of WBB on CBF in healthy humans, and has demonstrated improvements after intake of WBB (Bowtell et al., 2017). Nevertheless, the findings of Bowtell et al. (2017) cannot be compared with the results of this study as Bowtell (2017) was a chronic intervention study, whereby participants consumed a WBB intervention containing 378mg anthocyanins, and with cerebral blood flow assessed using arterial spin labelling

The lack of findings for CBF may be due to the technique not being sufficiently sensitive to measuring small changes in blood flow centrally. Moreover, it is possible that acute intervention lengths may not sufficient, to detect potential changes in CBF. It is also necessary to take into consideration that people in this study were healthy, free from any circulatory or vascular issue. Therefore, it could be the case that the WBB treatment in this study, or perhaps the dosage of anthocyanins, was not strong enough to modify blood flow.

In addition to this, blood flow to the brain is tightly regulated by homeostasis (Koch et al., 2018). Therefore, it is plausible that an acute dose of WBB polyphenols above and beyond the

participants' habitual consumption of polyphenols, was not strong enough to permanently have an effect on the regulatory processes involved in cerebral blood flow. It is important to recognise that this is the first study to measure the effects of WBB polyphenols on cerebral blood flow using TCD. Studies using similar interventions have previously used a more robust measure such as fMRI, or arterial spin labelling. As mentioned in chapter 3, TCD has many practical advantages such as being non-invasive, mobile, quick, and cost-effective. Nevertheless, TCD also carries a number of limitations and there is considerable scope for errors due to extrinsic factors which may affect the measurements such as room temperature, which may increase the risk of fluctuations of values between summer testing and winter testing. As well as this, it is sensitive to physical movements, such as participant slightly moving their head, which happens naturally as it can be difficult to maintain the same head posture for a given time.

No effects were seen for diastolic or systolic blood pressure at 2hrs following WBB consumption or placebo consumption across the age groups. This may suggest that blood pressure is unaffected after 2 hrs of a single serving of WBB polyphenols, and that further research is necessary to understand whether WBB may affect blood pressure acutely. Other reasons may be that no effects were seen across the younger cohorts due to having healthier levels of blood pressure and that in older adults who are known to have higher blood pressure, WBB polyphenols are not sufficient for any effects to be observed acutely. This is in line with previous studies who demonstrated no previous effects on BP after acute consumption of WBB in healthy populations (Wood et al., 2019; McAnulty et al., 2005; Zhu et al., 2017). This highlights the need for further research into the effects on blood pressure, using gold-standard measurements of blood pressure such as 24 h ambulatory blood pressure.

Findings for plasma metabolites showed significant increase for 13 metabolites following WBB consumption relative to placebo. The change was driven primarily by increases in cinnamic acids and benzoic acids derivatives. This supports previous finding by Feliciano et al. (2016) who also saw increased detection of these derivatives in plasma after acute WBB consumption. The number of metabolites detected in urinary excretion was double the amount detected in plasma but, similarly to plasma, was mainly driven by increases in cinnamic acids and benzoic acids derivatives. The profile of metabolites detected in urine was greater than plasma, with derivatives of hippuric acids, benzene diols and triols, and phenyl acetic acids also detected. Barfoot et al. (2021) recently conducted a similar investigation where the urinary excretion of metabolites following chronic consumption was assessed in children aged 8-10, using similar collection methods to the one in my study. Here the researchers did not find any significant changes in total polyphenols between WBB and placebo, however individual metabolite analyses observed a trend towards significance for dihydro caffeic acid 3-O-sulfate, with higher levels detected post consumption of WBB compared to placebo. Similarly, in the BluLife study, the levels of dihydro caffeic acid 3-O-sulfate were significantly higher in urine and plasma samples following WBB compared to placebo.

As well as this Barfoot et al. (2021) observed higher levels of hippuric acid following WBB consumption which was significantly different to levels detected in placebo. This supports the findings of this study, whereby the overall level of hippuric acid was significantly higher in urine sample following WBB intake versus placebo. Individual age group analysis revealed that this increase was also seen amongst the 8-10 year olds, 14-18 year olds, 22-28 year olds and 65-80 year olds. Although levels were higher for WBB compared to placebo, this was not significantly different amongst the 40-50 year olds. No other compounds were significantly

detected in Barfoot et al.'s study (2021); however, it should be taken into consideration that quantity and profile of metabolites quantified in the BluLife study is considerably greater than previous research and may therefore show an enhanced profile of the metabolites that are detected in plasma and urinary excretion in humans following acute consumption of WBB.

The significant findings for hippuric acid in my study is also supported by previous results by Feliciano et al. (2016) who found that hippuric acids derivatives were the greatest contributors of metabolites detected in urinary excretion following WBB intake. This may provide an indication of how metabolites are absorbed and excreted in the body and therefore the status of their bioavailability. This is particularly true with hippuric acids, which is metabolised by the gut microbiome. This may also provide an insight into the interactions of WBB polyphenols on the gut microbiome and the potential benefit that WBB polyphenols may have for gut microbiome health. Nevertheless, it should be taken into consideration that hippuric acid is a generic metabolite which arises from other metabolic processes in the body, not only from polyphenol metabolism. Furthermore, hippuric acid is common to all polyphenols, not just blueberry specific polyphenols (Toromanovic et al., 2008).

Moreover, it is believed that different factors such as age, gender, microbiome, and genotype may affect the absorption, and therefore the excretion, of polyphenols. In this study, some differences in metabolites were detected across different age groups. As a reminder, blood samples were not collected for under 18's in this study, therefore changes in metabolites across age groups is not as extensive as in the urine samples. Out of the 7 metabolites which exhibited a significant treatment x age interaction in plasma, individual age group analysis found higher levels for 4 metabolites following WBB intake for the 22-28 year olds, 2 metabolites for the 40-50 year olds and only 1 metabolite for the 65-80 year olds. Literature

suggest that the gut microbiome changes with age (Badal et al., 2020). This may affect the metabolism of polyphenols by the gut microbiota, and therefore affect excretion of metabolites by individuals across the life-course.

For urine, the majority of metabolites which exhibited a significant treatment x age interaction was driven by differences in metabolite levels between placebo and WBB amongst 65-80 year olds which saw significant higher concentrations following WBB consumption for nine metabolites. Although not many, significant increases in metabolite concentrations were also observed following placebo consumption amongst the 8-10 year olds and 65-80 year olds. Limitations do exist however, including the participants' habitual diet and meals consumed prior to the study days. Polyphenols exist in a wide range of products, not just fruit and vegetables; therefore, despite requesting the participants to follow a low polyphenol diet the day before, the risk of the participant accidently consuming and not reporting food items containing different polyphenol levels does exist. This is the nature of conducting RCTS looking at the effects of foods or nutritional products, as the ability to fully control the participants' diet during the study period is not entirely possible.

Lastly, a handful of studies have suggested that compounds such as cinnamic acid may significantly increase memory performance in mice with diabetes (Hemmati et al., 2018), as well as this, cinnamic acid derivatives such as ferulic acid have shown potential benefits for cognitive functioning in animal studies (Michels et al., 2018; Yang et al., 2016; Wang et al., 2021; Mhillaj et al., 2018). In the BluLife study, significant increases were found for cinnamic acid derivatives in plasma and urine, which may provide an insight into the potential mechanism of action regarding polyphenols and cognition.

5.9 Conclusion

Overall, the results from this study suggest that cognitive function may be enhanced 2 hrs after WBB treatment. Domains of cognitive function which were significantly improved included visuospatial working memory and episodic memory, as well as improvements in psychomotor processing. A significant improvement in endothelial function was observed following WBB consumption versus placebo across all ages, except the 8–10-year-olds, indicating that WBB may acutely improve vascular health. Nevertheless, the effects on CBF requires further investigation. Further research should be conducted to assess whether the bioavailability and metabolism of blueberry polyphenols changes through the life-course.

Chapter 6

The BluFlow Study: Investigating the effects of Chronic Wild Blueberry Supplementation on Cognitive Function and Cardiovascular Function in Healthy Older Adults

Statement- The design of the study was conceived jointly by our supervisory team, and me, Eleanor Wood. I designed and programmed the cognitive battery with some assistance from Dr Adrian Whyte. Cognitive testing on all participants was conducted by me, whilst all FMD procedures were conducted by Eleanor Wood, all other test day procedures (TCD, phlebotomy etc) were conducted jointly by myself and Eleanor Wood. For analysis, I analysed all cognitive data, Eleanor analysed all vascular data. Cerebral blood flow was analysed jointly by myself and Eleanor Wood. Gut microbiome samples collection was conducted by myself and Eleanor Wood, analysis of microbiome samples and DNA sequencing was conducted by Chaz Mein at Queen Mary University of London and microbiome bioinformatics was conducted by Robin Mesnage at King's College London.

6.1 Introduction

As outlined in Chapter two, a growing number of research studies have been conducted looking at the effects of blueberry polyphenols on cognitive function in older adults. This research is driven by the ever-increasing interest in the maintenance of cognitive health as we age, as well as reducing the risk of developing age-related neurological diseases such as Alzheimer's disease and vascular dementia. As mentioned in chapter one, the incidence of dementia is high and continues to increase, with figures predicting more than one million cases by 2025 (NHS, 2020). Therefore, understanding whether dietary habits, such as increasing polyphenol consumption by increasing intake of anthocyanin-rich blueberries,

leads to cognitive benefits, and other health benefits, such as vascular health, is critically important.

As covered in chapter 2, our current understanding of the effects of WBB supplementation in older adults is growing but remains limited, with scope for further investigation. Overall, the current literature suggests that WBB supplementation may have positive effects for executive functioning following 3 months and 6 weeks consumption respectively (Miller et al., 2017; Schrager et al., 2015) and episodic memory following 3 months consumption (Miller et al., 2017; Whyte et al., 2018) in healthy older adults. Acutely, no published studies have thus far demonstrated any treatment related effects for significant cognitive improvement in healthy older adults. However, the findings from the BluLife study indicated some interesting results which deserve further investigation. Overall, acute consumption of WBB polyphenols revealed cognitive benefits, particularly during the total word acquisition measure of the AVLT task, as well as an overall improvement in Corsi Blocks task accuracy. These findings indicate that a single dose of WBB polyphenols may positively enhance episodic and working memory. More related to older adults, age group analysis revealed significant improvements for total acquisition performance following WBB consumption relative to placebo; denoting improvements to the episodic memory domain (see Chapter 5).

The cognitive effects observed thus far may be attributed to changes to peripheral vascular function, as demonstrated in chapter 5, whereby significant improvement in FMD following acute WBB consumption relative to placebo were observed. It is widely documented that poor vascular health correlates with the incidence of developing neurological disorders as age increases (Satizabal et al., 2016). Although reasons for this is still unclear, evidence suggests that poor arterial health leads to a decrease in circulation, including cerebral blood flow. As a

result, may limit the amount of oxygen, nutrients and energy to the brain; negatively impacting the neurological functions, and chronically may lead to pathological changes which may causes neuro-vascular disorders such as vascular dementia (O'Brien and Thomas, 2015). Furthermore, it is known that CBF naturally decreases as we age, reasons which could be related to cardiovascular dysfunctions as ageing progresses, such as arterial stiffening and elevated blood pressure (Tarumi & Zhang, 2018). Similarly, the BluLife study showed lower levels of cerebral blood flow velocity amongst the 65-80 year olds relative to younger age groups in the BluLife Study (chapter 5, section 5.6.1).

The published evidence thus far for vascular health following WBB in healthy older adults is limited. To my knowledge, no studies have yet provided evidence to show that WBB intervention can improve FMD acutely or chronically in healthy older adults. However, in the BluLife study, a significant improvement was observed for FMD amongst the 65-80 year olds after acute consumption of WBB relative to placebo. Therefore, including FMD as a measure of vascular health in the BluFlow study will be critical to understand whether repeated daily consumption of WBB has any positive effects for endothelial function. Moreover, no significant effects of WBB were seen for diastolic or systolic blood pressure in the BluLife study. Previously published studies assessing chronic consumption of WBB for blood pressure have brought mixed findings. For instance, Rodriguez-Mateos et al. (2019) observed a significant decrease in 24-hour ambulatory systolic blood pressure following 1-month daily consumption of WBB intervention. Moreover, Dodd et al. (2019) observed a reduction in diastolic blood pressure following 3-month consumption of WBB relative to placebo, but this failed to reach significance (Dodd et al., 2019). Similarly, although a significant main effect of treatment was not found, Whyte et al. (2018) observed reduced systolic blood pressure 3 months post consumption of a WBB extract in healthy older adults, but this did not reach

significance. Consequently, further investigations of the effects of repeated daily dosing with WBB on blood pressure are warranted, therefore both office blood pressure and 24hr ambulatory blood pressure will be investigated in this study.

One proposed mechanism of action behind improvements in cognition following WBB interventions may be due to improved CBF mediated by improvements in vascular function. As mentioned previously, this cerebrovascular effect may subsequently promote neurogenesis, increase the expression of brain derived neurotrophic factor and enhance synaptic plasticity. Previous studies using fMRI have demonstrated significant increased observed increased neural activity during a working memory task following a daily consumption of WBB for 4 months relative to placebo, in older adults with MCI (Boespflug et al. 2018). Similarly, Bowtell et al. (2017) observed significant increase in cerebral blood flow assessed using arterial spin labelling, in healthy older adults following 3 months consumption of WBB. Despite the lack of treatment effects for CBF parameters documented in the BluLife study, it has not yet been ascertained whether chronic supplementation may induce changes in cerebral blood flow parameters assessed using transcranial doppler ultrasound (TCD), therefore the effects of chronic WBB consumption on CBF in healthy older adults will be assessed using TCD.

The importance of wellbeing is crucial across all stages of life. However, it is reported that wellbeing decreases as we age due to factors such as reduced physical health or increased loneliness (Steptoe et al., 2015). The evidence for improvements to mood following WBB consumption in older adults is lacking. In terms of chronic consumption, Whyte et al. (2018) failed to find any treatment-related effects for mood following WBB after 3 and 6 months consumption of WBB in healthy older adults. Similarly, Miller et al. (2017) did not find any

effects of treatment on mood following 3 months of WBB consumption. Nevertheless, a trend towards improved mood was observed by Krikorian et al. (2010), whereby older adults exhibiting mild memory complaints reported less depression-related symptoms following three months' consumption of a WBB intervention. In the BluLife study, there was a significant treatment-related reduction in negative affect following WBB consumption relative to placebo, when assessed prior to the task battery. Nevertheless, given the limited evidence to date, assessment of mood following WBB consumption in older adults requires further investigation and will be measured in the BluFlow study.

As demonstrated by the BluLife study, a number of circulating metabolites were significantly increased following WBB treatment compared to placebo following acute consumption. In this study, the collection of 24hr urine samples and blood samples will be repeated to understand if 3 months consumption affects these metabolite levels in a similar way.

Increasing evidence suggests that a healthy gut microbiome may positively influence human health, including cognitive health. Studies have demonstrated that people who consume a polyphenol-rich diet often have a healthy gut microbiota (Pei et al., 2020). Interestingly, it has been suggested that polyphenols act as a prebiotic, a source of feed for the microbiota (Alves-Santos, 2020), and therefore people who consume flavonoid-rich diets have healthier gut microbiotas which may lead to better health outcomes. Vendrame et al. (2011) conducted a crossover trial looking at how the consumption of WBB containing 375 mg, for six weeks, altered the microbiome. Results revealed that Bifidobacterium strain, a 'good' bacteria, significantly increased for the WBB-treated group and not for those treated with placebo. Further research is necessary to confirm these beneficial effects, however these studies suggest WBB polyphenols may have the potential to contribute towards creating a healthier

microbiome by increasing the quantity of healthier strains and reducing the number of harmful strains that may play a role in health and ultimately cognitive function. As an exploratory outcome, this study aims to collect faecal samples to assess whether a daily consumption of WBB polyphenols for three months alters the gut microbiome in terms of microbial composition and quantity. This may also give us an insight into whether different microbiome phenotypes may have an effect on how different people respond to WBB intervention. Correlation analysis between polyphenol metabolites, microbiome, cognitive function and vascular function will also be conducted to understand how these variables are influenced by each other.

In summary, the BluLife trial and previously published RCTs emphasize the need for further research investigating the effects of repeated daily WBB polyphenol supplementation on cognitive function, vascular health and CBF, in older adults. The general methodologies (as described in detail in Chapter 4) will be similar to those used in the BluLife to allow for a direct comparison of the acute and chronic effects of WBB polyphenols on cognitive performance, vascular function and cerebral blood flow. As before, the vascular data will be presented more comprehensively in a separate thesis (Wood, 2022), whilst the focus of this chapter will be cognitive change resulting from 3-months supplementation with WBB and linking these to any changes in flow-mediated dilation and blood pressure. It is anticipated that a 3 months consumption of WBB polyphenols, will lead to an improvement in cognitive function, as well as improved endothelial function. Relatedly, an increase in metabolites will be observed in both plasma and urinary samples for WBB-treated participants, which may correlate with improved cognitive performance. Finally, comparison of the gut microbiota profile from WBB-treated participants will show an increase in beneficial bacterial groups, an effect which may be mediated by an increase of circulating polyphenol metabolites in plasma.

6.1.1 Research Aims

1. To determine the effects of blueberry (poly)phenols vs placebo on cognitive performance in healthy older adults at baseline and 84 days after daily consumption, assessed using a cognitive battery consisting of 4 different tasks assessing different cognitive domains including the AVLT, Corsi blocks tapping task, Serial 3s and 7s and the Task-Switching Test.
2. To determine the effects of blueberry (poly)phenols vs placebo on flow-mediated dilation (FMD), office blood pressure and 24hr ambulatory blood pressure in healthy older adults at baseline and 84 days after daily consumption. This will be presented in a separate thesis (Wood, 2022)
3. To determine the effects of blueberry (poly)phenols on cerebral blood flow in healthy older adults at baseline and 84 days after daily consumption.
4. To measure the acute effects of blueberry (poly)phenols vs placebo on mood in healthy older adults at baseline and 84 days after daily consumption
5. To assess the effects of blueberry (poly)phenols vs placebo on the microbiome of healthy older adults at baseline and 84 days after daily consumption.
6. To assess the effects of blueberry (poly)phenols vs placebo on plasma and urinary polyphenols in healthy older adults at baseline and 84 days after daily consumption.

6.2 Methods and Materials

The methodology for cognitive testing, mood, FMD, TCD, office blood pressure, plasma and urinary sample analysis, and questionnaires administered, were described in detail in Chapter

4. In the sections below, additional methodological details specific to BluFlow that were not covered in Chapter 4 will be described.

6.2.1 Ethics

The BluFlow study was granted ethical approval by the BDM Research Ethics Subcommittee at King's College London. Study Reference: RESC reference: HR-18/19-9091

6.2.2 Clinical Trials Study Registration

The BluFlow study was registered on clinical trials.gov and allocated the following Clinical Trials.gov ID: NCT04084457.

6.2.3 Study design

The study design was a double-blind, placebo-controlled parallel RCT. Participants who expressed an initial interest in the study were invited for a screening and cognitive familiarisation session. The cognitive and physiological data collected during this visit were taken purely for determining whether the participant was eligible to take part, and therefore this data was not included in subsequent analyses. In total, four visits were scheduled post screening if the participant was eligible: pre study visit 1 (to start 24hr urine collection, and to have the 24hr ambulatory blood pressure monitor fitted), study visit 1, pre-study visit 2, and study visit 2 (Figure 6.1). A 1 month follow-up (without participant taking any treatment) was also scheduled as part of the original study design. However, this was disrupted due to the COVID-19 pandemic and only a small number of participants completed the 1 month follow-up. For this reason, the results collected at this time-point were not analysed and will not be discussed in this thesis.

Figure 6. 1 Flow diagram demonstrating the BluFlow study process

6.2.4 Study Population

The study population for this study was healthy older adults between the ages of 65-80. The sample size number was calculated based on a previous study (Whyte et al., 2018) who

calculated that 30 individuals per treatment group would yield a power of 0.96. Whyte et al.'s (2018) study consisted of a population of 60 healthy older adults aged 65-80 years old performing a wide array of tasks including the AVLT, Corsi Blocks, MANT, and Serial Subtractions Task, following a chronic consumption of WBB treatments containing either 1.35, 2.7 or 7 mg of WBB anthocyanins. Therefore, a minimum of 60 participants (30 allocated to each treatment group) will be recruited for this study.

The majority of participants recruited for this study derived from organisations such as the University of the Third Age, demographically speaking were white, spoke British English as their first language and lived in relatively affluent middle-class areas of London. A total of 66 people were recruited for this study, however, two discontinued their participation due to disliking the taste of their allocated treatment. One was excluded due to high fasting glucose levels, and one discontinued due to changes in their medication. Due to the COVID-19 pandemic outbreak, the study was halted prior to completion. In total, 53 participants completed visits 1 and 2, with 17 of those completing visit 3 studies prior to university closure due to COVID-19 lockdown.

6.2.5 Study Outcomes

Primary objectives included cognitive function to assess the effects of chronic WBB consumption on cognitive function. Secondary objectives included resting and active cerebral blood flow, FMD, 24hr blood pressure, and arterial stiffness. Tertiary objectives included the office diastolic and systolic blood pressure. A number of exploratory variables were also

assessed. This included mood using PANAS (see chapter 4 for description), microbiota, analysis of plasma and urinary polyphenol metabolites, habitual diet measured using food frequency questionnaires and food diaries, physical activity, AND baseline characteristics.

6.2.6 Treatments

The participants who were randomly assigned to the blueberry treatment group were given sachets containing 26g of WBB powder. Participants who were randomly assigned to the placebo group also received 26g sachets of placebo, which was matched for macronutrients (fibre and carbohydrate) to the active treatment but contained 0 mg of anthocyanins. The anthocyanin content in the WBB powder was calculated to be 10.17mg per 1g of powder, meaning that a consumption of 26g of WBB powder gave roughly 264mg of anthocyanin per 26g sachet (26x10.17=264.42). The ratio of the powder is 6.849: 1, which means that 6.849g of fresh weight blueberry equals to 1g of the WBB treatment powder used in this study. Therefore, the daily consumption of the 26g of WBB powder consumed by participants in the WBB treatment groups equalled to a daily consumption of 178g of fresh blueberries (6.849x26=178.074). The anthocyanin composition of the WBB treatment powder was analysed in our lab by Zhicheng Zhang as per the protocol outlined in chapter 4 section 4.12.4. The dose for this study was chosen based on previous WBB studies which demonstrated improvements for cognitive function using this dose (Hein et al., 2019). In total, it was found that malvidins contributes to almost the majority at 42%, followed by delphinidins, petunidins, cyanidins and peonidins (Figure 6.2). Constituents of powders can be seen in Table 6.1.

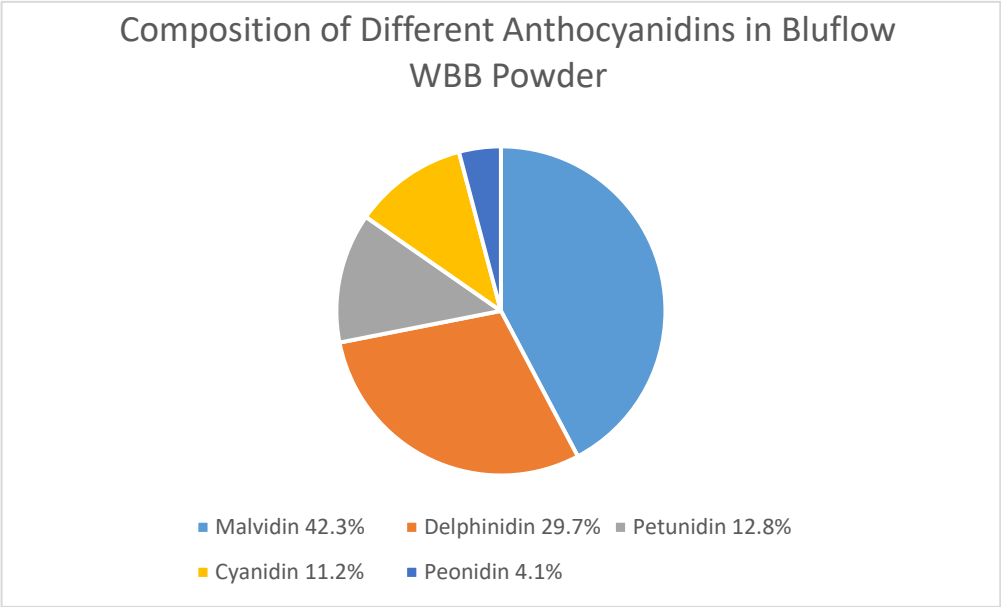


Figure 6. 2 The overall proportion of the anthocyanidins in the BluFlow WBB powder

Table 6. 1 BluFlow intervention and placebo powder composition

Ingredients	**Freeze-dried Wild Blueberry Powder (per 26 g)**	**Placebo (per 26 g)**
Anthocyanins (mg)	264.42	0
Glucose (g)	8.70	8.32
Fructose (g)	9.53	9.23
Fibre (g)	4.07	4.37
Vitamin C (mg)	*	*
Citric acid (g)	*	0.20
Silica (g)	0	0.26
Flavour %	0	2.31
Red colouring %	0	0.002
Blue colouring 1 %	0	0.036
Blue colouring 2 %	0	0.002
Purple colouring %	0	0.273

*not available

6.2.7 Blinding

The powders were shipped to the principal investigator at the clinical site. An independent party not involved with the research study blind-coded the treatments. This was blinded to

any researchers who have contact with participants in order to ensure double-blinding throughout the study procedure until statistical analysis was complete.

6.2.8 Drink Administration

Participants were randomly located into either the WBB or placebo group. Randomisation process was conducted at King's College London using a computerized research randomizer (www.randomizer.org). Participants enrolled on to the study were then assigned a screening number and a participant ID which was pre-assigned to either the blueberry or placebo treatment group. Treatments were labelled with two different codes 'AB' and 'YZ', ensuring that the process remined double-blinded throughout. Participants were provided with a three-month supply of the treatment, plus a number of spare sachets in case of accidents. Participants were requested to store treatments in a freezer throughout the intervention period.

6.2.9 Testing Procedure

All testing was conducted at the Metabolic Research Unit, Department of Nutritional Sciences, King's College London. Taking into consideration the study measurement timings, a maximum of two participants with staggered visit starting times for example 8am, and 8:30 am, were able to attend each day. Upon arrival to the clinic, the participant's 24 hr blood pressure monitor was removed, and their 24-hr urine jug collected, followed by a measure of body composition (TANITA). After this, the participants were taken into the vascular testing rooms and instructed to lie down for 10 minutes in order for their blood pressure and heart rate to stabilise. Three office blood pressure readings were then taken, followed by FMD, Sphygmocor and venepuncture. Before conducting the cognitive tasks, the participants were

provided with breakfast and asked to fill out a 24hr diet recall. Upon completion of breakfast the participants were taken into a room and resting TCD measurements were taken followed by the cognitive tasks and active TCD. Cognitive testing took place in separate private rooms so that the participants did not experience any distractions; however, the rooms were not soundproof. At the end of the tasks, the participants were provided with their allocated three-month long treatment sachets plus a food diary, and a return appointment was confirmed.

6.2.10 Twenty-Four-Hour Ambulatory Blood Pressure (ABPM) Measurement

The participants were asked to start 24hr blood pressure monitoring 24 hrs before each study visit. The type and brand of monitors used were A&D TM-2430 ambulatory blood pressure (ABP) monitoring devices. Before starting any measurements, the participants were shown how to use the monitors and what to do if it stopped functioning. A spare set of triple A batteries were also given in case the monitor became drained of battery. After this, the researcher chose a suitable cuff size (standard or large) depending on the width of the participant's upper arm. The cuff was placed roughly 2 cm above the inside of the elbow, and the air hose positioned around the back of the participants' neck. Once secured and comfortable, the participants had a choice of wearing the monitor either as a cross body bag or attached to the belt loop. Once satisfied with the fitting, a test blood pressure reading was taken so that the participants were aware of the sensations associated with each blood pressure reading. If the participant was happy to continue with the measurement, the researcher started the measuring process and programmed it to take measurements every 30 minutes during the day (7am-7pm) and hourly at night (7pm-7am). The participants were informed that if the monitor failed to take a reading for any reason for example, they were moving around too much, the monitor would attempt a second reading, so not to be alarmed

if they felt a second round of pressure. The participants were instructed to continue with their normal daily routine but to avoid any vigorous exercise. They were also asked to avoid the monitor getting in contact with water and to remove the monitors in order to take showers between the hourly breaks from 7pm onwards. When the participant arrived the following day, a researcher removed the monitor, and asked the participants for feedback on their experience with the measurements, collected the food and activity log sheets and the spare batteries. The data was then logged and saved into a secure online platform for analysis.

6.2.11 Food and Activity Log (24 hr ABPM)

To accompany the 24hr blood pressure monitoring, each participant was given a log where they were asked to record (1) the activity they were doing, such as walking, housework or sitting when a blood pressure reading was taking place and (2) any food and drink consumed and the time of consumption. This was in the case that the pressure was higher or lower than expected, the researcher can refer to the activity log to see what the participants was doing in that moment. In terms of food, it is known that processes involved in the act of eating, such digestion, can acutely affect blood pressure, as well as the types of food ingested, such as coffee (Papakonstantinou 2015). Therefore, it was important that food related information was available as well.

6.2.12 Seven-Day Food Diaries (EPIC)

All participants were asked to complete 7-day food diaries by EPIC (European Prospective Investigation of Cancer; University of Cambridge), at baseline and at three months in order

for their average macro-nutrient and micro-nutrient intake to be assessed and also to see if their habitual diet had significantly changed in any way during their intervention period. Participants were shown how to fill out the diaries and instructed to record their meals, snacks and drink including a breakdown of ingredients plus the weight of each food item. Each diary has clear sets of instruction to remind the participant how to complete their food logs in case they needed a reminder. Participants were told to include written recipes or food packaging if they found it helpful. The dietary compositions were assessed using Nutritics (Nutritics Professional Diet Analysis,V5.6 (Libro v0.9, Dublin). Phenol-Explorer database was used to assess the average daily polyphenol intakes based on the participants food diary entries (http://phenol explorer.eu). For this study the participants filled out the food diaries twice (at baseline and at 3 months), along with food frequency questionnaire and a 24hr dietary recall.

6.2.13 Arterial Stiffness

6.2.13.1 Pulse Wave Velocity (PWV)

PWV measurement was conducted to assess arterial stiffness, a measure that could indicate the development of cardiovascular disease in adults. The procedure consisted of the participants lying down for 10 minutes so that their blood pressure stabilised. The researcher then measured the carotid-to-heart and heart-to-femoral distance in centimetres, using a tape measure. Then, similarly to FMD, 3 ECG patches were placed in the 3-electrode system. Subsequently, applanation tonometry is conducted using the SphygmoCor® device (Smart Medical, Gloucestershire, UK) in the carotid artery (neck region), and femoral artery (upper thigh region). PWV was calculated by the time it takes for the pressure wave to move from

the aorta to the carotid artery, or the time it takes the pressure wave to go from the heart to the femoral artery.

6.2.13.2 Augmentation Index (AIx)

Augmentation Index measures the pressure waves in the radial artery located in the inside of the wrist. This periphery waveform differs from the waveforms in the central arteries measured in PWV because this waveform is lower at the periphery. Similarly, to PWV, applanation tonometry using the SphygmoCor® device is applied on the radial artery, situated in the inside of the wrist. The measurement is automatically captured based on the waveform shape. This ensures that all measurements are reliable and representative.

6.2.14 Microbiome Analysis

Collection of samples was conducted by myself and Eleanor Wood. DNA Extraction, Quantification, Normalisation and Sequencing was conducted by collaborators Chaz Mein and team at Queen Mary University of London and bioinformatics conducted by Robin Mesnage, King's College London.

6.2.14.1 Collection of Faecal Samples

Participants were asked to collect faecal samples 24 hr before each study visit, at baseline and at 3 months post intervention. For this, they were equipped with an OMNIgene®•GUT self-collection tubes (DNA Genotek, Ottawa, Canada). Each kit contained an instruction leaflet (see appendix P). When returning the sample at the study visit, the samples were immediately stored in a -80°C freezer until they were sent to an external laboratory for analysis (Queen Mary University of London).

6.2.14.2 DNA Extraction, Quantification and Normalisation

Microbial DNA was extracted from 0.25g of faecal product according to the PowerFecal protocol (Qiagen, Hilden, Germany). In order to quantify total microbial DNA, NanoDrop spectrophotometry (Thermo Fisher Scientific, MA,USA) and Qubit fluorimetry (Thermo Fisher Scientific, MA,USA) was used. A Biomek FX^{p} automated device (Beckman Coulter, CA, USA) was used to standardise the microbial DNA to 5ng/ml.

6.2.14.3 16S rRNA Sequencing of Faecal Microbiota

DNA amplification was conducted using PCR FastStart High Fidelity Taq (Roche) using forward and reverse primer and the amplification was verified on agarose gel (2%). A subsequent PCR cycle was conducted using 1ml diluted PCR product (dilution 1:100) for the addition of PCR adapters (Fluidigm, CA, USA) 0.5U of FastStart High Fidelity Taq (Roche), 4.5mM MgCl2, 0.1mM forward and reverse primer. A Tapestation D1000 tape (Agilent, CA, USA) to amplify the V3-V4 regions (Table 6.4).

Table 6. 2 v3-v4 16s RNA primer sequences.

16S V3-V4 Forward -ACACTGACGACATGGTTCTACACCTACGGGNGGCWGCAG (10µM)
16S V3-V4 Reverse - TACGGTAGCAGAGACTTGGTCTGACTACHVGGGTATCTAATCC (10µM)

6.2.14.4 Sequencing

The barcoded PCR products were pipetted and pooled and the overall pool was diluted to 4 nM. DNA sequencing was conducted according to the manufacturer's protocols using a MiSeq

(Illumina, CA, USA) for 300bp paired-end sequencing which generated a mean of 57,000 reads per sample.

6.2.14.5 Bioinformatics

The bioinformatics and data interpretation of the microbiota DNA sequences was conducted by Dr Robin Mesnage (King's College London) using QIIME (Quantitative Insights into Microbial Ecology) 2 Core 2021.2 (Bolyen et al., 2019). In order to identify and remove low quality bases, the sequences were trimmed to 19 bp and truncated to 290 and 260 bp. This resulted in a total of 15,394 ± 5,175 reads available to assign the taxonomy of each sample. The highly sensitive and specific VSEARCH algorithm (Rognes et al.., 2016) was used to categorize the sequences at 99% similarity. Further removal of non-bacterial variants or of sequences found in only one sample was conducted. The output derived from QIIME2 was then used for further analysis in R. Linear mixed model analysis of alpha diversity was conducting using sex, ethnicity and age as covariates. The total sum of beta diversity was calculated and analysed using a non-metric multidimensional scaling ordination of Bray-Curtis distance. Permutational Multivariate Analysis of Variance (PERMANOVA) with R package vegan 2.5-6 was used to evaluate statistical significance. MaAsLin 2 (Microbiome Multivariable Associations with Linear Models) was used to assess taxonomic composition differences. Lastly, random forest analysis was conducted using R package Caret (version 6.0-84) to predict if an individual is from the placebo or the WBB group. The outcome variables include (i) alpha diversity which is the diversity within a sample in terms of quantity and distribution of bacteria in a sample, and (ii) beta diversity which is diversity between samples and therefore gives an indication of how similar, or different, the diversity is between two samples. The impact of chronic WBB or placebo treatment on four main bacterial phylas

(*Firmicutes, Bacteroides, Proteobacteria and Verrucomicrobiota*) and how the composition of these phyla change between baseline and 3 months will also be assessed. Lastly, correlations between gut microbiome composition, cognitive function and vascular function, as well as correlations between the plasma polyphenol metabolites and cognitive function will be determined.

6.2.15 Statistical Analysis

Results (except where stated) were analysed using SPSS (Version 27.0, IBM, UK). Cognition, mood, CBF, FMD, blood pressure and metabolites data were analysed using linear mixed modelling (LMM). A linear model was performed for every dependent variable of each cognitive task: AVLT (total acquisition, immediate recall, proactive interference, retroactive interference, delayed recall, word recognition), Corsi Blocks (correct sequence, correct blocks), Serials (Serial 3s accuracy, serial 7s accuracy), TST (accuracy, RT), Mood (PANAS positive score pre-tasks, positive score post-tasks, negative score pre-tasks, negative score post-tasks). A linear model was performed for every dependent variable of vascular health (flow mediated dilation, blood pressure) and cerebral blood flow (blood flow velocity, pulsatility index). Lastly, a linear mixed model was performed for each of the polyphenol metabolites in plasma and in urine. Treatment was included as a fixed factors and baseline performance included as a covariate. In order to model repeated measures in TST, an unstructured covariance matrix was implemented. Switch Trial (0,1,2,3,4) and Task Type (high/low, odd/even), as well as interactions with treatment (Treatment x Switch trial, Treatment x Task Type, Treatment x Switch Trial x Accuracy) were also included in the model as fixed factors to understand how cognitive load differs between different treatment groups.

Post-hoc analysis using a Bonferroni correction to correct type 1 error, was applied to all pairwise comparisons where there were significant treatment-related effects (p<0.05).

The vascular results are presented more comprehensively in a separate thesis (Wood, 2022), however the results found for flow-mediated dilation and blood pressure will be presented here to help elucidate potential mechanisms of action behind any cognitive improvements observed in the BluFlow study.

6.3 Results

6.3.1 Baseline Demographics

Table 6. 3 Baseline Characteristics of the BluFlow study population. Values are mean (SD)

	Placebo group	**WBB group**	**p-value**
Gender (m/f)	12/17	12/20	N/A
Age	70.76 (3.81)	69.44 (3.48)	0.82
Ethnic Origin			
White British	80.65%	75%	N/A
White Other	9.68%	15.63%	N/A
White Irish	3.23%	3.13%	N/A
Caribbean	-	3.13%	N/A
Indian	3.23%	-	N/A
Cerebral Blood Parameters			
Blood flow velocity (cm/s)	53.6 (7.95)	54.9 (7.23)	0.597
Pulsatility index	1.18 (0.27)	1.02 (0.17)	0.065
Cognitive Measures			
AVLT			
Total Acquisition	44.95 (9.52)	44.72 (7.63)	0.260
Immediate Recall	5.23 (1.88)	5.28 (1.67)	0.473
Proactive Interference	-0.45 (2.28)	-0.16 (2.21)	0.959
Retroactive Interference	2.88 (1.92)	3.27 (2.09)	0.549
Delayed Recall	7.83 (3.13)	6.81 (3.16)	0.979
Word Recognition-List A (out of 15)	13.25 (1.39)	12.65 (1.65)	0.667

Word Recognition-List B (out of 35)	31.96 (3.06)	30.77 (3.87)	0.071
Corsi Blocks			
Correct Sequence	16.09 (3.87)	15.27 (3.38)	0.965
Correct Blocks	27.59 (3.80)	26.88 (3.33)	0.725
Reaction Time (ms)	1871.85 (383.79)	1862.09 (477.60)	0.706
Serials			
Serial 3s Accuracy	21.74 (9.69)	21.10 (9.72)	0.821
Serial 7s Accuracy	17.32 (8.04)	14.56 (7.70)	0.696
TST			
Overall Accuracy	0.88 (0.17)	0.82 (0.23)	<0.001*
Overall Reaction Time (ms)	987.54 (330.06)	1106.86 (400.39)	0.007*

Data are means (SD) or numbers (%)

**Denotes significant difference between groups (p<0.05)*

6.3.2 Cognitive Function

Table 6. 4 Pre-consumption and post-consumption raw scores for placebo and WBB for AVLT, Corsi Blocks, Serials, and TST. LMM fixed effect statistics for treatment show significantly better performance following wild blueberry treatment for AVLT immediate recall and TST overall accuracy. Significantly improved performance was observed following placebo for AVLT delayed recall.

Outcome Variable	*Baseline Placebo Mean (SD)*	*Baseline WBB Mean (SD)*	*Post-consumption Placebo Mean (SD)*	*Post-consumption WBB Mean (SD)*	*Treatment Effect*
AVLT					
Total Acquisition	44.95 (9.52)	44.72 (7.63)	46.75 (8.24)	45.50 (8.03)	*F(1,46)=0.036, p=0.851*
Immediate Recall	5.23 (1.88)	5.28 (1.67)	5.28 (1.49)	5.92 (1.71)	*(F(1,46)=4.321, p=0.043**
Proactive Interference	-0.45 (2.28)	-0.16 (2.21)	-0.48 (1.58)	0.04 (1.87)	F(1,47)=1.607, p=0.211
Retroactive Interference	2.88 (1.92)	3.27 (2.09)	2.12 (1.86)	2.64 (1.98)	*F(1, 49)=11.374, p=0.360*
Delayed Recall	7.83 (3.13)	6.81 (3.16)	9.43 (2.57)	7.48 (3.14)	*F(1,47)=5.042, p=0.029**
Word Recognition- (out of 15)	13.25 (1.39)	12.65 (1.65)	13.48 (1.69)	13.12 (2.17)	*F*(1,49)=0.0.29, *p*=0.886
Word Recognition-List B & distractor words (out of 35)	31.96 (3.06)	30.77 (3.87)	31.72 (4.18)	29.77 (5.61)	*F*(1,51)=0.742, *p*=0.393
Corsi Blocks					
Correct Sequence	16.09 (3.87)	15.27 (3.38)	15.40 (4.17)	14.92 (3.45)	*F(1, 48)=0.085, p=0.772*
Correct Blocks	27.59 (3.80)	26.88 (3.33)	26.52 (4.89)	26.42 (4.76)	*F(1,48)=0.189, p=0.666*
Serials					
Serial 3s Accuracy	21.74 (9.69)	21.10 (9.72)	25.17 (6.37)	21.16 (9.11)	*F(1,38)=0.0.748, p=0.393*
Serial 7s Accuracy	17.32 (8.04)	14.56 (7.70)	16.79 (6.94)	14.80 (7.40)	*F(1,43)=0.002, p=0.968*
TST					
Overall Accuracy	0.88 (0.17)	0.82 (0.23)	0.81 (0.25)	0.89 (0.17)	*F(1,90.12)=5.13, p=0.026**
Overall Reaction Time (ms)	987.54 (330.06)	1106.86 (400.39)	1063.39 (364.67)	1078.14 (359.73)	*F(1,89.5)=0.35, p=0.559*

*Significant at p< 0.05

6.3.2.1 Auditory Verbal Learning Task

As can be seen in Figure 6.3 A, the general performance of participants was as expected with a gradual increase in the number of words recalled over the first 5 recalls, followed by a decrease in recall when the interference list was introduced. Performance increased again when participants were asked to recall the original word list following both a short and long delay. A significant main effect of treatment was found for immediate recall (R1) with improved performance seen in the blueberry-treated group (mean words recalled= 5.92) compared to placebo (mean words recalled=5.28)) ($F(1,46)=4.321$, $p=0.043$; Figure 6.3 B). Interestingly, and contrary to previous work, a significant main effect of treatment was found for the delayed recall measure with the placebo group outperforming those treated with blueberry ($F(1,47)= 5.042$, $p=0.029$), indeed the placebo group recalled 9 (out of 15) words compared to the blueberry group who recalled only 7 words (Figure 6.3 C). No effects were observed for total acquisition, proactive interference, retroactive interference or word recognition.

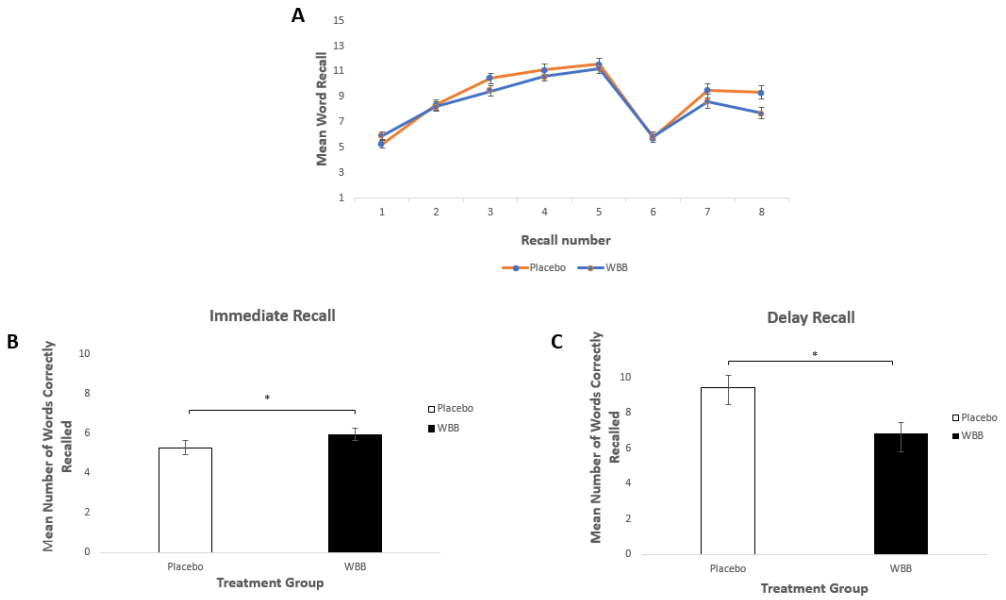


Figure 6. 3 **A)** Mean number of words (± SEM) correctly remembered per recall. Mean word recall increased between recall 1-5 for both interventions. This is to be expected as learning of the same word list increases. At recall 6 (list B) there is a decrease in the mean number of words recalled and there is also an expected decrease for delayed recalls 7 and 8. **B)** Mean immediate recall score (R1) (± SEM) following placebo and WBB consumption for 3 months. Significantly improved performance following WBB consumption in comparison to placebo (p=0.043). **C)** Mean delayed recall score (R8) (± SEM) following placebo and WBB consumption for 3 months. Significantly improved performance following placebo consumption relative to WBB (p=0.029)

6.3.2.2 Task Switching Test

6.3.2.2.1 Accuracy

For overall accuracy a significant effect of treatment was found, with the WBB performing significantly better (mean=0.89) versus the placebo group (mean=0.81) (F(1,90.12)=5.13, p=0.026; Figure 6.4). To see if performance was affected by task type or switch trial type, mean accuracy was calculated for the two task conditions (odd/even, high/low) and for each switch trial conditions (switch 1-4). Analysis revealed no significant difference between WBB and placebo for overall task type, meaning that performance was not different during the high/low or odd/even trial types following WBB or placebo treatment (F(1,90.29)=1.43, p=0.235). Similarly, no significant treatment x switch trial interaction was observed (F(3,90.92)=0.601, p=0.616) indicating that both groups of participants were equally affected by the switch as each other.

6.3.2.2.2 Reaction Time

For overall RT, it was observed that WBB performed quicker (mean=1078.14 ms) than the placebo group (mean=1106.86 ms); however, this failed to reach significance (F(1,89.5)=0.35, p=0.559). As with TST accuracy a treatment x switch trial interaction revealed no significant differences between WBB and placebo treatment on this measure (F(3,90.02)=1.06, p=0.372) and neither did a treatment x task type interaction (F(1,87.66)=0.124, p=0.725).

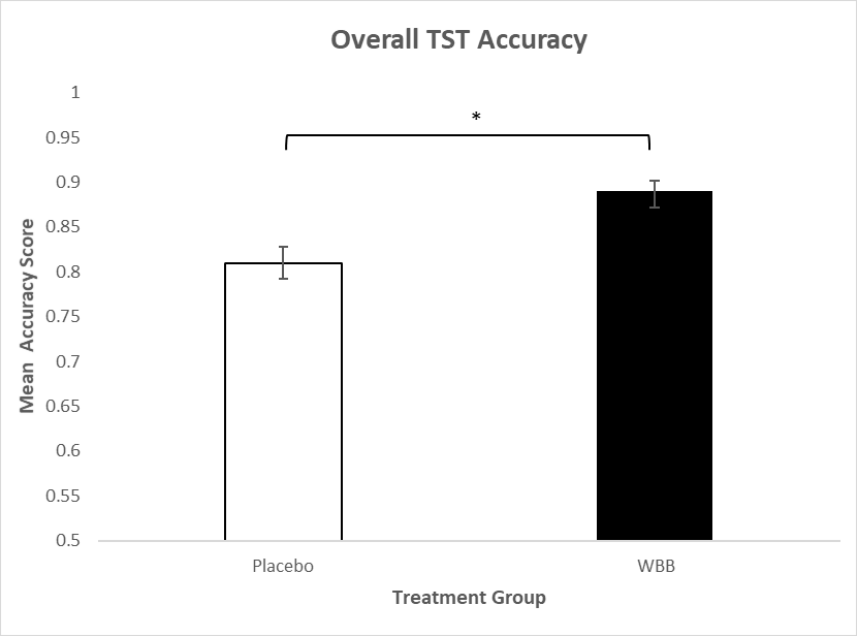


Figure 6. 4 Mean accuracy scores (+-SEM) showing significant effect of treatment, with higher overall accuracy for WBB compared to placebo $F(1,90.12)=5.13$, $p=0.026$.

6.3.2.3 Corsi Blocks

No significant effects of treatment were seen for the number of blocks correctly identified ($F(1,48)=0.189$, $p=0.666$), or the number of sequences correctly identified ($F(1, 48)=0.085$, $p=0.772$).

6.3.2.4 Serial Subtractions

No significant main effects of treatment were found for accuracy on either Serial 3s ($F(1,38)=0.748$,$p=0.393$) or serial 7s ($F(1,43)=0.012$, $p=0.914$).

6.3.3 Mood

The analysis revealed no significant main effects of treatment on positive or negative affect when assessed before or after completion of the cognitive battery (Table 6.5).

Table 6. 5 Positive and negative affect mood scores pre-cognitive tasks and post cognitive tasks at baseline and 3 months post-consumption

Dependent Variable	*Baseline Placebo Mean (SD)*	*Baseline WBB Mean (SD)*	*Post-consumption Placebo Mean (SD)*	*Post-consumption WBB Mean (SD)*	*Treatment effect*
Positive Affect					
Pre-cognitive Tasks	32.44(8.60)	35.92 (8.83)	34.08 (7.82)	34.16 (6.54)	$F(1,50)=0.297$, $p=0.588$
Post-cognitive Tasks	25.96 (8.44)	32.68 (11.03)	28.56 (9.82)	29.50 (8.77)	$F(1,50)=0.721$, $p=0.400$
Negative Affect					
Pre-cognitive Tasks	13.20 (3.40)	13.12 (4.56)	13.64 (4.02)	13.23 (3.35)	$F(1,51)=0.251$, $p=0.619$
Post-cognitive Tasks	13.24 (5.13)	13.64 (4.22)	13.84 (4.16)	13.65 (3.86)	$F(1,50)=0.275$, $p=0.602$

6.3.4 Cerebral Blood Flow

No differences were observed for either mean blood flow velocity or mean pulsatility index during the resting or active state, between the WBB or placebo group at 3 months. The results are outlined in Table 6.8.

Table 6. 6 Mean resting and active blood flow velocity and Pulsatility Index, pre- and post- consumption of WBB and placebo.

Dependent Variable	*Baseline Placebo Mean (SD)*	*Baseline WBB Mean (SD)*	*Post-consumption Placebo Mean (SD)*	*Post-consumption WBB Mean (SD)*	*Treatment Effect*
Resting					
Blood Flow Velocity	53.60 (7.95)	54.86 (7.23)	54.54 (9.72)	57.10 (6.45)	$F(1,24)=0.3.87$, $p=0.540$
Pulsatility Index	1.18 (0.27)	1.02 (0.17)	1.12 (0.20)	1.07 (0.22)	$F(1,24)=0.057$, $p=0.814$
Active					
Blood Flow Velocity	55.18 (10.14)	56.07 (4.80)	60.93 (10.92)	58.13 (6.89)	$F(1,7)=0.00$, $p=0.991$
Pulsatility Index	1.09 (0.19)	1.17 (0.16)	1.11 (0.23)	1.46 (0.36)	$F(1,24)=0.057$, $p=0.814$

6.3.5 Polyphenol Metabolites

6.3.5.1 Plasma Metabolites

Linear mixed modelling with baseline as a covariate revealed a significant effect of treatment for 7 compounds. Five of these metabolites significantly increased after a daily consumption of blueberries for three months, versus placebo. The 5 compounds included two pyrogallol derivatives: Pyrogallol-O-sulfate ($F(1,39)=6.17$, $p=0.017$) and 2-Methylpyrogallol-O-sulfate ($F(1,39)=4.40$, $p=0.042$), two benzene diols and triols: 4-Methylcatechol-O-sulfate ($F(1,44)=65.16$, $p=0.028$) and 4-Methylcatechol ($F(1,43)=18.70$, $p=0.011$) and one cinnamic acid derivative: Isoferulic acid ($F(1,44)=28.93$, $p<0.001$) (Table 6.7).

Furthermore, a significant effect of treatment was observed for two compounds following placebo consumption relative to WBB. These compounds included one benzoic acid derivative: vanillic acid ($F(1,5)=8.31$,$p=0.034$), and one phenylacetic acid derivative: phenylacetic acid ($F(1,16)=7.46$, $p=0.015$) (Table 6.7).

For total polyphenol concentration in plasma, a slight increase was observed at 3 months for the WBB-treated group alongside a concurrent decrease for the placebo group. However, this difference failed to reach significance ($F(1,44)=0.46$,$p=0.831$).

Table 6. 7 Mean ± SD plasma concentrations for pre- and post-intervention metabolites that showed significant effects

Plasma Concentration (nM)

(Poly)phenol Metabolite	Placebo Mean (±SD)		WBB Mean (±SD)		Treatment Effect
	Baseline	3 months	Baseline	3 months	
Benzoic Acid Derivatives					
Vanillic acid [b]*	1036±498	884±314	859±35	873±45	(F(1,5)=8.31,p=0.034)
Phenylacetic acid derivatives					
Phenylacetic acid [b]*	3355±5650	5242±2075	2898±2661	3171±2942	(F(1,16)=7.46, *p*=0.015)
Pyrogallol derivatives					
Pyrogallol-O-sulfate [a]*	343±284	172±239	166±176	250±345	(F(1,39)=6.17, *p*=0.017)
2-Methylpyrogallol-O-sulfate [a]*	441±541	266±323	607±1712	688±1620	(F(1,39)=4.40, *p*=0.042)
Benzene diols and triols					
4-Methylcatechol-O-sulfate [a]*	9769±6959	9196±7134	14164±19928	16764±17137	(F(1,44)=65.16, *p*=0.028)
4-Methylcatechol [a]*	1226±1001	1266±1044	1750±2002	2124±1736	(F(1,43)=18.70, *p*=0.011)
Cinnamic acid derivatives					
Isoferulic acid [a]*	847±2215	398±99	404±76	576±115	(F(1,44)=28.93, *p*<0.001)

Data is represented as (nM) ±SD

*Significance at the $p<0.05$ level for Treatment
[a]Higher metabolite concentration post WBB consumption
[b]Higher metabolite concentration post placebo consumption

6.3.5.2 Urine Metabolites

Linear mixed modelling with baseline as a covariate revealed a significant effect of treatment for 11 compounds. Ten of these metabolites significantly increased in urinary excretion after daily consumption of blueberries for three months, versus placebo. The ten compounds included one benzoic acid derivative: gallic acid ($F(1,50)=14.46$, $p<0.001$), one benzene diols and triols: Catechol-O-1-glucuronide ($F(1,50)=4.34$, $p=0.042$), seven cinnamic acid derivatives: Caffeic acid-3-glucuronide ($F(1,50)=11.21$, $p=0.002$), Isoferulic Acid 3-O-Sulfate ($F(1,50)=6.61$, $p=0.013$), Ferulic Acid 4-O- β -D-Glucuronide ($F(1,50)=4.97$, $p=0.030$), isoferulic acid ($F(1,50)=47.86$, $p<0.001$), p-coumaric ($F(1,49)=4.75$,$p=0.034$), Caffeic acid 4-O-sulfate ($F(1,48)=4.58$, $p=0.037$), Caffeic acid 3-O-sulfate ($F(1,48)=4.53$, $p=0.039$) and one hippuric acid derivative: Hippuric acid ($F(1,50)=9.43$, $p=0.003$) (Table 6.10). A significant effect of treatment was observed for one compound following placebo consumption relative to WBB with increases in one cinnamic acid derivative: 4-Feruloylquinic acid ($F(1,50)=6.89$, $p=0.011$) (Table 6.10). In addition to this, a trend towards significance was observed for four compounds following WBB consumption relative to placebo. These compounds included one benzene diols and triols: 4-Methylcatechol-O-sulfate ($F(1,46)=13.69$, $p=0.061$), one cinnamic acid derivative: Dihydro ferulic Acid 4-O-Sulfate ($F(1,50)=3.53$, $p=0.066$), one flavonol derivative: Quercetin-7-O-ß-D-glucuronide ($F(1,50)=2.90$, $p=0.095$) and one valeractone derivative: (4R)-5-(3',4'-Dihydroxyphenyl)-gamma-valerolactone-4'-O-sulfate ($F(1,30)=3.80$, $p=0.061$) (Table 6.10). Overall, the WBB-treated participants excreted higher levels of total polyphenols following 3 months treatment, whilst a decrease in total polyphenol excretion was observed for the placebo group ($F(1,50)=15.245$,$p<0.001$; Figure 6.5).

Table 6. 8 Mean ± SD urinary concentrations for pre- and post-intervention metabolites that showed significant effects

Total 24 h Urinary Excretion (µg)

(Poly)phenol Metabolite	Placebo Mean (±SD)		WBB Mean (±SD)		Treatment Effect
	Baseline	3 months	Baseline	3 months	
Benzoic Acid Derivatives					
*Gallic acid [a]**	254±301	119±90	940±528	242±197	(F(1,50)=14.46, p<0.001)
Benzene diols and triols					
4-Methylcatechol-O-sulfate [a#]	6575±4261	7383±4499	8448±10090	14612±12842	(F(1,46)=13.69, p=0.061)
*Catechol-O-1-glucuronide [a]**	1217±1120	1156±886	358±327	1844±1300	(F(1,50)=4.34, p=0.042)
Hippuric acid derivatives					
*Hippuric acid [a]**	217±187	29222288 ±936296	192±201	3031317±13245 334	(F(1,50)=9.43, p=0.003)
Cinnamic acid derivatives					
*Caffeic acid-3-glucuronide [a]**	417±405	350±245	6519±5559	495±218	(F(1,50)=11.21, p=0.002)
Dihydro ferulic Acid 4-O-Sulfate [a#]	5215±5896	4277±2076	82130±48174	5855±3912	(F(1,50)=3.53, p=0.066)
*Isoferulic Acid 3-O-Sulfate [a]**	2773±1322	2432±389	6481±2836	2673±426	(F(1,50)=6.61, p=0.013)
*Ferulic Acid 4-O- β -D-Glucuronide [a]**	32778±54139	30468±25617	4762±265	43554±25669	(F(1,50)=4.97, p=0.030)
*Isoferulic acid [a]**	93539±176406	101535±125850	522±644	556146±308779	(F(1,50)=47.86, *p*<0.001)
*p-coumaric [a]**	1644±1714	1249±992	40909±31237	1405±741	(F(1,49)=4.75,p=0.034)
*4-Feruloylquinic acid [b]**	3072±3298	4578±5570	26453±14071	4040±4759	(F(1,50)=6.89, p=0.011)
*Caffeic acid 4-O-sulfate [a]**	295±315	230±182	8529±4919	298±199	(F(1,48)=4.58, p=0.037)
*Caffeic acid 3-O-sulfate [a]**	3303±2760	3171±2135	44946±59166	4311±2505	(F(1,48)=4.53, p=0.039)
Flavonol derivatives					
Quercetin-7-O-ß-D-glucuronide [a#]	97±74	79±12	735±0	85±21	(F(1,50)=2.90, p=0.095)
Valeractone					
(4R)-5-(3',4'-Dihydroxyphenyl)-gamma-valerolactone-4'-O- sulfate sodium salt [a#]	5289±3330	4319±3458	1445±91	4929±3078	(F(1,30)=3.80, p=0.061)

Data is represented as (nM) ±SD
*Significance at the p<0.05 level for Treatment
[#]Trend towards significance at p< 0.10 for Treatment
[a]Higher metabolite concentration post WBB consumption
[b]Higher metabolite concentration post placebo consumption

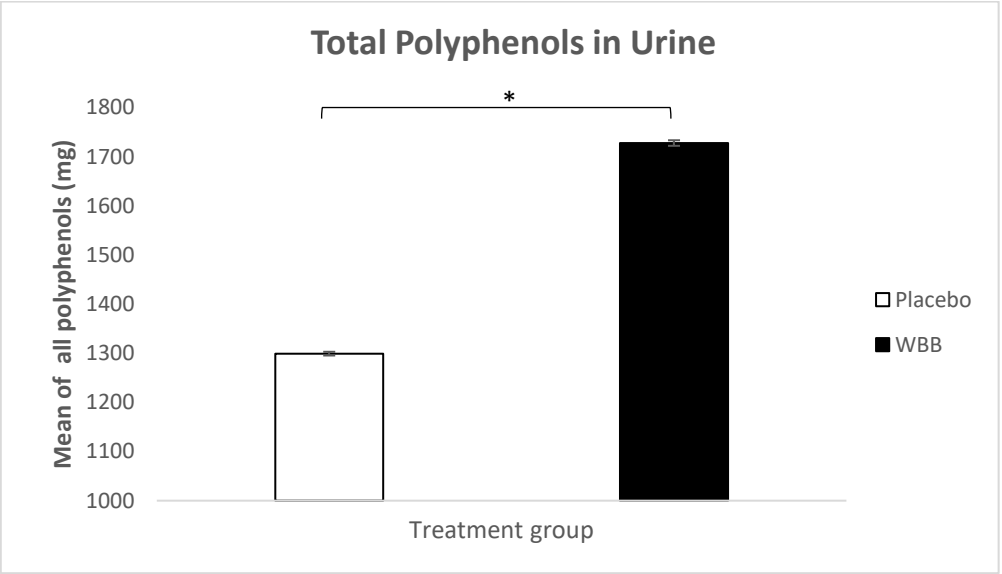


Figure 6. 5 Mean (± SEM) total polyphenol in urine 3 months post consumption of placebo and WBB participants.

6.3.6 Gut Microbiome

Following the daily consumption of WBB and placebo for 3-months, analysis of the overall microbiome diversity found a significant increase in alpha diversity (diversity within a sample) across the whole cohort at 3 months, irrespective of treatment (p=0.04; Figure 6.6 A). Notably, this increase in alpha diversity was mainly driven by increases in mean alpha diversity of the WBB group. Nevertheless, when the groups were individually analysed, no significance was observed for WBB alone. In addition to this, beta diversity remained unchanged after 3 months consumption of both WBB and placebo (Figure 6.6, B). When the four main bacterial phylas were analysed, no significant changes were observed after 3 months consumption of either WBB or placebo for *Firmicutes, Bacteroides, Verrucomicrobia* and *Proteobacteria* (Figure 6.6, C-F). However, a trend towards significance did exist for increased *Firmicutes* and decreased levels of *Bacteroidetes and Proteobacteria* following WBB.

To better understand the reasons for these effects, the amplicon sequence variants (ASV) were agglomerated in order for the taxonomic distribution in all the samples to be evaluated. The results showed that the faecal microbiome compositions were highly individualised as some individuals showed high levels of certain bacteria such as *Prevotella* and lower levels of bacteria such as *Bacteroides,* whereas some individuals demonstrated high *Bacteroides* levels but nothing detected for *Prevotella*. This may provide evidence as to why certain individuals microbiome composition were not affected by WBB composition, whereas others were. Nevertheless, our findings were in line with the microbiome composition of typical western individuals with microbiome composition in our cohort consisting of 52% *Firmicutes*, 34% *Bacteroidetes*, 9% *Proteobacteria* and 2% *Verrucomicrobia* (Figure 6.7). Subsequently, multivariable association analysis was conducted between clinical metadata and taxonomic

abundances to understand if quantity of any genus was changed following WBB consumption. The results found 3 genera increased including *Ruminiclostridium 9* (p = 0.0007), *Ruminiclostridium 5* (p = 0.002), *Parabacteroides* (p = 0.003). However, it must be taken into consideration that there was a high rate of false discoveries therefore further research needs to be conducted to confirm these observations.

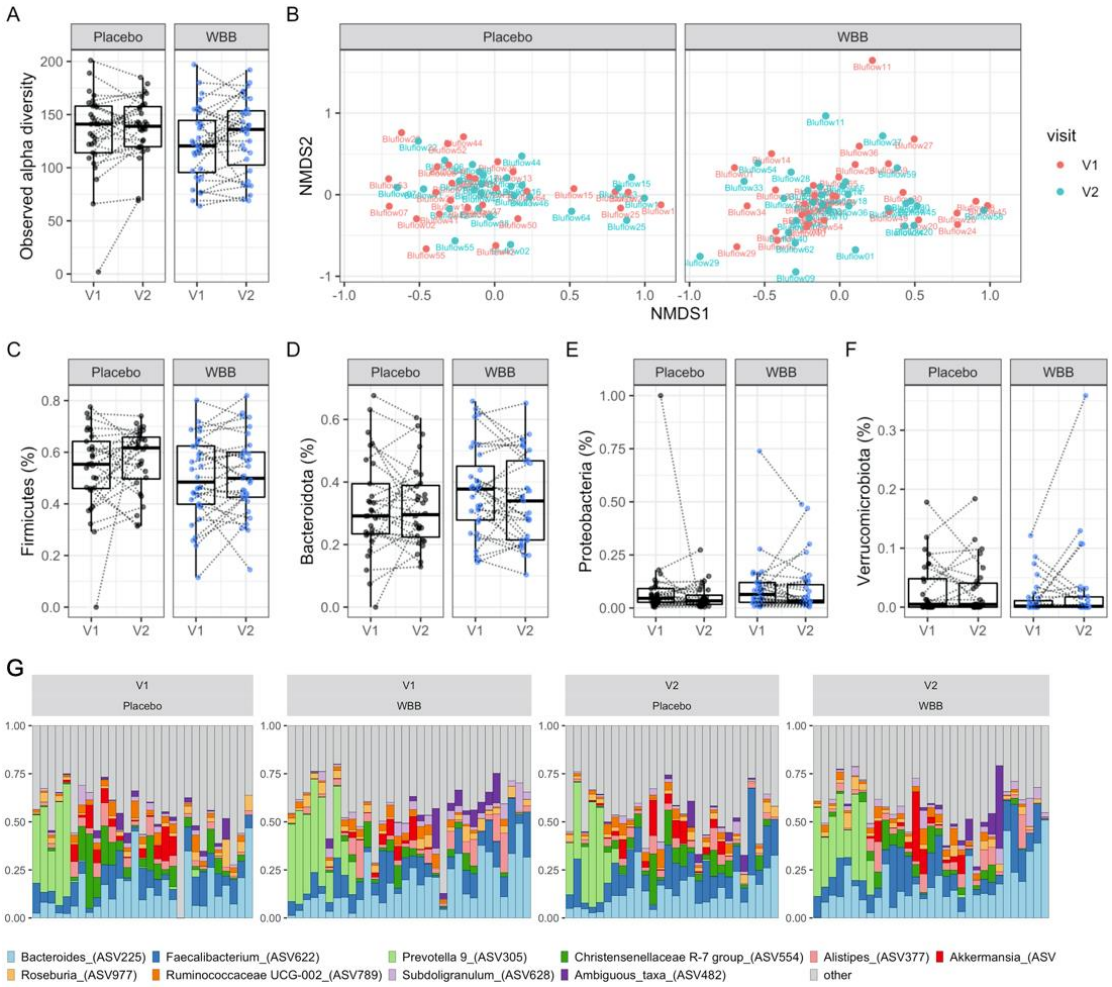


Figure 6. 6 Gut microbiome composition between WBB and placebo including A) Observed alpha diversity and B) Beta diversity. The abundance of C) *Firmicutes*, D) *Bacteroides*, E) *Proteobacteria* and F) *Verrucomicrobiota*, G) graphs to show the 10 most abundant bacteria genera for WBB and placebo at V1 and V2.

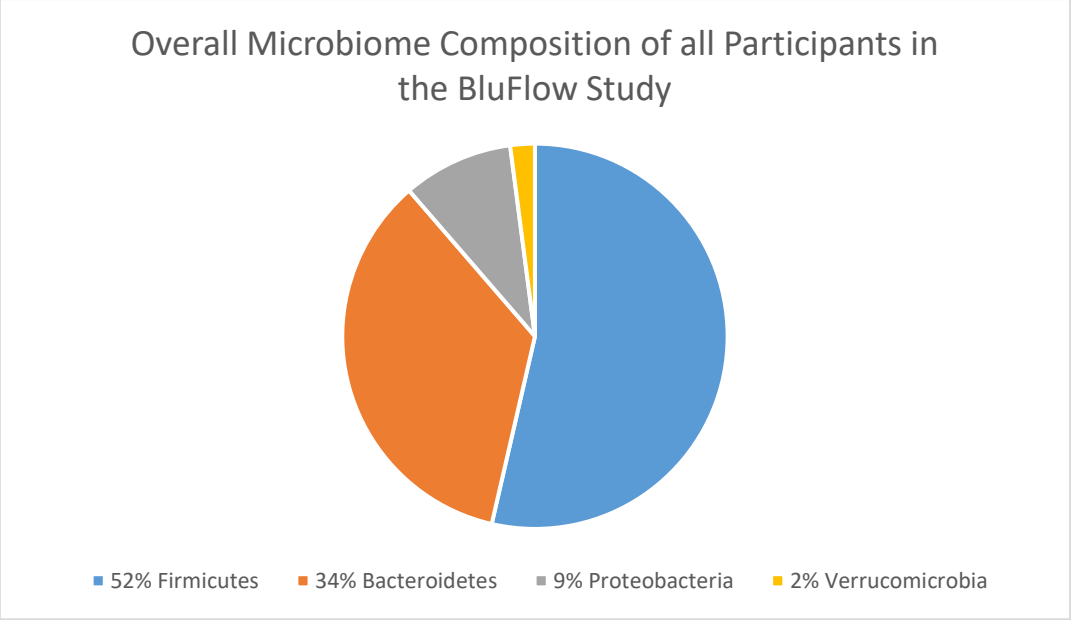


Figure 6. 7 The composition of four main bacterial phyla observed in the microbiome of participants in the BluFlow study. Values are means across visit 1 and 3 months post-consumption).

6.4 Discussion

The primary purpose of the BluFlow study, was to investigate how 3-months' daily intake of WBB affected the cognitive functioning. It also aimed to expand on what cognitive domains are affected, if any, by a chronic intake of WBB polyphenols in healthy older adults. For cognitive function, 4 tasks were employed, looking at a range of domains including episodic memory, working memory, attention, executive function, and processing speed.

The results from the AVLT tasks were mixed, with positive effects seen for immediate recall for the WBB treatment group and positive effects seen for delayed recall measure for the placebo group. No effects were observed for either treatment on total acquisition, proactive interference, retroactive interference or word recognition. This contradicts previous findings as previously beneficial anthocyanin effects from blueberries have been found for delayed

recall measure (Whyte & Williams, 2014) in younger age groups (8-10 years old) and also in older adults with MCI (Krikorian et al., 2010). For healthy older adults, positive effects of blueberry polyphenols were previously seen for the word recognition measure (Whyte et al., 2018), and also by McNamara et al. (2018) in adults suffering from mild cognitive complaints, but this was not replicated in this study. Nevertheless, this is the first study to my knowledge whereby positive effects were observed for the immediate recall measure (recall R1) following WBB intervention for three months which suggests that chronic consumption of WBB polyphenols in this case may enhance the memory encoding process after initial exposure to new information. However, it seems like this did not benefit long-term storage as no effects for the WBB-treated participants were observed for total acquisition or delayed recall.

No main effect of treatment was found for any of the Corsi Blocks measure, nevertheless a higher score for the number of sequences correctly memorised was observed for the WBB treatment group, which was accompanied by overall quicker performance, but this failed to reach significance. This is in line with previous findings by Whyte et al. (2018) who found a trend for higher number of sequences correctly recalled at 3 months for the WBB intervention group compared to placebo. To my knowledge, other than this study, only the study by Whyte et al. (2018) has previously tested the effects of WBB polyphenols on the performance of a Corsi Blocks task, and this was also in an older population cohort. This clearly highlights the need for further investigation into this cognitive test, and its sensitivity to WBB polyphenols.

In terms of the serial subtractions task, the analysis revealed no significant main effect of treatment for Serials 3s or Serial 7s. This is also in line with previous work by Whyte et al. (2018) who found no effects of a WBB intervention on the performance of this task in a group

of healthy older adults. Although not identical to the serials task, Bowtell et al. (2017) assessed the effects of blueberry polyphenols on the performance in a 1-back and 2-back memory task, which is similar to the serials subtraction task as it requires the participant to memorise a previous stimulus. Here, Bowtell et al. (2017) found a trend towards significance for improvements in performance after blueberry supplementation containing 378mg of anthocyanins in healthy older adults. This suggests that WBB polyphenols may still have the capacity to ameliorate working memory domain but further investigation is recommended as to whether the serials subtraction task is sufficiently sensitive to WBB intervention.

In chapter two it was suggested that it is in the most cognitively challenging tasks where the benefits of a polyphenol-rich intervention are observed. In this study we see exactly this, where there is a significant main effect of treatment for TST accuracy, with the WBB intervention group performing significantly better compared to the placebo group after a 3-month intake. The TST has been developed for its increased cognitive demand due to the requirement of switching between two different sets of instructions (High/Low), (Odd/Even), as well as its long task duration (20 minutes). Supporting this finding for improved TST performance, Miller et al. (2017) also found significant improvement in overall accuracy score when the task type (High/Low, Odd/Even) is switched over in healthy older adults who consumed a WBB intervention containing for 3 months, versus participants who consumed placebo.

Overall, this research holds great importance, as in a society where improvements in healthcare, standard of living allows us to live longer, therefore it is crucial that our quality of life also remains. According to a national survey of 999 subjects aged 65 years or older, researchers found that responses when asked what are the most important concepts related

to quality of life for these subjects responses included the maintenance of social relationships, health, financial situations, psychological wellbeing, independence and solo activities to name a few (Bowling et al., 2003). Successful ageing, and maintenance of the activities listed, requires maintenance of cognitive function, and this study suggests that we may improve certain cognitive aspects through the consumption of blueberry polyphenols.

When comparing the cognitive effects observed in the BluLife study with the effects seen in this study, I found benefits for episodic memory in terms of AVLT total acquisition and proactive interference following acute placebo intake relative to WBB. In the BluFlow study, benefits for episodic memory were also observed, however, improvements for delayed recall were also observed following placebo intake therefore further research is needed to understand how chronic WBB consumption may affect the episodic memory domain. Nevertheless, baseline characteristics revealed that although not significantly, the placebo group scored higher for delayed recall compared to the WBB at baseline, therefore may be a reflection of their ability of recalling words, and not the ability of WBB supplementation to improve delayed recall. In this study, I observed a significant improvement of mean TST accuracy following chronic WBB consumption relative to placebo. Although a slight increase in accuracy was observed for the 65-80 year olds following acute WBB consumption relative to placebo in the BluLife study, this effect was not significant. This suggests that acute consumption may not be sufficient to mediate any executive functioning effects in older adults, and that chronic consumption should be taken into consideration when testing the effects of WBB on executive functioning in older adults. Nevertheless, the cognitive effects observed between acute and chronic is not directly comparable as the acute study was a crossover study and effects for both WBB and placebo were tested in each participant. As well as this, the dosages administered varied, with the BluFlow participants receiving a daily WBB

treatment containing 264 mg and the BluLife participants consuming a single serving of WBB juice containing 133.2mg anthocyanins, highlighting the potential effect of dosages on the observed executive function findings.

For vascular health, a significant improvement was seen for FMD following WBB consumption relative to placebo. The results for vascular outcomes are presented in a separate thesis (Wood, 2022). This observation supports previous research by Rodriguez-Mateos et al. (2019) who observed a significant increase in FMD following 4 weeks consumption of WBB in a group of healthy young males. Although the study population is not comparable to the BluFlow study population, Curtis et al. (2019) found a significant increase in FMD following WBB consumption for 6 months in a group of adults with metabolic syndrome. In the BluFlow study a significant reduction was observed for 24hr systolic blood pressure. Previous studies have also observed similar results, including Rodriguez-Mateos et al. (2019) who found a reduction in 24hr systolic BP in a group of healthy young males, following WBB consumption compared to placebo for 4 weeks. Again, although the study population is not comparable to the BluFlow study, Johnson et al. (2015), observed a significant reduction in both 24hr systolic and diastolic blood pressure after 8 weeks consumption of WBB in a group of adults with clinically diagnosed hypertension. Similarly, Basu et al. (2010) found significant reductions in systolic and diastolic blood pressure after 8 weeks consumption of WBB in a group of adults with metabolic disorders. However, to my knowledge the BluFlow study is the first study to show such effects in healthy older adults. This finding is promising as it believed that improvement of 1% FMD may reduce the risk of developing cardiovascular disease by 10-13% (Ras et al., 2012; Shechter et al., 2014; Inaba et al., 2010).

Interestingly, a study conducted by Smith et al. (2011) found that in adults diagnosed with obesity and hypertension, increased levels of FMD, predicted greater executive functioning. This supports the findings observed in the BluFlow study, whereby significant improvements in both FMD and executive functioning (TST accuracy) were observed following WBB. In addition to this, evidence in literature suggest that hypertension is associated with poorer cognition (Novak & Hajjar, 2010; Goldstein et al., 2013). In this study, significant improvements in 24hr systolic blood pressure were seen following WBB treatment; therefore, this is a promising finding which suggests that chronic consumption of WBB may positively affects vascular health and therefore cognitive health, which are two very important health outcomes amongst the ageing population.

This observed improvement in vascular function may help us understand the mechanisms of action behind improvements in cognitive health. One proposed mechanism is through the improvements to cerebral blood flow, however this study failed to find any significant difference in cerebral blood flow parameters measured by TCD following 3 months consumption of WBB. This was also the case for the BluLife study, whereby no effects were observed for CBF following acute consumption. Reasons for this lack of effect have already been discussed in chapter 5 and included the limitations associated with TCD measurements such as high variability in readings due to factors such as room temperature, movement, and also homeostasis, which ensures consistent regulation of blood flow velocity. In terms of the values observed in this study relative to the BluLife study, a mean of 57.10 cm/s was observed for resting blood flow velocity following 3-months treatment with WBB, whilst a mean of 64.98 cm/s was observed for resting blood flow velocity in the 2 hour period following WBB ingestion in the BluLife study.

Effects were seen for polyphenol metabolites in both plasma and urine. However, metabolites were also significantly detected following placebo consumption. Generally, the number of metabolites observed were not as high as expected from a daily intake of WBB powder for 3 months in plasma and urine. A higher number of metabolites were detected to have significantly increased in plasma and urine following acute consumption in the BluLife study. Nevertheless, it has to be taken into consideration that in the BluFlow study, participants did not follow a low polyphenol diet the day each study visit before whereas in the BluLife study the participants did adhere to a low-polyphenol diet. As well as this, the plasma samples were taken fasted, 24 hrs after their last WBB consumption. Lastly, many of the participants joined because they reported to enjoy eating blueberries, and often ate blueberries regularly. Therefore, the effects observed in the BluFlow study may be diluted by the contribution of polyphenols from the participants' habitual diets pre, during and post intervention consumption. Nevertheless, the total 24hr polyphenol metabolites level was significantly higher following WBB consumption relative to placebo, which reflects better blueberry bioavailability compared to a fasted plasma sample. The findings for increased metabolite levels in plasma and urine following WBB consumption, relative to placebo, is in line with previous polyphenol investigations including Feliciano et al. (2016) who observed a correlation for increased plasma and urine concentrations following consumption of cranberry polyphenols for 1 month. Lastly, although cognitive parameters were not assessed, Rodriguez-Mateo et al. (2019) found 21 positive correlations between plasma blueberry metabolites in plasma and flow-mediated dilation in humans following one months' consumption of wild blueberry treatment containing 300mg anthocyanins.

The findings for gut microbiome in this study were mixed, and no significant differences between placebo and WBB for alpha diversity was observed. Instead, an overall significant

difference between baseline and 3 months was observed across the whole cohort. Generally, the studies investigating the effects of blueberry polyphenol on the gut microbiome is limited. Although not comparable, studies looking at the effects following 3 months consumption of a whole-fruit aronia berry treatment containing a low dose of 30mg anthocyanins, observed significant increases in *Bacteroides* (Istas et al., 2019). This observation was replicated in the study by Queipo-Ortuno et al. (2012) who revealed that significant increase for *Bacteroides* were observed following 4 weeks consumption of red wine polyphenols. Similarly, Mayta-Apaza et al. (2008) looked at how cherry polyphenols influenced the microbiome composition in healthy humans and demonstrated that *Bacteroides* were greatly influenced by tart cherry polyphenols. Here the researchers postulated that the increase may be influenced by the fermentation of carbohydrates, which are documented to be the main feed for *Bacteroides* (Flint et al., 2012).

Reasons for the lack of microbiome differences between WBB and placebo in the BluFlow study may be due to the fibre content in placebo. Fibre is known to enhance the microbiome as it acts as a prebiotic (Scott et al., 2008; Kumar et al., 2020. A study investigating the effects of alpha diversity and fibre revealed a positive association between the intake of soluble fibre and alpha diversity in the gut microbiome (Costabile et al., 2012). This may help explain why alpha diversity was increased following WBB, but also following the fibre-matched placebo in the BluFlow study.

Moreover, the cherry juice investigated in Mayta-Apaza et al.'s (2008) study, which was reported to contain no fibre content, revealed very little abundance of *Prevotella* and *Ruminococcus* specifically, which the authors attributed to the lack of fibre content in the intervention. Nevertheless, the levels of *Prevotella* in the BluFlow did not appear to be

affected by fibre content as varying levels were found following both treatments, which were matched for fibre. This highlights the need for further investigation into the effect of fibre on the gut microbiome. As well as this further research needs to be conducted to underpin whether the fibre effect observed in WBB intervention studies may influence the gut health of placebo-treated participants, as well as WBB-treated participants. Lastly, individual differences were observed between different strains, indicating that the gut microbiome is personalised, and may give us an insight into why differences were not observed between WBB and placebo-treated individuals. Different human metabotypes, and how they may vary upon consumption of polyphenols is discussed in the review by Cortés-Martín et al. (2020). Although inconclusive, the researchers suggest that the bacterial strains present in the microbiota, as well as the chemistry of each metabolite such as the location of the functional groups can all influence how polyphenols are metabolised in the microbiome and therefore the effects that they may exert may also vary. Moreover, it was discussed that the presence of certain enzymes in the human body capable of metabolising polyphenol compounds may also contribute towards inter-individual differences observed (Cortés-Martín et al., 2020). These factors highlighted are just a small number of reasons as to why people's microbiomes respond differently upon polyphenol consumption. One proposed method of testing how different metabotypes respond to polyphenols is by grouping participants with similar metabotypes and comparing how the microbiomes between these groups vary upon polyphenol consumption (Cortés-Martín et al., 2020). Overall, more research investigating how different individuals microbiomes respond to polyphenolic interventions, particularly wild blueberry polyphenols, is required.

6.5 Conclusion

Findings from the BluFlow study suggest that consuming WBB daily for 3-months, in addition to normal habitual diet, may positively enhance performance on some cognitive tasks including AVLT immediate recall, which assesses short-term episodic memory, but not AVLT delay recall, and executive functioning in healthy older adults aged 65-80 years old. Further research looking at working memory is recommended to see if a daily consumption of WBB polyphenols for 3 months enhances the performances in tasks including serial subtraction task, and Corsi Blocks tapping task. Nevertheless, findings from this study suggest that WBB polyphenols have the ability to maintain, and improve performance, during cognitively demanding tasks, supporting previous research. No change in mood was observed therefore further investigation into the positive and negative affects schedule in older adults is also recommended. It could also indicate lack of sensitivity of the PANAS measure when detecting changes in mood in older adults, as previous investigations using a similar cohort (Whyte et al., 2018) have also failed to observe any changes in this measure.

The findings in this study also demonstrate significant improvements in endothelial function, and 24hr systolic blood pressure in the WBB-treated older adults relative to those who consumed the placebo intervention. Moreover, significant metabolites associated with WBB consumption were found in plasma and urinary samples, which were correlated with the observed improvements in cognition and vascular health. This may give us insight into the improved effects observed for cognitive and vascular health. Lastly, analysis into the gut microbiome composition revealed that the microbiome may be personalised and that different metabotypes may be contributing to the way polyphenols are processed in the human body, and therefore may influence the health effects exerted by WBB polyphenols.

Chapter Seven

The Final Discussion

7.1 Overview of this PhD programme

The aim of this PhD was to investigate the effects of wild blueberry polyphenols on cognitive function and cardiovascular health in humans. To do this, investigations were conducted which acknowledged the research gaps that existed at the time of protocol development including further research into the effects in teenagers and middle-aged participants, as well as considering the impact of blueberry polyphenols across a range of cognitive domains.

The main aim was to increase the knowledge surrounding the effects of blueberry polyphenols on cognition in humans through a series of task assessing a range of cognitive domains (Figure 7.1). Alongside cognitive function, the assessment of vascular function took place in order to understand if a relationship exists between these two outcome variables through the rationale that better peripheral blood flow, may lead to increased cerebral blood flow, which may lead to improved cognitive performance. Further exploratory analyses on the levels of polyphenol metabolites in plasma and urine, pre- and post- consumption, were conducted in order to further my understanding regarding the absorption and metabolism of wild blueberry polyphenols in the human body. Lastly, the gut microbiome has gained much attention as recent evidence suggests that polyphenols may influence gut microbiome health in humans, as well as affecting the way polyphenols are metabolised and therefore the effects they exert. As an exploratory analysis, the effects of treatment on the gut microbiome were analysed, with the aim of further understanding whether the gut microbiome may influence the cognitive and cardiovascular effects observed in the BluFlow study.

In this chapter, the results observed from the two trials will be summarised and discussed relative to existing knowledge. Lastly, future directions based on the results and methodology of these studies will be suggested. A summary of the main findings per chapter are highlighted below.

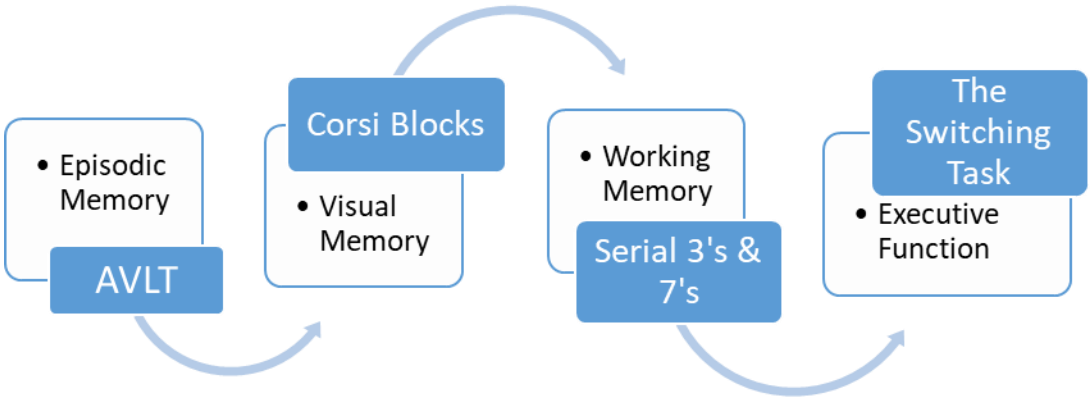


Figure 7. 1 Cognitive tasks used in the BluFlow study, and domains each task assessed.

7.2 Summary of Findings

7.2.1 Study one: The BluLife Study

As a reminder, the BluLife study followed a randomised double-blind, placebo-controlled, crossover design, whereby cognitive and vascular effects, alongside other exploratory variables, where tested at baseline and at 2 hrs following consumption of WBB and placebo. The participants of different age categories including 8-10, 14-18, 22-28, 40-50 and 65-80 years old, where exposed to the treatments only once and had a washout of between 2-7 days in between treatments. The WBB treatment had a content of 133.2 mg, equivalent to approximately 90g of fresh weight blueberries.

The purpose therefore was to investigate the acute effects of WBB polyphenols relative to placebo on cognitive function and cardiovascular health across the life course. The aims included investigating the effects of wild blueberry polyphenols on cognitive function, notably the cognitive domains of episodic memory, working memory and executive functioning. The investigation into the effects of wild blueberry polyphenols on vascular function, in terms of flow mediated dilation and blood pressure was also conducted. Study objectives also included assessment of changes in mood and cerebral blood flow in response to an acute dose of WBB polyphenols. Finally, the exploratory study outcomes were to assess the levels of polyphenols metabolites quantified in plasma and urinary samples pre-and post-consumption. It was hypothesised that acute consumption of WBB will significantly improve episodic memory and executive functioning across the life-course and that significant flow-mediated dilation improvements will be observed amongst the older age groups following WBB consumption versus placebo. It was also hypothesised that increased cerebral blood flow and increased levels of polyphenols metabolites in plasma and urine samples post consumption of WBB relative to placebo would be observed.

Main findings are as follows:

1. Consumption of whole freeze-dried blueberry powder containing 133.2mg anthocyanins maintained AVLT Total Acquisition performance (total numbers of words correctly recalled R1-R5) 2 hrs post consumption across the life-course, whereas performance declined following placebo consumption at 2hrs. Specifically, significant improvements in total acquisition following WBB consumption relative to placebo were seen in the 8–10 year-olds, and 65–80 year-olds.

2. Analysis revealed that although proactive interference performance worsened post-consumption for both treatments, a significant main effect of treatment found that placebo attenuated a larger decline in performance which was seen following WBB consumption. Individual age group analysis found that for the 8-10 year olds, placebo attenuated a larger decline compared to WBB, and for the 65-80 year olds performance at 2hrs was significantly better compared to baseline following placebo. In contrast to this, age-group analysis revealed that proactive interference was significantly reduced following WBB consumption relative to placebo for the 22–28-year-olds.

3. Consumption of WBB, compared to placebo, significantly improved the number of correct sequences correctly tapped in the Corsi Blocks task, as well as the correct number of blocks tapped, for all age groups, at 2 hrs post consumption.

4. Acute consumption of WBB relative to placebo significantly improved overall TST accuracy amongst the 14–18 year olds, and acute consumption of placebo significantly improved overall TST accuracy amongst the 8–10-year-olds, 2 hrs post consumption. In terms of reaction time, observations were seen for significant quicker performance following placebo compared to WBB for the 14-18 year olds, and significant quicker performance for following consumption of WBB relative to placebo the 8-10 year olds, 2 hrs post consumption.

5. Mean negative affect score at 2hr following WBB was significantly lower than placebo before commencement of cognitive tasks across the life-course. Age group analysis revealed significant reduction in negative affect mood scores following WBB relative to placebo before commencement of cognitive tasks.

6. Consumption of WBB, compared to placebo, significantly improved endothelial function 2 hrs post consumption for the 14-18 year olds, 22-28 year olds, 40-50 year olds and 65-80 year olds. However, no changes in cerebral blood flow were seen.

7. In plasma, a significant increase was found for 13 polyphenol metabolites following acute WBB consumption relative to placebo. Observations revealed a significant increase for one polyphenol metabolite following placebo consumption relative to WBB at 2hrs.

8. A significant increase was found for 26 polyphenol metabolites following WBB consumption relative to placebo over a 24hr urine collection and none following placebo consumption, relative to WBB.

9. Total polyphenols analysis revealed a significant increase in the total metabolites detected in urinary excretion following WBB consumption compared to placebo.

A significant effect of treatment was found for total acquisition (sum of R1-R5) following acute WBB consumption in the BluLife Study, with participants memorising a mean of 1.81 words more after consuming WBB powder compared to placebo consumption. In literature, the effects observed for AVLT following acute WBB consumption in children and adults is

consistent, indicating that this domain may be particularly sensitive to WBB polyphenols amongst these ages.

For instance, Barfoot et al. (2018) who conducted an acute, parallel study found a significant effect of treatment for total acquisition (R1-R5), whereby 7-10 year olds in the WBB treatment group recalled 2.5 words more than the placebo-treated group 2hrs post consumption. In support of this, Whyte, Schafer and Williams (2016) also observed significant improvement in total acquisition performance following a 253 mg of anthocyanin WBB consumption, whereby total acquisition recall was significantly improved following WBB relative to placebo at 1.15hrs post-consumption.

AVLT improvements following acute WBB consumption has also been observed for delayed recall (R6) (Barfoot et al., 2018). In accordance with this, Whyte and Williams (2015), who conducted a study with similar design with treatments consisting similar anthocyanin content to the content in the BluLife study (143 mg), also saw significantly improved performance for delayed recall following WBB consumption relative to placebo.

Acute WBB consumption has also shown effects for Word recognition measure, with Whyte et al. (2021) seeing the maintenance of performance following WBB consumption containing 725mg anthocyanins, but significant decrease in performance for placebo group, at 2, 6 and 8 hrs post-consumption in middle-aged adults. In addition to this, Whyte, Schafer & Williams (2016) observed significant improvements in delayed word recognition following WBB treatment containing 127 mg anthocyanins versus placebo 6 hrs post consumption, in children aged 7-10.

Surprisingly though, it appears that delayed recall, and word recognition, was unaffected in the BluLife study. The evidence thus far suggests that acutely, WBB polyphenols may have an effect for the delayed recall aspect of episodic memory, particular in children. Nevertheless, individual age group analysis revealed no treatment-related effects for the 8-10 year olds in my study. It has to be taken into consideration however that the sample size of the 8-10 year olds was considerable smaller (n=12) compared to samples sizes in literature; therefore, it may be the case that a larger sample size is required for any positive effects to be observed. Additionally, Whyte et al. (2021) observed delayed recall effect following WBB containing much higher doses of anthocyanins compared to the BluLife study (475mg vs 133.2 mg respectively) which may suggest a dose-dependency relationship exists, whereby higher doses may be necessary for effects to be observed for delayed recall following acute WBB consumption amongst the older age-groups in the BluLife study.

An effect for significantly reduced proactive interference (List A recall 1- List B) was observed following placebo consumption relative to WBB. This is a finding also shared by Whyte & Williams (2015), who saw this effect 2 hrs post-consumption of a placebo, relative to WBB. Individual age group analysis revealed that this effect was significant amongst the 8-10 year olds and the 65-80 year olds. Interestingly, these are the age groups which performed best during total acquisition following WBB consumption, meaning that the participants encoded list A more strongly, and list B less well. As a reminder, proactive interference is defined as reduced ability to memorise new material as result of previously encoded material. It could be argued that the significantly better performance observed for total acquisition, which is the total sum of List A words in trials 1-5, in the BluLife study, may have resulted in increased

proactive interference due to the participant encoding List A more strongly than List B following WBB consumption relative to placebo.

In the BluLife study, a significant improvement in performance during the Corsi Block task was observed, with participant tapping a significantly higher number of correct sequence and blocks following WBB consumption relative to placebo. In literature, no studies thus far have shown an effect of acute WBB consumption versus placebo on visuo-spatial working memory performance in terms of accuracy score. To my knowledge, this is also the first study to demonstrate significant effects for this domain following acute WBB consumption; however, the effect of acute WBB consumption for visuospatial working memory requires further investigation. The Corsi Blocks tapping task has not been as widely used in RCTs investigating the effects of WBB, or indeed berry polyphenols, on cognitive function, compared to AVLT for example, with only one other study in literature having used the task (Whyte et al., 2018). Before it is decided whether the Corsi Blocks task is sufficiently sensitive to test the effects of WBB, further testing is required.

In addition to this, no effects for the Serials Subtraction tasks, assessing working memory, were observed. This is in line with previous studies who also failed to observe significant improvements in similar tasks, such as the n-back task, following acute WBB consumption containing 507.79mg anthocyanidins (Dodd et al., 2019). Although not directly comparable, Philip et al. (2019) found significantly better performance for serial 3s, but not serial 7s, following the acute consumption of a grape and blueberry extract (anthocyanin content in mg not reported) relative to placebo in a group of young adults aged between 18-25 years old. An effect has also previously been observed following acute cocoa consumption in young adults for serial 3s (Scholey et al., 2010) and for the more cognitively demanding serial 7s

(Massee et al., 2015). This might suggest that acute WBB polyphenol may not be effective enough to mediate any significant beneficial effects for the working memory domain in this instance, or maybe that the serial 3s and 7s subtraction task is not sufficiently sensitive to detect small changes in cognitive performance following WBB consumption.

TST findings were mixed, in the BluLife study we observed a significant treatment x age interaction with the 8-10 year olds performing significantly better following placebo and the 14-18 year olds performing significantly better following WBB consumption relative to placebo. This effect can be explained through the analysis of their reaction time, as it was found that the 8-10 year olds performed significantly quicker and the 14-18 year olds performed significantly slower following WBB, which suggests that a trade-off between speed and accuracy exists. Nevertheless, these effects were only observed amongst younger age categories, and the performance during Task-Switching Test remained unchanged amongst the middle-aged adults and older adults. To my knowledge, no studies thus far have used the TST as a way of assessing executive functioning in acute WBB studies. Nevertheless, previous studies assessing executive functioning via tasks such as Modified Flanker Task (Whyte, Schafer, & Williams, 2016) have also shown significant improvement in accuracy scores during an executive function task following acute WBB consumption relative to placebo in young children. Improvement in reaction time during executive function tasks following acute WBB consumption have also been previously observed in children (Whyte, Schafer & Williams, 2017; Barfoot et al. 2018; Whyte et al., 2020). Moreover, Whyte et al. (2021) observed significant benefits in accuracy scores following acute WBB consumption versus placebo during both the MANT and The Cued Go/No-Go task in middle-aged adults. As with the effect seen for delayed recall improvement in executive functioning following acute WBB

consumption has been primarily observed in children and middle-aged adults. Individual age group analysis did not reveal any effects for older age categories in either accuracy or reaction time for TST. Nevertheless, observations for improvements to executive functioning in middle-aged adults used higher doses of anthocyanins compared to the BluLife study (475mg vs 133.2 mg respectively) which may again suggest a dose-dependency relationship exists, whereby higher doses may be necessary for effects to be observed for executive functioning following acute WBB consumption amongst the older age-groups in the BluLife study.

Overall, this study supported the hypothesis that cognitive function significantly improves following acute WBB consumption relative to placebo across all of the age groups recruited to this study. Significant improvements were also observed following WBB for episodic memory and working memory. Executive function was also influenced by acute WBB treatment, but the effects here were dependent upon the age of the participants, specifically for the 14-18 year olds. Although the results from this study did not support the hypothesis regarding significant effect of WBB for cerebral blood flow, a significant improvement for endothelial function was observed following WBB consumption relative to placebo at 2 hrs. In addition to this, it is worth mentioning that the anthocyanin content in the WBB powder was 133.2mg, which is lower compared to levels used in previous research investigating the acute effects of WBB including 253mg anthocyanins (Whyte, Schafer & Williams, 2017; Barfoot et al., 2018; Whyte et al., 2020; Barfoot et al., 2021), 475 mg (Whyte et al., 2021), and 578.8 mg (Dodd et al., 2019). Therefore, the benefits observed in the BluLife study are promising as they may indicate that relatively achievable amounts (119g fresh weight blueberries) are sufficient for improvements to be observed, and that recommendations including regular and realistic portions can be considered.

7.2.2 Study Two: The BluFlow Study

The BluFlow study followed a randomised double-blind placebo-controlled parallel design, whereby cognitive and vascular effects as well other exploratory variables were tested at baseline and at 3-month post consumption. The participants in this study who were aged between 65-80 year olds, consumed either a placebo or a WBB treatment containing 264mg of anthocyanin (equivalent to 178g of fresh weight blueberries) daily for 3 months.

The primary study outcomes included cognitive function, notably the cognitive domains including episodic memory, working memory and executive function. Additionally, the study assessed the effects of treatment on vascular function, in terms of flow mediated dilation and blood pressure, as well as cerebral blood flow and mood analysis. Exploratory study outcomes included the levels of polyphenol metabolites quantified in plasma and urinary samples pre- and post-consumption, as well as gut microbiome composition pre- and post-consumption of either WBB or placebo for 3 months. It was hypothesised that chronic consumption of WBB would significantly improve episodic memory, and that significant flow-mediated dilation improvements will be observed following WBB consumption versus placebo. It was also hypothesised increased cerebral blood flow velocity and increased levels of polyphenols metabolites in plasma and urine sample post consumption of WBB relative to placebo.

Main findings are as follows:

- Daily consumption of whole freeze-dried blueberry powder containing mg 264 mg anthocyanins for 3 months, relative to placebo, significantly improved AVLT immediate recall, whereas daily consumption of placebo for 3 months, relative to WBB, significantly improved AVLT delayed recall

- Daily consumption of WBB for 3 months, relative to placebo, significantly improved mean TST accuracy

- Daily consumption of WBB for 3 months, relative to placebo, significantly improved endothelial function (FMD) and significantly reduced 24h systolic blood pressure, but no effect for cerebral blood flow parameters were seen

- Daily consumption of WBB for 3 months significantly increased the levels of 5 metabolites in plasma, whereas placebo for 3 months significantly increased the levels of 2 metabolites in plasma, relative to WBB

- Daily consumption of WBB for 3 months significantly increased the levels of 10 metabolites in 24hr urine whereas significant increases were seen for 1 metabolite following placebo

- For total polyphenols concentration, a significant increase was observed at 3 months following WBB consumption, relative to placebo, in 24hr urine collection

- For gut microbiome, no significant differences were observed for alpha diversity (diversity within a sample) or beta diversity (diversity between samples) following 3 months consumption of WBB or placebo, and no significant changes were observed after 3 months consumption of either WBB or placebo for *Firmicutes, Bacteroides, Verrucomicrobia* or *Proteobacteria*

In the BluFlow study, a significant effect for immediate recall was observed following WBB consumption relative to placebo for 3 months. A review by Lamport & Williams (2020) highlight that cognitive benefits following polyphenolic dietary interventions were mainly seen for the verbal memory domain. Indeed, the observations observed thus far for episodic following chronic WBB consumption have been consistent. In addition to episodic memory benefits seen in children following WBB intervention, findings showing the effects of WBB for episodic memory have also been repeated amongst healthy older adults. For instance, Whyte et al. (2018) observed word recognition benefits following supplementation with a WBB extract containing 7mg anthocyanins, compared to the placebo after 3 months, but not after 6 months. Note, as mentioned in chapter 2, the WBB extract was formulated with L-cysteine and L-glutathione in order to facilitate the stabilisation of their anthocyanin content and, in turn, allow a higher rate of absorption than might be possible at doses used in previous studies. Miller et al. (2017) also found significant effects for episodic memory following WBB consumption containing 460 mg anthocyanins, for 3 months in healthy older adults whereby the participants in the blueberry group made fewer repetition errors on day 90 compared to day 0, whereas the opposite effect for the placebo-treated participants were seen. In addition to this, Krikorian et al. (2010) observed improvement in V-PAL cumulative learning and delayed word recall during the CVLT following consumption of WBB juice (anthocyanin dosage varied according to body weight) in adults with mild cognitive impairment. Similarly, McNamara et al. (2018) observed improvements in HVLT recognition memory discrimination performance following 6 months consumption of WBB treatment which provided 269 mg of anthocyanins per serving. Lastly, although not comparable, findings by Bensalem et al. (2019) revealed that a combined grape and blueberry intervention benefitted immediate recall

amongst older adults, with increased benefits observed amongst the participants with higher levels of cognitive impairment.

No effects were observed for proactive interference, or retroactive interference. Unexpectedly, delayed recall was significantly improved following placebo relative to WBB. This finding does not support previous observations by (Whyte & Williams, 2015; Barfoot et al., 2018) who found improvements for delayed recall following acute WBB consumption. Nevertheless, it is worth noting that these effects have all been previously observed in young children, which could suggest that this delayed recall is particularly sensitive to WBB consumption in this age group, and that further investigation is required to understand the effects of chronic WBB consumption on delayed recall in healthy older adults.

These results, together with the observations for improved immediate recall in the BluFlow study, suggests that the episodic memory domain is particularly sensitive to WBB after chronic consumption, rather than acute in older adults. However, it has to be taken into consideration that four out of the five studies published to date looking at the effects of WBB on cognition in older adults all adopted a chronic RCT design (Schrager et al. (2015); Miller et al. (2017); Bowtell et al. (2017); Whyte et al. (2018), and only one followed an acute RCT design (Dodd et al., 2019) whereby no cognitive effects were observed. This presents the need for further studies investigating the acute effects of WBB on cognitive function in older adults, particularly after the significant effects of total acquisition observed in the BluLife study. In addition to this, as reported in Lamport & Williams review (2020), although verbal memory this may also be a reflection of the frequency of its usage in randomized clinical trials, and not just it sensitive to polyphenol consumptions.

The effect of WBB on visuospatial working memory has previously been documented in Whyte et al.'s (2018) study, whereby a trend (<0.07) towards significance for correct sequences was observed in healthy older adults following WBB consumption for 3-months. Nevertheless, this effect was not observed amongst the older adults following WBB consumption in the BluFlow study. Generally, the Corsi blocks tapping task has not been as widely used in RCTs investigating the effects of WBB on cognitive function, compared to AVLT, with only one other study in literature having used the task (Whyte et al., 2018). Therefore, further testing is recommended before it can be concluded that this task is not sufficiently sensitive to test the effects of WBB.

Similarly, no effects for the Serials Subtraction tasks were observed in either in the BluFlow study. This is in line with Whyte et al. (2018) who also failed to observe any significant effects for Serials 3'or 7s following chronic WBB consumption in healthy older adults. Nonetheless, although limited, there has been previous observations for the benefits of WBB for working memory following chronic consumption. For instance, the n-back task which has previously shown to be sensitive to WBB polyphenols, with healthy older adults performing significantly better following 3 months consumption compared to placebo (Bowtell et al., 2017). Similarly, to Corsi Blocks, the serial subtraction tasks have not been as widely used WBB interventions therefore, it would be useful to conduct further testing to ascertain its sensitivity to WBB polyphenols.

Lastly, significant improvements in TST accuracy were observed amongst the participants who consumed WBB for 3 months versus the placebo group. This is in line with observations by Miller et al. (2017) who also found a significant main effect of treatment for TST accuracy, with significantly improved performance following chronic WBB consumption relative to

placebo in healthy older adults. To my knowledge, no other studies thus far have shown any significant improvements in performance during executive functioning tasks in older adults. For instance, Whyte et al. (2018) who assessed executive function using the MANT in older adults following chronic WBB consumption also failed to find any significant effects of treatment. Similarly, Bowtell et al. (2017), who assessed executive function through the administration of the Groton maze timed chase test, the Groton Maze Learning test and the identification task also did not observe any positive effects for this domain following chronic WBB consumption. Lastly, Schrager et al. (2015) failed to observe any beneficial effects for performance of the trail making test part B but did observe a significant improvement in the Dual-Task Adaptive Gait Test (DTAG) whereby participants conducted less step errors in comparison to the placebo condition following WBB consumption relative to placebo.

Furthermore, it is evident that assessment for executive function using TST is not as widely used compared to AVLT which assesses episodic memory in older adults and may therefore be a reflection of its infrequent use as a task rather than its sensitivity to WBB polyphenols.

Lastly, as documented in chapter 2, executive function effects were observed amongst the more cognitively demanding switch trials of the switching task for Miller et al.'s (2017) study and the Dual Task Adaptive Gait Test in Schrager et al.'s (2015) study. Overall, this study supported the hypothesis that cognitive function significantly improves after 3 months daily WBB consumption relative to placebo in healthy older adults, as significant improvements in episodic memory and executive functioning were observed following WBB. The lack of observations for CBF in this study mimic those seen in the BluLife study and, once again, suggest that TCD may not be sufficiently sensitive to detect the changes in CBF following consumption of WBB polyphenols. Nevertheless, a significant improvement for endothelial function was observed following WBB consumption relative to placebo 3 months post

consumption, as well as significant improvement 24hr systolic blood pressure for the WBB treated group versus the placebo-treated group. Lastly the hypothesis regarding increased plasma and urinary metabolites following WBB consumption, as well differences between gut microbiome composition of WBB versus placebo should be kept pending and requires further research.

7.2.3 Physiological Effects

Significant effects for FMD following WBB consumption relative to placebo was found in both the BluLife study and BluFlow study, demonstrating that WBB polyphenols have significant benefits for endothelial function (results are discussed in Wood, 2022). This is in line with previous observations by Rodriguez-Mateos et al. (2019) who conducted a double-blind parallel trial with both acute and chronic time-points. Here, Rodriguez-Mateos et al. (2019) observed an increase in FMD by 1.5% following 2hrs consumption and an increase by 2.3% following 4 weeks consumption of a WBB treatment containing 150mg anthocyanins in healthy young males. Similarly, although not comparable due to a longer dosing length of 6 months, Curtis et al. (2019) observed improvements in FMD by 1.45% following a WBB treatment consisting of 364mg of anthocyanins for 6 months in older adults with metabolic syndrome.

In the BluLife study FMD increased by 1.12% following acute dosing, whilst the increase for FMD in the chronic BluFlow study was 0.86% amongst older adults aged 65-80 (Wood, 2022). Although both were statistically significantly different when compared to placebo, the increase following acute consumption was larger. Reasons for this may be because participants are older, or that the effects of WBB for endothelial function is time-dependent. In the BluLife study, the participants' measurements took place 2 hrs post consumption. However, in the BluFlow study the participants were asked to take their last treatment sachet on the morning of the pre-visit, meaning that FMD, and all the measurements, were assessed over 24hrs post consumption. Previous investigations looking into the time-dependency of

WBB on FMD found significant increases in dilation of the brachial artery between 1-2hr and 6 hr post consumption of WBB (Rodriguez-Mateos et al., 2013).

These observations were also accompanied by reduction of neutrophil NADPH oxidase activity, as well as increases in plasma metabolites at these time points. This might suggest that polyphenols exert their peak benefits for vascular health approximately 2 hrs post consumption, which might help explain the higher dilation increase (%) in the BluLife study compared to the BluFlow study post consumption of WBB.

The BluLife study failed to find any significant improvement following acute WBB consumption for office blood pressure, which agrees with the existing lack for significant improvements observed for blood pressure following acute WBB consumption in the literature. Although not comparable, Del Bo et al. (2013) assessed the effects of acute consumption of a WBB intervention containing 348mg anthocyanins, on blood pressure in a group of young male smokers. Although they did not see a significant decrease in blood pressure, they did observe a maintenance of systolic pressure, whereas in the placebo group, systolic pressure was increased. This suggests that WBB may have positive effects for blood pressure, but in people who have compromised vascular systems due to tobacco smoking.

As with the BluLife study, no treatment-related effects for office blood pressure were seen following chronic WBB consumption in the BluFlow study. To my knowledge no studies thus far have shown improvements of WBB consumption on blood pressure in healthy adults. Nevertheless, previous studies have observed blood pressure improvements following chronic consumption in adults with metabolic syndrome. For instance, Johnson et al. (2015) observed a significant reduction in systolic pressure by -7 mmHg and diastolic pressure by -5 mmHg following 8 weeks consumption of a WBB intervention containing 469mg anthocyanins

in a group of postmenopausal women with pre- and stage-1 hypertension. Similarly, Basu et al. (2010) observed a significant reduction in systolic pressure by -7.8 mmHg and diastolic pressure by 2.5 mmHg following 8 weeks consumption of a WBB intervention containing 842mg anthocyanins in a group of middle-aged adults. More similar to the findings of the BluFlow study, McAnulty did not observe any changes in blood pressure following 3 weeks consumption of 250 g fresh WBB (anthocyanin content not reported) in a group of smokers.

Nevertheless, this may not be due to a lack of positive effects for office blood pressure but more a lack of sensitivity of the actual measurement. For instance, a significant improvement was found for 24hr systolic blood pressure in the BluFlow study, whereby systolic blood pressure was reduced following WBB consumption relative to placebo. This may indicate that 24hr systolic blood pressure measurements over time may give us an improved representation of the participant's blood pressure. The office blood readings may be affected by short-term factors such as the white-coat effect, which is when the presence of a clinician or researcher induces a rise in blood pressure (Celis & Fagard, 2004; Pioli et al., 2018). In support of this, Rodriguez-Mateos et al. (2019) also reported a significant decrease of in 24hr systolic blood pressure by -5.6 mmHg, in healthy males, following a daily consumption of WBB treatment containing 300 mg anthocyanins, for 4 weeks. Nevertheless, to my knowledge, this is the only study assessing the effects of 24hr ambulatory blood pressure following chronic consumption of WBB, indicating that further research is needed to better understand the effects for blood pressure.

7.2.4 Transcranial Doppler Ultrasound (TCD) Findings

In both the BluLife study and BluFlow study, cerebral blood flow was assessed using TCD. The first parameter included blood flow velocity, which refers the rate at which blood travels, not the quantity. The second parameter included pulsatility index which is a method of assessing vascular resistance and is defined as the peak systolic velocity (V_{max}) minus the minimum diastolic velocity (V_{min}), divided by the mean velocity. No effects of treatment were observed for any cerebral blood flow parameters in the BluLife study or BluFlow study. Nevertheless, this is expected considering that unlike for peripheral circulation, cerebral autoregulation ensures that arterial diameter remains unchanged and therefore cerebral blood flow velocity remains stable (Van Beek et al., 2008).

Lack of observed treatment related CBF effect in the BluLife and BluFlow study may suggest that TCD not be an appropriate choice of measurement if the interest was to observe changes in cerebral blood perfusion or neural activity. Previous studies investigating flavonoids on cerebral blood flow used fMRI techniques including ASL to assess cerebral blood perfusion, all which observed findings indicating increased perfusion following flavonoid consumption relative to placebo (Lamport et al., 2015; Lamport et al., 2016; Francis et al. 2006; Bowtell et al. 2018). Similarly, previous investigations using fMRI to conduct blood oxygen level dependent (BOLD) imaging, such as Boespflug et al.'s (2017) study, observed increased neural activity during a working memory task following a daily consumption of WBB containing 269 mg of anthocyanins for 4 months relative to placebo, in older adults with MCI. In addition to this, Brickman et al. (2014) revealed increased neural activity in the dentate gyrus, detected using fMRI, following chronic consumption of a high flavanol (900mg) cocoa drink relative to

a low flavanol drink in healthy older adults. On the other hand, studies demonstrating the positive effects of flavonoids on CBF parameters using TCD are limited. For instance, Sorond et al. (2008) did not observe any differences between a high-flavanol and a poor-flavanol cocoa intervention for cerebral blood flow velocity assessed using TCD. Similarly, Evans et al. (2017) did not observe any significant difference between a resveratrol intervention and a placebo for cerebral blood flow velocity assessed using TCD. On the other hand, Marsh et al. (2017), who assessed the effects of acute cocoa flavanols consumption on cerebral blood flow velocity with TCD, found significantly lower cerebral blood flow velocity following consumption of milk and dark chocolate, but not in white chocolate. To explain this, Marsh et al. (2017) suggested that the decreased cerebral blood flow velocity may indicate an improvement to vascular health leading to increased efficiency of CBF responses.

Further research is required to ascertain whether TCD is a reliable technique for assessing changes to CBF in nutritional intervention studies, and if so, what the expected observation would be. Sorond et al. (2010) assessed in parallel, the changes in CBF following cocoa consumption using TCD versus arterial spin labelling (ASL). Their findings revealed significant positive correlation between blood flow velocity in the middle cerebral artery detected using TCD with regional perfusion detected by MRI imaging techniques. This suggests that, although TCD is unable to assess the diameter of the middle cerebral artery, cocoa flavanols did also affect cerebral perfusion as assessed concurrently using MRI. Nevertheless, a previous study conducted by Lunt et al. (2004) who investigated the effects of caffeine on cerebral blood flow velocity in the middle-cerebral artery using TCD, concluded that changes in blood flow velocity does not necessarily imitate changes in cerebral blood flow, therefore further research is required to understand the suitability of TCD as an assessment of CBF.

Treatment aside, observation for increased blood velocity was seen for the younger age groups, relative to older age groups, and findings from the BluLife study revealed a decline in blood flow velocity as age increases. These findings are supported by results observed in Demirkaya et al.'s (2008) study, which aimed to establish reference values for expected blood flow velocity per age group. Here, Demirkaya et al. (2008) found that mean blood flow velocity decreased with age and observed similar values per age group as to the values observed in the BluLife study. Proposed reasons as to why a decrease in blood flow velocity is associated with increased age may be due increased incident of atherosclerosis, leading to stiffening of arteries and therefore reduced elasticity (Yamauchi et al., 2019). As well as this, it is documented that ageing influences factors related to cerebral hemodynamics including increased arterial pressure, reduced cardiac output and increased pulsatility index (Tarumi & Zhang, 2018).

7.3 Possible Mechanisms of Action

The proposed mechanism of action relevant to my findings and measurements used will be discussed. As mentioned previously, previous studies have shown significant improvements for flow mediated dilation following WBB consumption (Wood et al., 2019) and it has been proposed that this improvement in endothelial function may lead to improvements to cognitive function due to increases in oxygen availability and nutrient uptake in the brain. Previous investigations have shown cognitive improvements in healthy participants supplemented with oxygen, particularly during tasks assessing episodic memory (Moss & Scholey, 1996; Moss et al., 1998; Scholey et a; 1998; Scholey et al., 1999; Chung & Lim, 2008) visuospatial memory (Chung et al., 2004[a]; Chung et al., 2004[b] ; Sohn et al., 2005; Chung et al., 2006; Chung et al., 2008[a]; Choi et al., 2010), and working memory (Chung et al., 2007; Chung

et al., 2008[b]). In my studies, effects for episodic memory (AVLT Total Acquisition and Immediate recall) and visuospatial working memory (Corsi Blocks) were observed which could have been facilitated by the increase of oxygen availability in the brains of WBB supplemented participants. The proposed mechanism of action behind improvements in cognitive function therefore is the increased arterial dilation leading to increased cerebral blood flow and therefore increased delivery of oxygen to the brain. In both my studies, a lack of significant results was found for cerebral blood flow velocity, however, as mentioned previously, this may be a reflection of cerebral autoregulation ensuring that cerebral blood flow velocity remain stable, rather than the proposed physiological effect which could be occurring.

The proposed mechanism of action relevant to improvements in FMD may be attributed to a reduction in neutrophil NADPH oxidase activity as demonstrated by Rodriguez-Mateos et al., 2013) where plasma levels revealed significant reductions in neutrophil NADPH oxidase activity following WBB consumption relative to placebo. Interestingly multivariate regression analysis revealed that certain changes in metabolites, notably vanillic, hippuric and homovanillic acid predicted improvements to FMD as well as reduction in NADPH oxidase activity which as a result may increase the uptake of nitric oxide. This leads me nicely to the circulation of WBB metabolites as a proposed mechanism of action. Although not conclusive, it is suggested that polyphenol metabolites may be able to permeate through the blood brain barrier (Andres-Lacueva et al., 2005; Schaffer & Halliwell, 2012; Vazour, 2012; Del Rio et al., 2012) leading to benefits for brain health by exerting anti-oxidant properties, which may help protect the central nervous system from oxidative stress and neuroinflammation. The knowledge surrounding polyphenols metabolites and their effects is still very young; however, the detection of WBB metabolites in plasma and urine samples in my studies are promising and may help confirm these proposed mechanisms of action. Although no increases

for cerebral blood flow velocity was observed, beneficial neural effects may not necessarily be caused the rate of blood flow, but instead the presence of metabolites circulating to the brain. Lastly, the interest surrounding the effect of gut microbiome for cognitive and vascular health is increasing, as evidence continues to support the findings regarding the microbiota's association with non-communicable disease including cardiovascular disease (Yamashita et al., 2016; Emoto et al., 2016; Yu et al., 2018) and dementia (Vogt et al., 2017; Saji et al., 2019; Stadlbauer et al., 2020

The ideal composition of gut microbiota is still under investigation but is undeniably complex, due to factors that affect the composition and dysfunction in the gut-brain axis including habitual diet, ingestion of nutritional supplements, the environment and stress, to name a few (Rojo et al., 2017). In addition to this, evidence in literature suggest that the gut microbiome may influence the production of active metabolites produced during the metabolism process (Pasinetti et al., 2018). This is critical as polyphenols are not fully absorbable in the small intestine therefore certain species present in the microbiota can help facilitate the bio-availability of polyphenolic metabolites (Manach et al., 2005). Ultimately this may influence the production of metabolites that have the potential to cross the blood-brain barrier (Filosa et al., 2018), and may therefore influence cognitive performance. The presence and abundance of certain strains can also influence the biosynthesis of neurotransmitters vital for cognitive health including serotonin (O'Mahony et al., 2015) dopamine, Gamma-Aminobutyric Acid (GABA) and acetylcholine (Strandwitz, 2018). Although the microbiome data from the BluFlow study was somewhat inconclusive, there was indication that polyphenols can influence gut microbiome composition, and in particular, beneficial gut microbiota.

7.4 Limitations

This section will consider a few limitations identified from the study series. Firstly, recruitment was often very difficult and obtaining the correct number of participants took a long time, particularly of children. Numerous reasons for this include the unavailability of children for study days due to school commitment, which meant that study days could only be hosted on weekends or during half term. However, this often also brought complications due to parents not being available to bring their children to the study days due to work commitments, family holidays, or extra-curriculum activities that children often attended on weekends. Posters and leaflets were handed out at train stations and with permission, put up in supermarkets. However, the location of the research unit was in central London (Franklin-Wilkins Building, Stamford Street, Waterloo, London) and therefore the audience of the study posters was often commuters and working professionals, who often do not have the time to commit to scientific research participation. Although local primary schools do exist, these schools were often too preoccupied and not interested in taking on more responsibilities such as handing out study flyers to students and their parents. One success in terms of recruitment of children was through a family-orientated magazine (*Families, London SW Magazine)* which resulted in multiple bookings over the Easter half-term 2020; however, this was quickly disrupted and discontinued due to the coronavirus pandemic. For this reason, recruitment was extensive and highly time-consuming and the number of child participants required was not met. In addition to this, the recruitment of older participants was often difficult as many people who expressed an interest in the study were on medications or had existing health problems which made them ineligible to take part.

Secondly it was often reported by participants that the cognitive battery was long and tedious. Indeed, the battery which lasted an average of 45 minutes, was designed in this way to induce increased cognitive demand. Within this time period participants can quickly become bored, fatigued and unmotivated. For this reason, a number of participants did not want to attend further sessions after having experienced the full battery at screening. In addition to this, due to the multiple measurements which took place, including vascular health, and cognitive testing, the study days were often very long and discouraging. More specifically for the crossover study (BluLife) participants often reported that they did not have 6 hrs in the day to commit to attending study sessions.

Thirdly, there were report of participants disliking the WBB and placebo intervention. Nevertheless, the perception of drinks consumed by the participants was not reported, therefore any differences between the WBB and placebo drinks are unknown. Concerns regarding drink palatability have also been acknowledged by Barfoot et al.'s (2021) study, whereby participant reported to enjoy the placebo significantly more than the WBB intervention. This was also the case in Whyte, Schafer and Williams (2012) whereby concerns regarding the differences in texture and taste between the WBB treatment drink and placebo were reported as a limitation. This should be taken into consideration for future investigation, especially since studies have reported that food palatability may influence mood (Singh, 2014; Pribic et al., 2018), and mood may in turn affect cognitive functioning (Khalid et al., 2017; Tyng et al., 2017). To counteract this for future investigations, it is recommended that negative responses in relation to test rinks are formally reported and quantified, to understand whether the dislike of drinks may have an effect on the cognitive function test scores.

Other limitations worth highlighting were the external factors which influence the performance during cognitive tasks. The rooms where participants conducted cognitive testing were not soundproof. This is because the testing rooms were not originally designed for cognitive testing, but instead designed for vascular measures. One solution to this would be to ask the participants to wear an over-the-ear headphone to minimise the external noise. However, the participants also needed to wear the TCD headband for CBF measurements therefore this could not be an option. Instead, the participants wore an in-ear headphone, but these are often not as sound-proof.

Lastly, although participants in the BluLife study were given thorough guidelines and recommendations regarding how to follow a low-polyphenol diet 24hr prior to study days, it can often be hard to control and ensure that participants did not accidently consume food items that unknowingly contain polyphenols. I suggest that future RCTs requiring a low-polyphenol diet prior to study days give the participants options of purchasing a range of ready meals they can purchase from supermarkets and asking participants to bring the receipts in as evidence, and also for the participants to be re-imbursed. It also worth mentioning that in the BluLife study, the participants consumed breakfast upon arrival, which is not best practice when measuring vascular related measurements. In the BluFlow study this was recognised, whereby vascular measures were taken whilst the participant were in a fasted state.

7.5 Future recommendations

The findings form the studies in this thesis are promising and demands future research in order to further consolidate our knowledge surrounding the effects of WBB polyphenols on cognitive function and vascular health. The variables assessed in both the studies were thorough, however a number of measurements which could be incorporated in future trials will be proposed.

Firstly, I suggest that future studies incorporate the assessment of brain-derived neurotrophic factor (BDNF) in serum, in order to broaden our understanding regarding the mechanism of actions. As outlined in chapter 1 and 2, BDNF is a neurotrophin that may have benefits for neuronal health by improving hippocampal plasticity as well as increasing neurogenesis (Cunha et al., 2010). Previous studies in animals have demonstrated blueberry's ability to increase BDNF levels in the hippocampal area of the brain (Williams et al., 2008; Rendeiro et al., 2012). Similarly, investigation into the effects of acute WBB consumption on BDNF levels in humans revealed blueberry's ability to attenuate BDNF levels, whereby BDNF levels decreased following placebo consumption in healthy older adults (Dodd et al., 2019).

Secondly, I propose that future studies assessing the effects of cerebral blood flow should utilise a more robust measurement such as fMRI as, although transcranial Doppler ultrasound offer many practical advantages, it does not show which area of the brains are activated or affected by changes in cerebral blood perfusion, if any. Furthermore, literature suggest that approximately 10-15% of people may not have detectable transtemporal window (Purkayastha & Sorond 2013), which may risk inadequate number of TCD measurements within the study cohort.

To date, dose-response investigations on cognitive effects with WBB is limited, with one study looking at different doses in children (Whyte, Schafer & Williams (2016) whereby significant effects following both doses were found (127 mg and 253 mg anthocyanins) and one in healthy older adults (Whyte et al., 2018) who observed significant effects following a purified WBB extract containing a low dose of 7mg anthocyanins. Krikorian et al. (2010) was the first to use doses calculated according to the participants body weight. Although the aim was not to understand what dose is optimal, the aim was to ensure all participants received a dose suitable for their weight. Moreover, previous studies have observed dose-dependent effects for metabolic health. One example includes Bell et al.'s study (2017) who observed different postprandial glucose responses following three different doses of WBB, with the highest dose, providing 724mg anthocyanin, maintained glucose levels for longer compared to the sugar-matched placebo, which suggests here that higher anthocyanin does mediate better metabolic effects in terms of glucose responses. Therefore, further research should be conducted to investigate the possibility that cognitive effects may be dose-dependent, and future studies investigating effects across the life-course should considers appropriate doses depending on age, and body size in order to understand whether an optimum dosage exists per age group.

7.6 Final Conclusion

It can be concluded that acute and chronic WBB consumption improves cognitive function across different age groups, notably episodic memory, visuo-spatial working memory, executive functioning and reaction time. Chronic consumption of WBB polyphenols improves episodic memory and executive function in healthy older adults. These effects were observed following relatively realistic doses of polyphenols, which make it feasible to recommend the

consumption of blueberries as part of a healthy balanced diet in order to enhance cognitive function. This is a benefit which can be useful in the school environment, such for studying and conducting exams, and also in the working environment. Further research should be conducted to build on these findings, in order to understand how WBB affects cognition across the life-course, and in older adults.

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Appendices

Appendix A

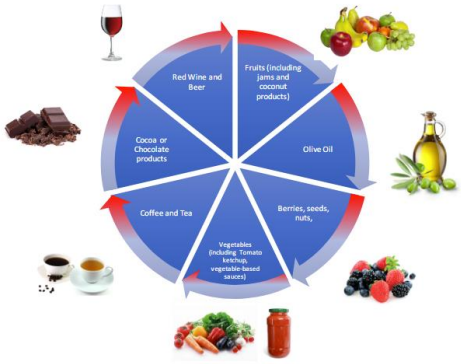
A.1 Participant Low Polyphenol Diet Guidance

DIET GUIDANCE

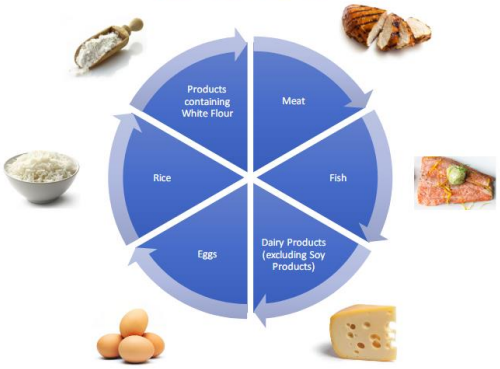
KING'S College LONDON

24 hours (1 day) prior to visit day 1 and 2, please refrain from eating products containing high polyphenol content. Please refer to the charts below for guidance:

CAN'T EAT



CAN EAT



Appendix B

B.1 Product Description of Low-polyphenol Breakfast

1. Philadelphia 'Light' cream cheese:

Ingredients:

Medium Fat Soft Cheese, Salt, Stabilisers (Locust Bean Gum, Carrageenan), Acid (Citric Acid).
(Details sourced from www.philadelphia.co.uk).

2. DANONE Activia Fat free yoghurt, vanilla flavour

Ingredients:

Fat free yogurt (milk), Oligofructose (Fibre), Modified maize starch, Carrot concentrate, Natural vanilla flavouring, natural flavouring, Bourbon vanilla powder, concentrated lemon juice, Thickener (carrageenan), Sweeteners (acesulfame K , sucralose), Acidity regulator (sodium citrate).

(Details sourced from https://www.danoneactivia.co.uk/).

Appendix C

Examples of recruitment advertisements

C1: BluLife Study Recruitment Poster

HEALTHY VOLUNTEERS WANTED

Are you aged:

8-10 **14-18** **22-28** **40-50** or **65-80**?

We might need your help to investigate the benefits of Blueberries on cardiovascular health and cognitive function!

What we ask from you:

- *Come to King's College London on **5** separate days (2 of them lasting 10 mins)*
- *Consume a **blueberry** drink on two of the days*

In return, you will receive:

- *Some medical information about your **health***
- *A **financial compensation** for your time*

Interested?

Contact us and we will provide you with all the info!

Email: blulifestudy@kcl.ac.uk

Telephone: 02078484162

C.2 BluFlow Study Poster

KING'S College LONDON

VOLUNTEERS NEEDED FOR THE BLUFLOW STUDY

We are running a study looking at the effects of blueberries on ***vascular health*** and ***cognitive function***.

What we ask from you:

- Come to King's College London on 4 separate days
- Consume a blueberry drink daily for three months

In return you will receive £150 and a free health check!

ARE YOU?

Aged between 65-80 years old?

Healthy, non-smoking?

Want to contribute to scientific research?

If so, then we need you!

Interested? Get in touch!

Email: bluflowstudy@kcl.ac.uk

Telephone: 02078484162

Appendix D

Study Case Report Forms (CRF)

D.1: Example of Study Screening CRF for BluLife Study. The screening CRF for BluFlow Study followed similar format:

CASE REPORT FORM

SCREENING

Randomised, double-blind, placebo controlled, crossover trial investigating the acute effects of blueberry (poly)phenols on vascular function and cognitive performance in healthy individuals across the life course.

Principal Investigator: Ana Rodriguez-Mateos, Ph.D. Claire Williams, Ph.D

Sponsor: WBANA

Name of site: Kings College London

CRF Version Number: 04

Participant Initials □□□ Subject screening No. □□□

Subject study No. □□□

CRF Completion Instructions

General

Complete the CRF using a black ballpoint pen and ensure that all entries are complete and legible.

Avoid the use of abbreviations and acronyms.

The CRF should be completed as soon as possible after the scheduled visit.

Do not use subject identifiers anywhere on the CRF, such as name, hospital number etc., in order to maintain the confidentiality of the subject. Ensure that the header information (i.e. subject's initials and ID number) is completed consistently throughout the CRF. Missing initials should be recorded with a dash (i.e. D-L).

Each CRF page should be signed and dated by the person completing the form.

The 'completed by' Name in the footer of each page must be legible and CRFs should only be completed by individuals delegated to complete CRFs on the Site Delegation log (and signed by the PI).

Ensure that all fields are completed on each page:

If a test was Not Done record ND in the relevant box(es)

Where information is Not Known write NK in relevant box(es)

Where information is not applicable write NA in the relevant box(es)

Corrections to entries

If an error is made, draw a single line through the item, then write the correct entry on an appropriate blank space near the original data point on the CRF and initial and date the change.

Do NOT

Obscure the original entry by scribbling it out

Try to correct/ modify the original entry

Use Tippex or correction fluid

Medications taken by the subject during the trial should be recorded on the "Concomitant Medications Log" using the generic name whenever possible,

except combination products which will be recorded using the established trade name. All non-IMPs mentioned in the protocol should also be recorded on the "Concomitant medication Log" for the duration of the trial.

Verbatim Adverse Event terms (initial medical term) should be recorded as the final diagnosis whenever possible.

Complete all dates as day, month, year i.e. 13/NOV/2008. Partial dates should be recorded as NK/NOV/2008.

All times are to be recorded in 24 hour format without punctuation and always use 4-digits; i.e. 0200 or 2130. Midnight is recorded as 0000.

Weights should be recorded to the nearest 0.1 kg.

Source documents such as lab reports, ECG reports etc. should be filed separately from the CRF (if not in the medical notes) for each subject and be signed and dated by a delegated Investigator as proof of review of the assessment during the trial. Questionnaire should be considered as the CRF appendices (except standard approved questionnaire e.g. EQ-5D)

If a subject prematurely withdraws from the trial a single line must be drawn across each uncompleted page to correspond with the last visit of the subject as mentioned on the “Trial Completion” page.

The protocol deviation/violation/serious breach log should be used to record comments relating to each CRF visit that cannot be captured on the page itself. This includes reason for delayed or missed protocol visits or trial assessments, unscheduled visits etc.

The Chief Investigator (for lead site)/Principal Investigator is responsible for the accuracy of the data reported on the CRF. The CI/PI must sign and date the Principal Investigator’s Sign Off page to certify accuracy, completeness and legibility of the data reported in the CRF.

Serious Adverse Events (SAEs)

SAEs should be faxed within 24 hours of the site being aware of the event using the trial specific SAE report form to rosie.calokatsia@kcl.ac.uk

Storage

CRF documents should be stored in a locked, secure area when not in use where confidentiality can be maintained. Ensure that they are stored separately to any other documents that might reveal the identity of the subject

Date of Assessment: __ __ / __ __ __ / __ __ __ __

(DD / MMM / YYYY)

Informed Consent:	
Date participant signed written consent form: __ __ / __ __ __ / __ __ __ __ (DD / MMM / YYYY)	Date of first trial-related procedure: __ __ / __ __ __ / __ __ __ __ (DD / MMM / YYYY)
Name of person taking informed consent: _____	

Demographic Data:				
Date of Birth & Age: __ __ / __ __ __ / __ __ __ __ (DD / MMM / YYYY) _____ yrs				
Ethnicity:				
White	White British ☐	White Irish ☐	White Other ☐	
Mixed race	White & Black Caribbean ☐	White & Black African ☐	White & Asian ☐	Other mixed background ☐
Asian or Asian British	Indian ☐	Bangladeshi ☐	Pakistani ☐	Other Asian background ☐
Black or Black British	Caribbean ☐	African ☐	Black Other ☐	

Chinese or other ethnicity	Chinese ☐	Other ☐ (please specify)
Sex: ☐ Male ☐ Female		

Has the particpant had any relevant medical history?	☐ No ☐ Yes, Complete below		
Condition / illness /surgical procedure	Start date (DD/MMM/YYYY)	Stop date (DD/MMM/YYYY)	Or tick if ongoing at Screening Visit?
Known allergies and reactions to medications	____/_____/_____	____/_____/_____	☐
Medical/psychiatric illnesses	____/_____/_____	____/_____/_____	☐
Past surgeries/injuries/hospitalizations	____/_____/_____	____/_____/_____	☐

Immunizations (last 6 months)	____/_____/_____	____/_____/_____	☐
Current or past excessive alcohol/drug use	____/_____/_____	____/_____/_____	☐
	____/_____/_____	____/_____/_____	☐
	____/_____/_____	____/_____/_____	☐
	____/_____/_____	____/_____/_____	☐
	____/_____/_____	____/_____/_____	☐
	____/_____/_____	____/_____/_____	☐
	____/_____/_____	____/_____/_____	☐

The following criteria MUST be answered YES for participant to be included in the trial (except where NA is appropriate):		Yes	No
1.	Healthy men or women aged 22-28, 40-50 or 65-80 years old	☐	☐
2.	Willing to maintain their normal eating/drinking habits and exercise habits to avoid changes in body weight over the duration of the study	☐	☐
3.	Able to understand the nature of the study	☐	☐
4.	Able to give signed written informed consent	☐	☐
5.	Signed informed consent form	☐	☐
6.		☐	☐
7.		☐	☐
8.		☐	☐
9.		☐	☐
10.		☐	☐
If any of the above criteria is answered NO, the participant is NOT eligible for the trial and must not be included in the study. Please list reason(s) for ineligibility for screen failure on Participant Eligibility Review page.			

Blood Pressure (seated):	1. ___ ___ ___ / ___ ___ ___ mmHg 2. ___ ___ ___ / ___ ___ ___ mmHg 3. ___ ___ ___ / ___ ___ ___ mmHg AVERAGE: ___ ___ ___ / ___ ___ ___ mmHg
Pulse:	1. ____ ___ ___ beats/min 2. ____ ___ ___ beats/min 3. ____ ___ ___ beats/min AVERAGE: ____ ___ ___ beats/min
Height: ___ ___ ___ cm	
Weight: ___ ___ ___ . ___ kg	BMI: ___ ___ ___ . ___ kg/m²
Body Fat ___ ___ ___ . ___ %	BMR ___ ___ ___ ___ . ___ KCal

The following criteria MUST be answered NO for the participant to be included in the trial:		Yes	No
1.	Manifest cardiovascular disease including coronary artery disease, cerebrovascular disease and peripheral artery disease	☐	☐
2.	Hypertensive, as defined as SBP ≥ 140 mmHg or DBP ≥ 90 mm Hg	☐	☐
3.	Obese participants, defined as BMI ≥ 30	☐	☐
4.	Diabetes mellitus, metabolic syndrome, acute inflammation, terminal renal failure, malignancies or abnormal heart rhythm (lower or higher than 60-100 bpm)	☐	☐
5.	Allergies to berries or other significant food allergy.	☐	☐
6.	Under medication or on vitamin/dietary supplements (within 2 weeks of baseline).	☐	☐
7.	Lost more than 10% of their weight in the past 6 months or are currently in a diet	☐	☐
8.	Reported participant in another study within one month before the study start	☐	☐
9.	Smoke cigarettes	☐	☐
10.	MCI or dyslexic or unable to complete the cognitive function tasks for any reason such as visual impairments.	☐	☐
11.	Subjects who require chronic antimicrobial or antiviral treatment.	☐	☐
12.	Subjects with unstable psychological condition.	☐	☐
13.	Subjects with history of cancer, myocardial infarction, cerebrovascular incident.	☐	☐
14.	Any reason or condition that in the judgment of the clinical investigator(s) may put the subject at unacceptable risk or that may preclude the subject from understanding or complying with the study's requirements.	☐	☐

If any of the above criteria is answered YES, the participant is NOT eligible for the trial and must not be included in the study. Please list reason(s) for ineligibility for screen failure on Participant Eligibility Review page.

Does the participant currently smoke? ☐ No ☐ Yes, exclude from study

Has the participant ever smoked? ☐ No ☐ Yes, Complete below

☐ Former smoker

Smoked for ___ ___ months/years

Date when smoking ceased: __ __ / __ __ __ / __ __ __ __

(DD / MMM / YYYY)

When smoking, participant's average daily use:

Participant's average alcohol consumption per week (units):

Has the participant taken vitamins/minerals/dietary supplements in the last year? ☐ No ☐ Yes

Type of supplement:

Amount and frequency:

For how long and until when:

Willing to stop taking them before and during the study (ensure 2 weeks washout): ☐ No ☐ Yes

Date of Assessment: __ __ / __ __ __ / __ __ __ __

(DD / MMM / YYYY)

Is the participant taken any concomitant medications at screening or visits 2 and 3					☐ No ☐ Yes, Complete below		
Medication (Record Generic or trade name)	Reason for use (Medical History diagnosis or other reason, e.g. Prophylaxis)	Dose and units	Frequ e-ncy	Route	Start Date (DD/MMM/YYYY)	Stop Date (DD//MMM/YYY)	Or tick if ongoing at Screening Visit
1.					____/_____/____	____/_____/____	☐
2.					____/_____/____	____/_____/____	☐
3.					____/_____/____	____/_____/____	☐
4.					____/_____/____	____/_____/____	☐
5.					____/_____/____	____/_____/____	☐
6.					____/_____/____	____/_____/____	☐
7.					____/_____/____	____/_____/____	☐
8.					____/_____/____	____/_____/____	☐
9.					____/_____/____	____/_____/____	☐
10.					____/_____/____	____/_____/____	☐

End of Screening Visit Checklist:			
		Yes	No
1.	Does the participant satisfy the inclusion and exclusion criteria to date?	☐	☐
2.	Have all Screening Visit procedures been completed?	☐	☐
3.	Have the Medical History and Concomitant Medication pages been completed?	☐	☐
4.	Is the participant still willing to proceed in the trial?	☐	☐
5.	Have all the cognitive tasks been practiced successfully?		

Participant's eligibility Investigator Sign-Off:	
Is the participant eligible to take part in the Clinical Trial? Investigator's Signature: _____ Date : __ __ / __ __ __ / __ __ __ __ (DD / MMM / YYYY) Investigator's Name: _____	☐ Yes ☐ No, Please give reason for screen failure below ☐ Yes ☐ No ☐ Yes ☐ No
Reason(s) for screen failure:	
1.	
2.	
3.	

D.2 Example of Study Visit 2 CRF for BluLife Study. The study visit CRF for BluFlow Study followed similar format but has been amended to not include acute testing points:

CASE REPORT FORM

VISIT 2

Randomised, double-blind, placebo controlled, crossover trial investigating the acute effects of blueberry (poly)phenols on vascular function and cognitive performance in healthy individuals across the life course.

Principal Investigator: Ana Rodriguez-Mateos, Ph.D. Claire Williams, Ph.D

Sponsor: WBANA

Name of site: Kings College London

CRF Version Number: 04

Participant Initials ☐☐☐	Subject screening No. ☐☐☐
Subject No. ☐☐☐	

Date of visit: __ __ / __ __ __ / __ __ __ __ (DD / MMM / YYYY)	
Participant Randomisation number allocated:	_____
Date of Randomisation/Enrolment:	__ __ / __ __ __ / __ __ __ __ (DD / MMM / YYYY)

Treatment code (drink):	
Cognitive randomisation code (pre-consumption):	
Cognitive randomization code (post-consumption):	
Breakfast consumed at:	_____:_____ (HH:MM)

Time of treatment consumption:	_____:_____ (HH:MM)

Room temperature	_____ ˚C

Peripheral blood pressure and heart rate performed?	☐ No (Comment Below) ☐ Yes, Complete below Comment*:_____
Time of measurement 1 (baseline)	_____:_____ HH:MM
Time of measurement 2 (post-intervention):	_____:_____ HH:MM
Position: Supine Device: Automatic Sphygmomanometer	

BLOOD Pressure	SBP (mmHg)	DBP (mmHg)	HR (bpm)
0h replicate 1			
0h replicate 2			
0h replicate 3			
Average (0h)			
2h replicate 1			
2h replicate 2			
2h replicate 3			
Average (2h)			

FMD performed?	☐ No (Comment Below) ☐ Yes, Complete below Comment*:_____
ECG normal?	☐ No (Comment Below) ☐ Yes, Complete below Comment*:_____
Time of measurement 1 (baseline):	_____:_____ HH:MM
Time of measurement 2 (post-intervention):	_____:_____ HH:MM
Position: Supine Device/probe (if different than usual): Vivid i	
Comments	

FMD results can be found at:

External BLULIFE hard drive in folder “BLUELIFE” → “FMD-results” → “BluLife_FMD results.xcl”

Room temperature	_____ºC
TCD successfully performed?	☐ No (Comment Below) ☐ Yes, Complete below Comment*:_____ _____
Time of measurement 1 (baseline):	_____:_____ HH:MM
Time of measurement 2 (post-intervention):	_____:_____ HH:MM
Position: 45-60° upright Device: Transcranial Doppler sonography (EZ-Dop, DWL)	

	TCD time point 1 (baseline 0 h)					
Measurement (minutes)	Y/N	Mean blood flow velocity (cm/s)	Mean ap blood flow (cm/s)	Pulsatility index (cm/s)	Maximum velocity (cm/s)	Minimum velocity (cm/s)
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						

	TCD time point 2 (post-intervention 2 h)					
Measurement (minutes)	Y/N	Mean blood flow velocity (cm/s)	Mean ap blood flow (cm/s)	Pulsatility index (cm/s)	Maximum velocity (cm/s)	Minimum velocity (cm/s)
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						

Temperature of room:	_____ ºC
Sound recording on?	☐ Yes ☐ No
TCD successfully performed during task switching?	☐ No (Comment Below) ☐ Yes, Complete below Comment*:_____ _____
Time of measurement 1 TCD (baseline):	_____:_____ HH:MM
Time of measurement 2 TCD (post-intervention):	_____:_____ HH:MM
Position: 45-60° upright Device: Transcranial Doppler sonography (EZ-Dop, DWL)	

Cognitive task	Performed (baseline)	Completed (post-intervention)
PANAS	☐ No ☐ Yes	☐ No ☐ Yes
Verbal episodic memory (as measured by RAVLT)	☐ No ☐ Yes	☐ No ☐ Yes
Corsi Block task	☐ No ☐ Yes	☐ No ☐ Yes
Serial 3s and 7s	☐ No ☐ Yes	☐ No ☐ Yes
Switching task + TCD	☐ No ☐ Yes	☐ No ☐ Yes
PANAS	☐ No ☐ Yes	☐ No ☐ Yes

	TCD time point 1 (baseline 0 h)					
Measurement (minutes)	Y/N	Mean blood flow velocity (cm/s)	Mean ap blood flow (cm/s)	Pulsatility index (cm/s)	Maximum velocity (cm/s)	Minimum velocity (cm/s)
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						

	TCD time point 2 (post-intervention 2 h)					
Measurement (minutes)	Y/N	Mean blood flow velocity (cm/s)	Mean ap blood flow (cm/s)	Pulsatility index (cm/s)	Maximum velocity (cm/s)	Minimum velocity (cm/s)
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						

Cognitive tasks were all completed	☐ No (Comment Below) ☐ Yes, Complete below Comment*:_____ _____
Time cognition 1 start (baseline)	_____:_____ HH:MM
Time cognitive task 1 completion (baseline):	_____:_____ HH:MM
Time cognitive task 2 start (post-intervention)	_____:_____ HH:MM
Time of cognitive task 2 completion (post-intervention):	_____:_____ HH:MM
Device: Cognitive software : Eprime	

Cognitive task notes

Cognitive performance results can be found at:

BLULIFE external hard drive in folder “BLULIFE” → “Cognition” → “BluLife_cognition results”

Blood collection performed?	☐ No (Comment Below) ☐ Yes, Complete below Comment*:_____ _____
Time of Sample 0h:	_____:_____ HH:MM
Time of Sample 2h:	_____:_____ HH:MM

BLOOD COLLECTION (68.5 mL)	ANALYSIS	0h	2h
10 ml EDTA (purple large)	(Poly)phenols	☐ No ☐ Yes	☐ No ☐ Yes
12 ml heparin (green) x2	(Poly)phenols	☐ No ☐ Yes	☐ No ☐ Yes
4 mL EDTA (purple small)	FBC	☐ No ☐ Yes	
7 mL Serum (red)	Lipids, Liver fct, Urea	☐ No ☐ Yes	
3 mL Na/F K/oxalate (grey)	Glucose	☐ No ☐ Yes	

BLOOD COLLECTION Laboratory Parameter	0h	2h	Number of Eppendorf tubes 0h / 2h		Stored in eppendorf box (-80°C)
0.6 ml plasma EDTA with 2% FA	☐ No ☐ Yes	☐ No ☐ Yes			
0.6 ml plasma EDTA no FA	☐ No ☐ Yes	☐ No ☐ Yes			
0.6 ml Plasma heparin with 2% FA	☐ No ☐ Yes	☐ No ☐ Yes			
0.6 ml Plasma heparin no FA	☐ No ☐ Yes	☐ No ☐ Yes			
1 ml full blood	☐ No ☐ Yes	☐ No ☐ Yes			

Naf (glucose)	☐ No ☐ Yes	☐ No ☐ Yes			
Serum (lipids)	☐ No ☐ Yes	☐ No ☐ Yes			

Blood results can be found at:

BLULIFE external hard drive in folder “BLULIFE” → “Blood-results” → “BluLife_blood results”

Epic FFQ filled?	☐ No (Comment Below) ☐ Yes, Complete below Comment*:_____
Reading FFQ filled?	☐ No (Comment Below) ☐ Yes, Complete below Comment*:_____
24 h pre-study diet diary filled?	☐ No (Comment Below) ☐ Yes, Complete below Comment*:_____
Exercise questionnaire filled?	☐ No (Comment Below) ☐ Yes, Complete below Comment*:_____
Began collecting 24 h urine sample and willing to drop this off the following day?	☐ No (Comment Below) ☐ Yes, Complete below Comment*:_____
Preference of time to drop 24 h urine sample off:	
What drink do you think you had today?	
Visit 3 date confirmed?	__ __/ __ __ __ / __ __ __ __ (DD / MMM / YYYY)

AE No	Event Name (Please give Diagnosis if known)	Start date (DD/MMM/YYYY)	Stop date (DD/MMM/YYYY)	Serious? If serious, please complete a JRO SAE form	Con-comitant Medication given	Severity 0 - Mild 1- Mode-rate 2 - Severe	Study Drug Action 0 - None 1 - Temporarily Interrupted 2 - permanently withdrawn	Outcome 0 - Resolved 1- Resolved with sequelea 2 - Not resolved	Relationship to Study Drug 0 - Definitely 1 - Probably 2 - Possibly 3 - Unlikely 4 - Not related 5 - Not assessable
1		____/____/____	____/_____/____	☐ No ☐ Yes	☐ No ☐ Yes				
2		____/_____/____	____/_____/____	☐ No ☐ Yes	☐ No ☐ Yes				
3		____/_____/____	____/_____/____	☐ No ☐ Yes	☐ No ☐ Yes				

4		____/_____/____	____/_____/____	☐ No ☐ Yes	☐ No ☐ Yes				
5		____/_____/____	____/_____/____	☐ No ☐ Yes	☐ No ☐ Yes				
6		____/_____/____	____/_____/____	☐ No ☐ Yes	☐ No ☐ Yes				

I have reviewed the AEs on this page and have assessed them for seriousness, causality, severity and outcome and confirm that, to the best of my knowledge, it accurately reflects the study information obtained for this participant

PI signature _____ _____ Date:_____ ☐ Please check box if this is the last page used

Appendix E

Participant Information Sheet (PIS)

E.1 BluLife Study PIS for participants over 16yrs:

02/01/2018 Version 2

PARTICIPANT INFORMATION SHEET

***BDM RESC Protocol Number* HR-17/18-5338**

Randomised, double-blind, placebo controlled, crossover trial investigating the acute effects of blueberry (poly)phenols on vascular function and cognitive performance in healthy individuals

We would like to invite you to participate in this nutritional research project undertaken as part of a PhD program. You should only participate if you want to; choosing not to take part will not disadvantage you in any way. Before you decide whether you want to take part, it is important for you to understand why the research is being done and what your participation will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information.

What is the purpose of the study?

The study primarily aims to investigate the effects of blueberries on cognitive performance and vascular function. We measure cognitive performance using various tasks put together on a computer system. We then measure vascular function using a non-invasive ultrasound technique called endothelium-dependant flow-mediated dilation (FMD). We will also measure the blood flow supplying the brain using the same non-invasive ultrasound equipment. These measurements will be taken before and then after the consumption of two blueberry drinks on separate study days. A total of 100 volunteers of various ages (between 8 and 80 years old) will be recruited for the study. Polyphenols, a natural plant chemical found in fruits, vegetables, cocoa and tea, are thought to be partly responsible for the health benefits that have previously been observed. Therefore, to measure the polyphenol content and better understand the mechanisms of action, we will collect blood samples and 24 h urine from volunteers. The results of the study will help us

to understand whether blueberry polyphenols can improve cognitive function across all ages, and whether this is caused by an increased blood flow to the brain. Furthermore, it will add to our knowledge on the effects of these polyphenols on vascular function. Improving vascular function would lead to a decreased risk of cardiovascular diseases and improvements cognitive function could slow the natural course of cognitive decline as we age.

Why have I been invited to take part?

You have been invited to take part as you have expressed interest in our research.

We would like to investigate 100 individuals, 20 from each age category: 8-10, 14-18, 22-28, 40-50, 65-80, who can answer 'yes' to the following questions:

- I am aged 8-10, 14-18, 22-28, 40-50 or 65-80.
- I do not suffer from peripheral artery disease, cerebrovascular disease, hypertension, obesity, acute inflammation, abnormal heart rhythm or terminal renal failure.
- I have no previous diagnosis of cognitive impairment, dyslexia, or significant visual impairment.
- I have never had a heart attack, stroke, diabetes mellitus, cancer, allergies to berries or other significant food allergy.
- I do not have a history of excessive alcohol or substance abuse.
- I do not take medications, vitamins or dietary supplements, or have not done for at least 2-weeks prior to participating in this study.

Do I have to take part?

No. The choice to participate is entirely yours. If you do decide to take part, we will go through this information sheet in more detail with you and answer any questions you may have. You will be given this information sheet and be asked to sign two copies of a consent form. However, you still may withdraw from the study at any time by informing one of the researchers, you are not obliged to give a reason. If you decide to withdraw once the study has started, the data may be used in the final report unless you request to withdraw your data. Data cannot be withdrawn once the study has been submitted as a study report which will be after the 1st

January 2019. If you do decide to participate, please inform us of any other research you have been involved in during the previous year.

What will happen to me if I take part?

If you answer 'yes' to the above questions and remain interested in participating, you will be invited to attend a screening visit to confirm your eligibility, then you will be enrolled onto the study if you remain eligible. The study will last 1-2 weeks depending on your availability for a practice day and two study days. Therefore, including the screening visit you will attend King's College London for 5 visits, 2 of which will be short visits to drop off 24 h urine collections. On the screening day we will familiarise you with the cognitive function tests and go through any questions that you might have, on the two test days you will consume a blueberry drink and placebo drink and we will take measurements before and after this drink is consumed, these two test days will take around 5 hours.

Screening visit:

If we think you are potentially suitable for the study you will be invited to attend a clinic screening appointment (approx. **2 h** including a cognitive test practice) in the **Metabolic Research Unit on 4th Floor, Corridor A, Franklin-Wilkins Building, 150 Stamford Street, London, SE1 9NH** (close to Waterloo Station).

On arrival, the study will be explained in detail and you will have the opportunity to ask any questions to ensure you will be giving fully informed consent. Once consent is provided, your height, weight, body fat percentage and blood pressure will be measured and we will ask questions about your medical history. In addition, you will practice a cognitive battery test lasting around 45 minutes, twice. We will also be using ultrasound to locate your middle cerebral artery on the right side of your head for the purpose of cerebral blood flow measurements. This should not take more than 10 minutes.

Study visits:

Following screening, if your results comply with the study inclusion criteria you will be invited to attend the Metabolic Research Unit on 2 further occasions lasting **5 h** each. These study visits will consist of taking measurements before, then 1.5 hours after a blueberry or placebo drink. Measurements include: non-invasive ultrasound measures of your brachial artery and your cerebral arteries, cognitive function

tasks, collections of 24 h urine samples (information and equipment will be provided at screening), blood pressure, and a small blood sample. There will be an approximate 1.5 h gap between measurements where you will be free to do some work or watch the television provided in our metabolic research unit.

For **24 h before you attend the two study visits we will ask you to follow a low polyphenol and alcohol free diet**, which means avoiding alcohol, fruits, vegetables, berries, wine, cocoa, chocolate, coffee and tea. You will be able to eat foods such as meat, fish, eggs, dairy, rice and products containing white flour. More detailed information on what is allowed will be provided on your screening visit.

About the measurements:

- Flow-mediated dilation: Elasticity of the brachial artery will be measured through echography. With an ultrasound probe we will measure the diameter of the brachial artery in the upper arm. To test the flexibility of the artery, a cuff will be placed on the forearm and will be inflated during 5 min. After deflation an increased blood flow will expand the artery by 5-8%. This expansion is called flow-mediated dilation.
- Cognitive function: This will be assessed by using a set of cognitive tests on a laptop, including tests for executive function and other tasks for memory, as well as a mood questionnaire. We will familiarise you with the cognition tasks when you attend the first visit for screening.
- Cerebral blood flow: Measured by placing an ultrasound probe onto the transtemporal window to identify the middle cerebral artery. This will be held in place using a headband device to allow us to take 10 minutes of measurements, then an additional 10 minutes of measurements during the cognitive task performance.
- Blood pressure: An office blood pressure cuff will be placed around the upper arm and the machine will take your blood pressure 3 times by tightening for a short period of time.
- Blood tests: This only applies to participants over the age of 18, if you are comfortable with this. On the two study visit days we will take blood samples, before and after drinking the blueberry drink, using a small needle, a small amount of blood will be taken (around 44ml overall).
- Urine collection: We will ask that you start collecting urine into a container provided by us as you arrive at each study visit. We then ask that you take the container home in a small cool bag with ice blocks provided then

- continue to collect urine until the following morning. Then you will be asked to drop the sample off that day.

Summary of the visits:

- **Following the screening visit, if eligible you will be invited to attend the metabolic research unit in the Franklin-Wilkins Building on 2 further occasions.**
- **The day after attending each study visit you will be asked to drop off a 24 h urine collection (equipment and information will be provided).**
- **Before the two study visits we will ask you to avoid alcohol, vegetables, fruits, wine, cocoa, chocolate, tea and coffee 24 h prior to each visit. On the study days you will be asked to fill a form with what you ate and drank 24 h prior to attending.**
- **We will also ask that you attend each of the two study days fasted for 12 h. We will provide breakfast at the study day as well as the blueberry drink.**
- **The study days will involve initial measurements: Blood pressure, Flow-mediated dilation, cognitive function, cerebral blood flow and blood tests.** **see above for more info on measurements.*
- **You will then consume the blueberry drink or a matched placebo drink. This will alternate depending on the study day.**
- **After a short break (around 1.5 h) the same measurements will be repeated.**
- **We will aim to leave 72 h (± 24 h) between the three visits. Study visits will be in the mornings and the screening visit will be in an afternoon.**

What are the benefits and risks of you taking part?

Benefits;

- You will be compensated with a payment of £75 for completing the study.
- Information on your health including cholesterol levels, glucose levels and body fat composition will be given to you on request.
- You will be contributing to invaluable research on food and how it can improve different aspects of our health and possibly slow inevitable factors of aging such as cognitive decline and reduce cardiovascular risk.

Risks;

- The blood sample venepuncture may cause a brief discomfort and could leave a small bruise.
- The blood pressure cuff or the cuff used during flow-mediated dilation measurements may cause short discomfort or a tingling sensation in your arm. However, the cuff is not reported to cause any damage to the arm.
- In the unlikely event that we discover that you have a medical condition (i.e., high blood pressure or elevated blood cholesterol) that you were unaware of, we will inform you and discuss informing your GP.
- If you are unaware of any allergies to berries and if an allergic reaction should occur this could result in vomiting, diarrhoea or nausea.

Will my information and participation be confidential?

Yes. All personal information collected during the study will be kept confidential and in password protected software or in locked cabinets/rooms. We will assign you to a subject code which will be used in place of names to store and collect data. Only the investigators have access to this data and only anonymised data will be shared with other researchers. Should you wish to find out any personal results such as health information please feel free to contact the PhD students running the study: Eleanor Wood and Sabine Hein (contact details below).

How is the project being funded?

The study is organised by the Diabetes and Nutritional Sciences Division, King's College London and is funded by the Wild Blueberry Association of America.

What will happen to the results of the study?

The study results will be presented in a report and will be published in a scientific journal. You will not be identified in the results of the study or any publication that might arise from this study. We will be happy to discuss the results with you when the study is completed, and will let you know how you can get a copy of the published results if you wish.

Who should I contact for further information?

Eleanor Wood or Sabine Hein via the study email address:
BluLifestudy@kcl.ac.uk

Ask for Sabine or Eleanor on: **+44 20 784 84162**

What if something goes wrong?

If this study has harmed you in any way or if you wish to make a complaint about the conduct of the study you can contact King's College London using the details below for further advice and information:

The Chair, BDM Research Ethics

Subcommittee (RESC), rec@kcl.ac.uk

Thank you for reading this information sheet and for considering taking part in this research

_____	_____	_____
Name of Participant	**Date**	**Signature**
_____	_____	_____
Name of Researcher	**Date**	**Signature**

**we will ask you to sign this after the screening visit and provide you with a copy.*

E.2 BluLife Study PIS for participants under 16yrs:

Child Information sheet

Information about the blueberry experiment

Title of study: **Investigating the effects of blueberry polyphenols on vascular function and cognitive performance in healthy individuals**

Would you like to take part in a cool experiment?

In this experiment we are trying to find out whether blueberries help you memorise better and increase the flow of blood to the brain, which is something that is really good for your brain!

If you would like to help us, then that will be great! 😊

The experiment will start in the morning, so we will give you some breakfast and then you will do some puzzles and tasks on a computer. After that we will measure the blood flowing to your brain using a really cool device. Then we will give you a blueberry drink and a break of about 2 hours, where you get to watch the television! Then we will do the same thing again after the break. You will only need to do this on two separate days 😊

We won't tell anyone else about what you say or do in the experiments, but it is okay for you to tell other people if you want to.

If you don’t want to have the drink then you don’t have to and you don’t need to say why. If you decide you don’t want to do any of the puzzles then you can stop at any time and you don’t need to say why.

I hope that you will want to take part!

From,

Sabine and Ellie at King’s College London

E.3 BluLife Study information sheet for parent

Study: *Investigating the effects of blueberry (poly)phenols on vascular function and cognitive performance in healthy individuals*

Researchers:

Sabine Hein: Tel +447593710575, Email: Sabine.hein@kcl.ac.uk)

Eleanor Wood: Tel +44207848 4162, Email: Eleanor.m.wood@kcl.ac.uk

Dr Claire Williams: Tel +44 (0) 118 378 7540, Email: Claire.williams@rdg.ac.uk)

Dr Ana Rodriguez-Mateos: Tel +447397552344, Email: ana.rodriguez-mateos@kcl.ac.uk

Blueberry Study Information For Parents

We to hope to provide you with all the information you will need about the study in order for you to make an informed decision about whether you would like your child to participate. However, if you have any further queries or would like to discuss any aspect of the study, please do not hesitate to contact the researchers using the contact information at the top of this sheet.

Background to the study

We are interested in finding out about the effects of a wild blueberry supplementation on cognitive performance and vascular function. Blueberries naturally contain high amounts of natural plant compounds called (poly)phenols that are found in a number of foods including vegetables, fruits and fruit juices. Recent research have shown that blueberries improve executive functioning and memory in both healthy adults and children. To date no study has directly linked alterations in blood flow, particularly in arteries supplying the brain, with improvements in cognitive function. In this study we aim to directly link a wild blueberry treatment with increased vascular and cerebral blood flow and positive cognitive outcomes in healthy individuals of various age groups. We will use individuals throughout the ages to determine whether the natural decline in cerebral blood flow and cognitive function as we age influences the effects shown by blueberry (poly)phenols.

Who is running the study?

The study is run by research staff in the Department of Nutritional Sciences, Kings College London and the School of Psychology & Clinical Language Sciences at the University of Reading. The study has been reviewed by the University Research Ethics Committee and has been given favourable ethical approval; which means that an independent group did not raise any objections to the study on ethical grounds and have permitted the study to proceed. In addition to this all researchers involves are in possession of appropriate food hygiene certificates.

What are the study's objectives?

To determine the effects of blueberry (poly)phenols vs placebo on cognitive performance 1.5hr post-consumption.

To determine the effects of blueberry (poly)phenols vs placebo on cerebral blood flow 1.5hr post-consumption.

To determine the effects of blueberry (poly)phenols vs placebo on flow-mediate dilation of the brachial artery as a measure of endothelial function, 1.5hr post-consumption.

To determine the effects of blueberry (poly)phenols vs placebo on office blood pressure (SBP and DBP) 1.5hr post-consumption.

To collect 24-hour urine samples in order to assess changes in urine (poly)phenol metabolites.

What does the study involve?

Session 1

You and your child will visit Kings College London at least one week before the study and will be briefed on what the study entails. You will also be asked to answer some questions on your child's health. After this your child will be asked to complete a 'practice' of all the cognitive tests that they will encounter at each future test phase. This is so your child can get used to the tasks and ask questions if they are unsure of what to do.

From 8.00am a day before study day 1 your child must not consume any of the following:

All fruit and vegetables (except bananas, carrots and sweetcorn)

Chocolate

Fruit juice

Tea and coffee

Fizzy and energy drinks

Pain relievers (e.g. paracetamol, ibuprofen, aspirin)

From 8 pm the day before session 2 your child must refrain from eating or drinking anything apart from water until the study begins the next day. We will provide your child with breakfast upon arrival.

Session 2 (Study day 1)

You will come into Kings College London with your child in the morning and will be provided with breakfast. After this, their blood pressure will be measured, and their cerebral blood flow recorded using a quick and non-invasive ultrasound device for 10 minutes. They will then take part in the previously practised cognitive test battery lasting approximately 45 minutes. Your child will then receive a blueberry drink to consume and then will take a break lasting roughly 1.5 hours. After the break your child will be invited to conduct the repeat the measurements as before. Your child will also be asked to begin a 24 hr urine collection on this day.

Session 3

You will come into Kings College London to drop off the 24 hr urine collection. There will be a chance to speak to the researchers about how the study day went and answer any questions if you may have any. The researchers will remind you about the next study day.

From 8.00 am on the day before the study day 2 your child must not consume any of the following:

- All fruit and vegetables (except bananas, carrots and sweetcorn)
- Chocolate
- Fruit juice
- Tea and coffee
- Fizzy and energy drinks
- Pain relievers (e.g. paracetamol, ibuprofen, aspirin)

Physical activity: They must also not partake in vigorous exercise. However, 2 x 10 minute mild-moderate-paced walks are acceptable.

From 8 pm the day before session 4 your child must refrain from eating or drinking anything apart from water until the study begins the next day. We will provide your child with breakfast on arrival.

Session 4 (Study day 2)

This session will take place at the same time as session 3 but 3-7 days later. It will run in exact the same manner as the first study day (session 2).

Session 5

You will come into Kings College London to drop off the 24 hr urine collection. There will a chance to speak to the researchers about how the study day went and answer any questions if you may have any.

About the blueberry drinks…

Your child will receive a blueberry drink containing a safe and specific dose of polyphenols. All drinks will be prepared hygienically within the university. With the exception of the ingredients already listed, no additives or any other items will be added. A full breakdown of the ingredients can also be made available to you upon request. In order to ensure that it is truly the drinks that are having an effect, we will provide you with a list of foods that are high in polyphenol content, which we will ask your child to

avoid eating for 24 hours before each day. A list of optional healthy alternative food will also be provided. Full details of this will be made available to you in advance.

What happens to the data?

All information collected will remain fully confidential and no results from any of the tasks your child performs will be shared. All the information you provide us with will be assigned an anonymous number and no name will appear on any of the documents. All data will be kept safely locked at King's College London and only the named researcher at the top of this sheet will have access. The data will be used only for research purposes, and in accordance with the Data Protection Act of 1998, they will be destroyed 5 years after the completion of the study.

On completion of this study there is a possibility that that the results will be published in an academic journal. Only the overall results will be referred to in this publication with no direct reference being made regarding your child or the school they attend. Additionally, if you so

request, the results of the study will be forwarded to you upon its completion either by post or email.

Does my child have to participate?

No. Participation in this study is entirely voluntary and you are under no obligation to agree to participate. Also, you may withdraw your child at any point during the study without giving any reason.

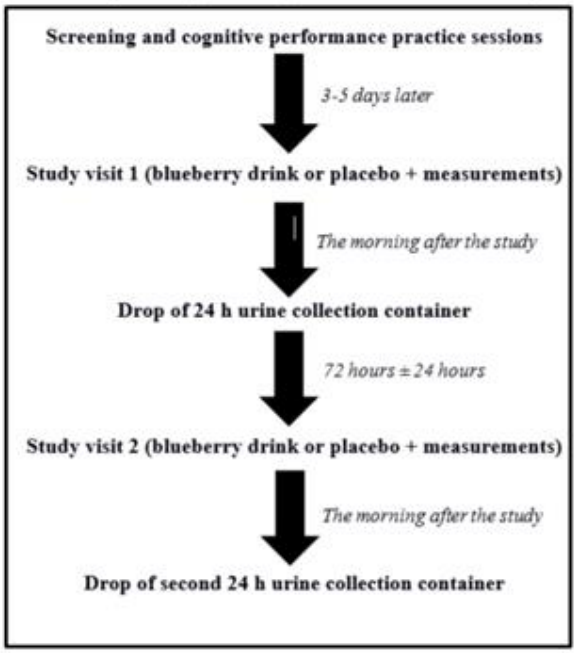
Will my child be paid to participate?

A reimbursement of £75 will be offered to you and your child.

I'd like my child to participate, what happens next?

Please contact one of the researchers above, indicating the information enclosed in the accompanying letter. A member of the research team will then contact you to arrange the study visits and to answer any further questions you may have.

Summary of visits to King's College London, Metabolic Research Unit (Waterloo Campus)



Thank you for your help!

E.4 BluFlow Study Participant Information Sheet

INFORMATION SHEET FOR PARTICIPANTS

Randomised, double-blind, placebo controlled, parallel trial investigating the effects of daily consumption of blueberry (poly)phenols on vascular function and cognitive performance

We would like to invite you to participate in this nutritional research project undertaken as part of a PhD program. You should only participate if you want to; choosing not to take part will not disadvantage you in any way. Before you decide whether you want to take part, it is important for you to understand why the research is being done and what your participation will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information.

What is the purpose of the study?

The study primarily aims to investigate the effects of daily consumption of blueberries on cognitive performance and vascular function. This dietary daily intervention will be investigated for 12 weeks. We measure cognitive performance using various tasks put together on a computer system. We then measure vascular function of the brachial artery (running along the bicep) using a non-invasive ultrasound technique called flow-mediated dilation (FMD). We will also measure the blood flow supplying the brain using similar non-invasive ultrasound equipment.

The health effects of blueberries are of particular interest to us due to a natural compound they contain known as polyphenols. These plant chemicals can be found in fruits, vegetables, cocoa and tea, and are thought to be partly responsible for the health benefits that have previously been observed. Therefore, to measure the polyphenol content and better understand the mechanisms of action, we will collect blood samples, faecal samples and full 24 hour urine from volunteers. The results of the study will help us to understand whether blueberry polyphenols can improve cognitive function and whether this is caused by an increased blood flow to the brain. Furthermore, it will add to our

knowledge on the effects of these polyphenols on vascular function. Improving vascular function would lead to a decreased risk of developing cardiovascular diseases in the future and improvements in cognitive function could slow the natural course of cognitive decline as we age.

Why have I been invited to take part?

You have been invited to take part as you have expressed interest in our research.

We would like to individuals aged between 65-80, who can answer 'yes' to the following questions:

I am aged between **65** and **80** years.

I **do not suffer** from peripheral artery disease, cerebrovascular disease, hypertension, obesity, acute inflammation, abnormal heart rhythm or terminal renal failure.

My BMI is **below 30.**

I have **no previous diagnosis of** cognitive impairment, dyslexia, or significant visual impairment.

I have **never had** a heart attack, stroke, diabetes mellitus, cancer, allergies to berries or other significant food allergy.

I do not have a history of **excessive** alcohol or substance abuse.

I am a **non-smoker.**

Do I have to take part?

No. The choice to participate is entirely yours. If you do decide to take part, we will go through this information sheet in more detail with you and answer any questions you may have. You will be given this information sheet and be asked to sign two copies of a consent form. However, you still may withdraw from the study at any time by informing one of the researchers, you are not obliged to give a reason. If you decide to withdraw once the study has started, the data may be used in the final report unless you request to withdraw your data. Data cannot be withdrawn once the study has been submitted as a study report which will be after the 1st January 2020. If you do decide to participate, please inform us of any other research you have been involved in during the previous year.

What will happen to me if I take part?

If you answer 'yes' to the above questions and remain interested in participating, you will be invited to attend a screening visit to confirm your eligibility, then you will be enrolled into the study if you remain eligible. Including the screening visit you will attend King's College London for 7 visits, 1 of which will be a one-month follow-up post the intervention period. On the screening day we will familiarise you with the cognitive function tests and go through any questions that you might have. Additionally, you will be required to come into the research unit a day before your first day for us to attach a 24-hour blood pressure monitor in preparation for the study day. On the three test days you will attend the facilities where we will undergo vascular health measurements and cognition tests.

Screening visit:

If we think you are potentially suitable for the study you will be invited to attend a clinic screening appointment (approx. **2 hours** including a cognitive test practice) in the **Metabolic Research Unit on 4th Floor, Corridor A, Franklin-Wilkins Building, 150 Stamford Street, London, SE1 9NH** (close to Waterloo Station).

On arrival, the study will be explained in detail and you will have the opportunity to ask any questions to ensure you will be giving fully informed consent. Once consent is provided, your height, weight, body fat percentage and blood pressure will be measured, and we will ask questions about your medical history. In addition, you will practice a cognitive battery test lasting around 45 minutes, twice. We will also be using ultrasound to locate your middle cerebral artery on the right side of your head for the purpose of cerebral blood flow measurements. This should not take more than 10 minutes.

Study visits:

Following screening, if your results comply with the study inclusion criteria you will be invited to attend the Metabolic Research Unit on 3 further occasions lasting **3 hours** each. Measurements include non-invasive ultrasound measures of your brachial artery and your cerebral arteries, cognitive function tasks, blood pressure, and a small blood sample. Additionally, you will be asked to come to the research unit **24 hours prior** to each of the 3 study visits to have a 24-hour blood pressure monitor fitted and to receive your urine collection kit to start the 24-hour urine collection. Study visits will not be too close

together: Study visit 2 will be 12 weeks after study visit 1, and study visit 3 will be 4 weeks after study visit 2.

About the measurements:

Flow-mediated dilation: Elasticity of the brachial artery will be measured through echography. With an ultrasound probe we will measure the diameter of the brachial artery in the upper arm. To test the flexibility of the artery, a cuff will be placed on the forearm and will be inflated during 5 min. After deflation an increased blood flow will expand the artery by 5-8%. This expansion is called flow-mediated dilation.

Cognitive function: This will be assessed by using a set of cognitive tests on a laptop, including tests for executive function and other tasks for memory, as well as a mood questionnaire. We will familiarise you with the cognition tasks when you attend the first visit for screening.

Cerebral blood flow: Measured by placing an ultrasound probe onto the temple to identify the middle cerebral artery. This will be held in place using a headband device to allow us to take 10 minutes of measurements, then an additional 10 minutes of measurements during the cognitive task performance.

Arterial stiffness: A pressure-sensitive probe is applied on the skin to measure the pulse from the carotid (neck), femoral (thigh) and brachial (wrist) arteries.

24-hour ambulatory blood pressure monitoring (ABPM). This is a small digital blood pressure machine that is attached to a belt around your body and which is connected to a cuff around your upper arm. It is small enough and won't interfere with your daily routine, including sleep. This will be attached to measure your blood pressure 24 hrs before your first study day.

Blood tests: On the two study visit days we will take blood samples, before and after drinking the blueberry drink, using a small needle, a small amount of blood will be taken (around 50 ml overall). Included in blood analysis will be, health parameters (glucose, lipids and liver function), polyphenol analysis and the analysis of DNA and RNA samples.

Faecal samples: We will also ask you to collect a faecal sample (approx. ½ tsp) the day before each study visit using a very easy stool sample collection kit which we will provide more detail on how to do.

Urine collection: We will ask that you start collecting urine into a container provided by us the day before each study day. We then ask that you take the container home in a small cool bag with ice blocks provided then continue to collect all urine excreted over the 24-hour period until the following morning where you will come in for the study visit.

Summary of the visits:

Screening visit (approx. 30 mins): If eligible you will be invited to attend the metabolic research unit in the Franklin-Wilkins Building on 6 further occasions.

24 hours prior to study visit 1 (30 mins): To have a 24-hour blood pressure monitor fitted and collect equipment for 24-hour urine collection.

Study visit 1 (approx. 3 hours): All measurements described above will be performed before you take home enough blueberry drink for 12 weeks which you will consume once a day. You will then return to the research unit 12 weeks later.

24 hours prior to study visit 2 (30mins): To have a 24-hour blood pressure monitor fitted and collect equipment for 24-hour urine collection.

Study visit 2 (approx. 3 hours): 12 weeks after study visit 1 you will return to the research unit for all of the measurements again, mentioned above. You will then return 4 weeks later after not consuming any blueberry drink to see if any health benefits remain.

24 hours prior to study visit 3 (30mins): To have a 24-hour blood pressure monitor fitted and collect equipment for 24-hour urine collection.

Study visit 3 (approx. 3 hours): 4 weeks after study visit 2 you will return for the final visit and all measurements mentioned above will be done once more.

What are the benefits and risks of you taking part?

Benefits;

You will be compensated with a payment of £150 for completing the study **(£50 per study visit).**

Information on your health including cholesterol levels, glucose levels and body fat composition will be given to you on request.

You will be contributing to invaluable research on food and how it can improve different aspects of our health and possibly slow inevitable factors of aging such as cognitive decline and reduce cardiovascular risk.

Risks;

The blood sample venepuncture may cause a brief discomfort and could leave a small bruise.

The blood pressure cuff or the cuff used during flow-mediated dilation measurements may cause short discomfort or a tingling sensation in your arm. However, the cuff is not reported to cause any damage to the arm.

In the unlikely event that we discover that you have a medical condition (i.e., high blood pressure or elevated blood cholesterol) that you were unaware of, we will inform you and discuss informing your GP by providing you a letter template for you to take to them.

If you are unaware of any allergies to berries and if an allergic reaction should occur this could result in vomiting, diarrhoea or nausea.

Will my information and participation be confidential?

Yes. All personal information collected during the study will be kept confidential and in password protected software or in locked cabinets/rooms. We will assign you to a subject code which will be used in place of names to store and collect data. Only the investigators have access to this data and only anonymised data will be shared with other researchers. Should you wish to find out any personal results such as health information please feel free to contact the PhD students running the study: Eleanor Wood and Sabine Hein (contact details below).

How is the project being funded?

The study is organised by the Diabetes and Nutritional Sciences Division, King's College London and is funded by the Wild Blueberry Association of America.

What will happen to the results of the study?

The study results will be presented in a report and will be published in a scientific journal. You will not be identified in the results of the study or any publication that might arise from this study. We will be happy to discuss the results with you when the study is

completed and will let you know how you can get a copy of the published results if you wish.

Who should I contact for further information?

Eleanor Wood or Sabine Hein via the study email address: **BluFlowStudy@kcl.ac.uk**

Ask for Sabine or Eleanor on: **+44 20 784 84162**

What if something goes wrong?

If this study has harmed you in any way or if you wish to make a complaint about the conduct of the study you can contact King's College London using the details below for further advice and information:

The Chair, BDM Research Ethics

Subcommittee (RESC), rec@kcl.ac.uk

Thank you for reading this information sheet and for considering taking part in this research

_____	_____	_____
Name of Participant	**Date**	**Signature**
_____	_____	_____
Name of Researcher	**Date**	**Signature**

**we will ask you to sign this after the screening visit and provide you with a copy.*

Appendix F

Consent Forms

F.1 BluLife Study Consent form for participants over 16

years:

CONSENT FORM FOR PARTICIPANTS IN RESEARCH STUDIES

Please complete this form after you have read the Information Sheet and/or listened to an explanation about the research.

Title of Study: Randomised, double-blind, placebo controlled, crossover trial investigating the acute effects of blueberry (poly)phenols on vascular function and cognitive performance in healthy individuals.

King's College Research Ethics Committee Ref:

Thank you for considering taking part in this research. The person organising the research must explain the project to you before you agree to take part. If you have any questions arising from the Information Sheet or explanation already given to you, please ask the researcher before you decide whether to join in. You will be given a copy of this Consent Form to keep and refer to at any time.

	Please tick or initial
I confirm that I understand that by ticking/initialing each box I am consenting to this element of the study. I understand that it will be assumed that unticked/initialed boxes mean that I DO NOT consent to that part of the study. I understand that by not giving consent for any one element I may be deemed ineligible for the study	
I confirm that I have read and understood the information sheet dated 02/01/2018 Version 2 for the above study. I have had the opportunity to consider the information and asked questions which have been answered satisfactorily.	

1. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason. Furthermore, I understand that I will be able to withdraw my data up to 01/01/2020	
2. I consent to the processing of my personal information for the purposes explained to me. I understand that such information will be handled in accordance with the terms of The General Data Protection Regulation (GDPR) (EU) 2016/679.	
3. I understand that my information may be subject to review by responsible individuals from the College for monitoring and audit purposes.	
4. I understand that confidentiality and anonymity will be maintained and it will not be possible to identify me in any publications	
5. I agree to be contacted in the future by King's College London researchers who would like to invite me to participate in follow up studies to this project, or in future studies of a similar nature.	
6. I agree that the research team may use my data for future research and understand that any such use of identifiable data would be reviewed and approved by a research ethics committee. (In such cases, as with this project, data would/would not be identifiable in any report).	
7. I understand that the information I have submitted will be published as a report and I wish to receive a copy of it.	
8. I understand that I must not take part if I fall under the exclusion criteria as detailed in the information sheet and explained to me by the researcher.	
9. I agree that my GP may be contacted if any unexpected results are found in relation to my health.	
10. I agree to the self-collection of urine samples and allow these to be stored anonymously and analysed by 01/07/2020.	
11. I agree to the collection of blood samples and allow these to be stored and analysed by 01/07/2020.	

Name of Participant	**Date** **/ /**	**Signature**
Name of Researcher	**Date** **/ /**	**Signature**

F.2 BluLife Study Consent form for participants under 16 years

Consent Form

An experiment on how blueberry can help us to pay attention, remember things and increase blood flow to the brain

I ……am happy to take part in the study called 'Investigating the effects of blueberry polyphenols on vascular function and cognitive performance'

☐ I have been given a piece of paper with information about the study.

☐ The researcher has read this to me and explained any parts I didn't understand.

☐ I have been asked if I have any questions about the study and the researcher answered these.

☐ I understand that the researchers won't tell anybody else about what I say or do during the study, but I am able to tell other people what I did if I want to.

☐ I understand that I don't need to take part if I don't want to.

☐ I understand that if I want to stop taking part during the study I can and I don't have to say why.

Signature ……Date…….

Name (in capitals) ……

Birthday (date,month,year) ……

F.3 BluLife Study Parental Consent Form For Parents Of A Child Under 16

Parental Consent Form

Investigating the effects of blueberry (poly)phenols on vascular function and cognitive performance in healthy individuals

I ……parent/guardian of ……

agree to my child participating in the study 'The effects of blueberry (poly)phenols on vascular function and cognitive performance in healthy individuals' at the Department of Nutrition and Dietetics, King's College London. This study has been reviewed by Kings College London Research Ethics Committee and has been given ethical approval.

(Please initial as appropriate)

I have seen and read a copy of the Study Information Sheet and have been given the opportunity to ask questions about the study and these have been answered to my satisfaction.

I understand that all personal information will remain confidential to the researcher and arrangements for the storage and eventual disposal of any identifiable material have been made clear to me.

The contents of the drinks have been explained to me and I am happy for my child to consume them.

I understand that participation in this study is voluntary and that I can withdraw my child at any time without having to give an explanation.

I believe that my child understands what is required of them during the study

I am happy for my child to proceed with participation.

Signature …….

Name (in capitals) …….

Date ……

F.4 BluFlow Study Consent Form

KING'S College LONDON

CONSENT FORM FOR PARTICIPANTS IN RESEARCH STUDIES

Please complete this form after you have read the Information Sheet and/or listened to an explanation about the research.

Title of Study: Randomised, double-blind, placebo controlled, parallel trial investigating the effects of daily consumption of blueberry (poly)phenols on vascular function and cognitive performance

King's College Research Ethics Committee Ref:

Thank you for considering taking part in this research. The person organising the research must explain the project to you before you agree to take part. If you have any questions arising from the Information Sheet or explanation already given to you, please ask the researcher before you decide whether to join in. You will be given a copy of this Consent Form to keep and refer to at any time.

	Please tick or initial
1. I confirm that I understand that by ticking/initialing each box I am consenting to this element of the study. I understand that it will be assumed that unticked/initialed boxes mean that I DO NOT consent to that part of the study. I understand that by not giving consent for any one element I may be deemed ineligible for the study	
2. I confirm that I have read and understood the information sheet dated 11/03/2019 Version 3 for the above study. I have had the opportunity to consider the information and asked questions which have been answered satisfactorily.	
3. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason. Furthermore, I understand that I will be able to withdraw my data up to 01/04/2020	

4. I consent to the processing of my personal information for the purposes explained to me. I understand that such information will be handled in accordance with the terms of The General Data Protection Regulation (GDPR) (EU) 2016/679.	
5. I understand that my information may be subject to review by responsible individuals from the College for monitoring and audit purposes.	
6. I understand that confidentiality and anonymity will be maintained and it will not be possible to identify me in any publications	
7. I agree to be contacted in the future by King's College London researchers who would like to invite me to participate in follow up studies to this project, or in future studies of a similar nature.	
8. I agree that the research team may use my data for future research and understand that any such use of identifiable data would be reviewed and approved by a research ethics committee. (In such cases, as with this project, data would/would not be identifiable in any report).	
9. I understand that the information I have submitted will be published as a report and I wish to receive a copy of it.	
10. I understand that I must not take part if I fall under the exclusion criteria as detailed in the information sheet and explained to me by the researcher.	
11. I agree that my GP may be contacted if any unexpected results are found in relation to my health.	
12. I agree to the self-collection of urine and fecal samples and allow these to be stored anonymously and analysed by 01/07/2020.	
13. I agree to the collection of blood samples and allow these to be stored and analysed by 01/07/2020.	

Name of Participant	**Date** / /	**Signature**
Name of Researcher	**Date**	**Signature**

Appendix G

G.1 Auditory Verbal Learning Task Word Lists

Version 1A	**Version1B**	**Version 2A**	**Version 2B**	**Version 3A**	**Version 3B**
Cane	Sword	Latch	Fawn	Swamp	Herd
Needle	Pipe	Metal	Bowl	Penny	Sheet
Spike	Vest	Fox	Doll	Fudge	Rake
Ferry	Kennel	Armour	Beehive	Woodland	Camel
Owl	Cliff	Brunch	Duck	Sail	Rod
Package	Napkin	Measles	Barrel	Arrow	Mansion
Pearl	Deck	Brick	Shore	Gun	Mud
Bullet	Lemon	Linen	Hammer	Kitten	Soldier
Lung	Frost	Tool	Bush	Board	Sweat
Shoulder	Pepper	Chicken	Motor	Rubber	Piano
Church	Cloth	Star	Land	Pine	Rug
Jar	Stone	Ball	Glove	Neck	Soup
Picture	Mirror	Garden	Button	Oven	Ticket
Dog	Seat	Meat	Ring	Pot	Sock
Bag	Door	Spoon	Tree	Fruit	Plant
SCORE	**SCORE**	**SCORE**	**SCORE**	**SCORE**	**SCORE**

Version 4A	**Version 4B**	**Version 5A**	**Version 5B**	**Version 6A**	**Version 6B**
Inn	Sword	Hose	Lime	Scout	Beast
Doorway	Pipe	Prison	Tack	Palace	Shield
Barn	Vest	Tank	Calf	Pit	Crow
Sardine	Kennel	Shepherd	Marble	Lilly	Farmyard
Worm	Cliff	Seed	Oak	Shell	Heel
Slipper	Napkin	Chapel	Rifle	Gravel	Laundry
Soil	Deck	Bone	Weed	Blade	Shark
Valley	Lemon	Jewel	Tractor	Gravy	Movie
Stew	Frost	Card	Van	Flag	Chin
Candy	Pepper	Hotel	Office	Wire	Uncle
Coin	Cloth	Wood	Blood	Oil	Throat
Film	Stone	Bin	School	Tray	Sleeve
Pocket	Mirror	Toilet	Orange	Building	Pillow
Soap	Seat	Clock	Rain	Brush	Lamp
Men	Door	Salt	Face	Book	Girl
SCORE	**SCORE**	**SCORE**	**SCORE**	**SCORE**	**SCORE**

Appendix H

H.1 Positive and Negative Affect Schedule (PANAS-NOW) Mood adjectives

TableX1: Ten positive and ten negative mood objectives assessed in the PANAS

Positive Adjectives	**Negative Adjectives**
Active	Afraid
Alert	Ashamed
Attentive	Distressed
Determined	Guilty
Enthusiastic	Hostile
Excited	Irritable
Inspired	Jittery
Interested	Nervous
Proud	Scared
Strong	Upset

Appendix I

I.1 Phlebotomy full list of biomarkers

The following parameters were analysed in blood pertaining to safety:

- Creatinine
- Urea
- Uric acid
- Bilirubin
- Total Cholesterol
- HDL-cholesterol
- LDL-cholesterol
- Triglycerides
- Lactate dehydrogenase
- Aspartate aminotransferase (GOT or AST)
- Alanine aminotransferase (GPT or ALT)
- Gamma-glutamyl-transpeptidase (GGT)
- Alkaline phosphatase
- Glucose
- Full blood count:
    - Total white blood cells (neutrophil granulocytes, lymphocytes, monocytes, eosinophil and basophil granulocytes)
    - Total red blood cells
    - Hemoglobin
    - Hematocrit
    - MCV (mean corpuscular volume)
    - MCH (mean corpuscular hemoglobin)
    - MCHC (mean corpuscular hemoglobin concentration)
    - RDW (red blood cell distribution width)
    - Thrombocytes or platelets
    - MTV (mean thrombocyte volume)
    - Neutrophils
    - Lymphocytes
    - Monocytes
    - Eosinophils
    - Basophils
    - Reticulocytes

Appendix J

J.1 Phlebotomy full list of biomarkers

The following parameters were analysed in blood pertaining to safety:

- Creatinine
- Urea
- Uric acid
- Bilirubin
- Total Cholesterol
- HDL-cholesterol
- LDL-cholesterol
- Triglycerides
- Lactate dehydrogenase
- Aspartate aminotransferase (GOT or AST)
- Alanine aminotransferase (GPT or ALT)
- Gamma-glutamyl-transpeptidase (GGT)
- Alkaline phosphatase
- Glucose
- Full blood count:
    - Total white blood cells (neutrophil granulocytes, lymphocytes, monocytes, eosinophil and basophil granulocytes)
    - Total red blood cells
    - Hemoglobin
    - Hematocrit
    - MCV (mean corpuscular volume)
    - MCH (mean corpuscular hemoglobin)
    - MCHC (mean corpuscular hemoglobin concentration)
    - RDW (red blood cell distribution width)
    - Thrombocytes or platelets
    - MTV (mean thrombocyte volume)
    - Neutrophils
    - Lymphocytes
    - Monocytes
    - Eosinophils
    - Basophils
    - Reticulocytes

Appendix K

K.1 Polyphenol Analysis Standard Operating Procedure

Standard operating procedure (SOP)

Title: Polyphenol Analysis

Author: Yifan Xu (PhD student)

PI Name	Ana Rodriguez-Mateos	PI Signature	
Author Name		Author Signature	

Version	3
Effective from	June 220
Review date	June 2021
Author	Yifan Xu

Purpose

The purpose of this SOP is to describe the steps and procedures for polyphenol analysis in human plasma and urine samples using triple quadruple mass spectrometer.

Scope

The scope of this SOP is to describe the procedure of polyphenol analysis involving sample processing though SPE and analysis by UPLC-MS.

Revision status

Amendment number: date	Version number discarded	Insert version number	Page	Section involved	Amendment

1. Responsibilities

It is the responsibility of the user to ensure that:

Equipment is used responsibly and stored/kept correctly in a secure room.

Participants are aware of the procedures for the measurement in case of any discomfort during the occlusion of the arm.

2. Materials

- Reagents needed for sample preparation:

Water (HPLC grade)

Phosphoric acid (85%)

Acetic acid (100%)

Formic acid (LC-MS grade)

Ammonium formate (powder, eluent additive for LC-MS, >=99%)

Acetone for HPLC (Chromasolv)

Methanol for HPLC (Chromasolv)

Acetonitrile for UPLC MS

- Solutions needed for sample preparation:

- 4% phosphoric acid: pipette 4720 µL of H_3PO_4 (85%) in a 100 mL volumetric flask containing Milli Q water and fill up the mark with Milli Q water. Final concentration = 0.725 M) and pH = 1.17.

- 0.2% acetic acid: pipette 230 µL of CH3COOH (100%) in a 100 mL volumetric flask containing Milli Q water and fill up the mark with Milli Q water. Final concentration = 0.04 M and pH = 3.08.

Eluent solution: MeOH +0.1%FA+10mM Ammonium formate: pipette 100uL Formic acid (LC-MS grade) in a 100ml volumetric flask, weigh 63.1mg Ammonium formate powder(>=99%) and add to the flask and fill up the mark with HPLC grade MeOH (>99.9 %)

- UPLC grade MeOH (>99.9 %)
- UPLC grade water
- UPLC grade HCOOH (>99.9 %)

3. Preparation

Master standard mix calibration curve: 13 mixes are prepared using the excel template “standard mix preparations.072020”. Carefully prepare 600 uL per eppy for each mix. Aliquote 60 uL of each mix into vials add 6uL of elution solution and 5uL of isotope internal standards mix and 5uL of taxifolin (1:80 diluted) and put in -20C freezer. Prepare one curve-set per sample batch.

Internal standard solutions:

Internal standard solution1: 1:4000 diluted taxifolin stock solution

Internal standard solution2: 1:10 diluted C13 labelled standard 156/157 mix(1:1)

Quality check samples: three types of quality checks will be designed for the sequence run: QC solvent, QC pool-spike and QC pool-non spike. QC solvent is a HPLC vial filled with mobile phase A (1.5 mL). QC pool are pooled plasma/diluted urine samples of all volunteers that ran that day. About 10 uL per sample will be pooled. The QC pooled samples will then be treat as any other volunteer sample (will be diluted in H3PO4, spiked with IS and undergo uSPE).

Post-spiked blank mixes: The QC plasma/diluted urine sample will be spiked with mix 9 after SPE for reference samples and put into vials for multiple injections.

Check which samples you will need at least two days beforehand. We are analyzing plasma from blood collected in EDTA with formic acid and the urine is also the one collected with formic acid. If the EDTA plasma looks like jelly then Heparin sample can be replaced. Samples should be organized in specific boxes.

4. Procedure Sample preparation

The day before:

Prepare microcentrifuge Eppendorf tubes (safe lock tubes that can handle high centrifugation forces) and label them with the numbers ascribed in the last item (from 1 to x). This will make easy to keep track of the pipetting scheme.

Add 353µL of 4% phosphoric acid to each tube

Put everything in the fridge (4°C)

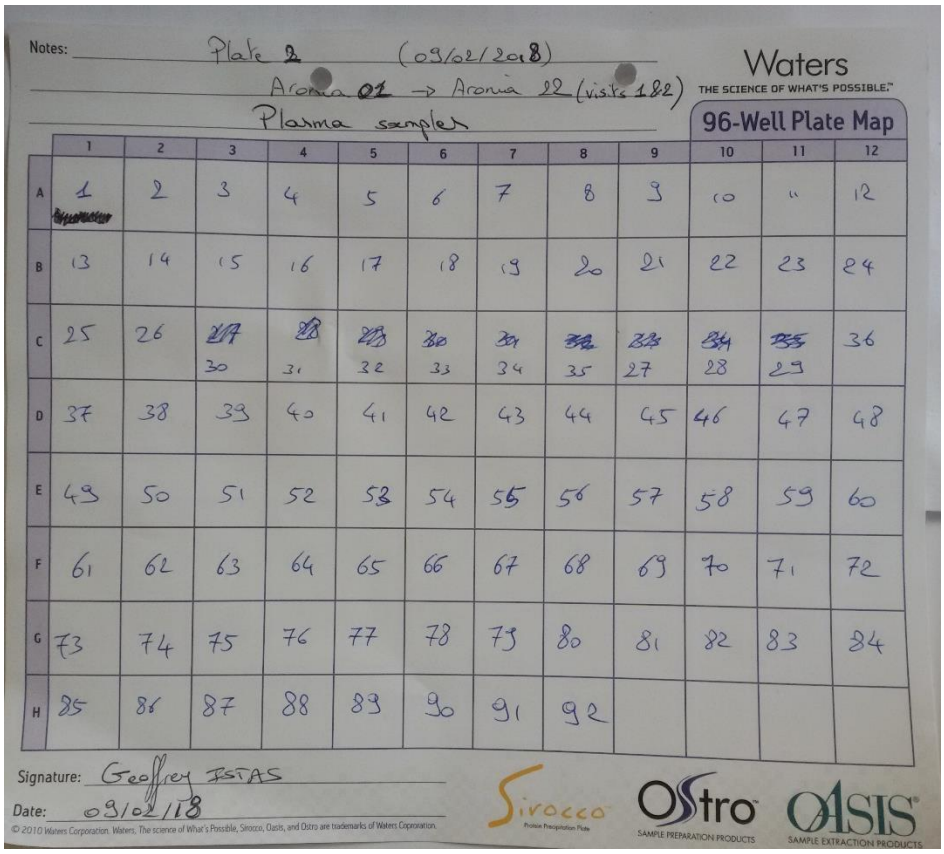
Prepare the sequence run: number the samples starting from Aronia 1_visit 1_0h = 1 and continue with Aronia 1_visit 1_2h = 2… This is the numbering you will use during sample prep. However, samples need to be injected in a randomized fashion. Use an online randomizer tool or MS Excel o randomize your sequence list. In that way the auto-sampler will inject the samples randomly even though the 96-well plate has samples in a logic order (see excel file "sequence run template-SOP-202006").

On the day:

Pre-cool the centrifuge at (4°C) 30 min in advance.

Take out the plasma/urine samples from the -80 and put them immediately in ice to thaw. Tip: don't push the eppies in the ice, just lay them horizontally on the ice to speed up thawing (appr. 45 min)

Use a default sheet from Waters to write which samples will go on each wells on the 96-well plate and label using the same numbers as written on the eppies (starting from 1). Keep these papers in the blue folder above the SPE extractor for future reference. Also, on the notes section write the number of the plate, which should be sequential, and write down the volunteers' numbers and visits that were analyzed and sample type.



Notes: Plate 2 (09/02/2018)
Aronia 01 → Aronia 22 (visits 1&2)
Plasma samples

Waters
THE SCIENCE OF WHAT'S POSSIBLE.™

96-Well Plate Map

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	2	3	4	5	6	7	8	9	10	11	12
B	13	14	15	16	17	18	19	20	21	22	23	24
C	25	26	30	31	32	33	34	35	27	28	29	36
D	37	38	39	40	41	42	43	44	45	46	47	48
E	49	50	51	52	53	54	55	56	57	58	59	60
F	61	62	63	64	65	66	67	68	69	70	71	72
G	73	74	75	76	77	78	79	80	81	82	83	84
H	85	86	87	88	89	90	91	92				

Signature: Geoffrey ISTAS
Date: 09/02/18

Sirocco Protein Precipitation Plate
Ostro SAMPLE PREPARATION PRODUCTS
OASIS SAMPLE EXTRACTION PRODUCTS

When the samples are thawed, dilute the urine with HPLC water in 1:5.

Centrifuge the samples at 15000g (RCF) for 15 min at 4C

For Eppendorf Centrifuge on 3rd floor: 11800 RPM

For the Biofuge Fresco Heraeus on 4th floor: 12600 RPM

For the centrifuge on 3rd floor 3.130: 15000g

Pipette 350 µL of urine or plasma into the phosphoric acid containing tubes

Vortex the urine/plasma diluted with phosphoric acid

NOTE: If the volume of urine or plasma is < 350 µL, pipette the supernatant with the P100 and register the volume. Add up the remaining volume with HPLC water and write down the exact volume of sample that was

used because the sample dilution factor will be different.

Vortex each tube.

In the collection plate: add 35uL HPLCwater, 5uL isotope standard mix (you can do this while the samples are loading/washing to prevent evaporation).

Solid phase extraction: “Waters Positive Pressure-96 Processor”

RP based columns (vinylpyrrolidone (hydrophilic) and polyvinylbenzene (lypophylic) that retain phemolic acids

Put the washing 10 mL plate in the PP96 and add the white adaptor on the top.

Add the microelution plate on the top.

Load 600 µL of each sample in the corresponding wells (follow the water 96 well template)

Spike 5ul of taxifolin (1:4000) into the wells using multi-channel pipette. Spike the position of QC-spike with 30uL of mix9.

Open the nitrogen cylinder using the metal key provided and turn on the valve by turning towards right (it feels like you are closing the valve but you are actually opening it). Check the pressure on the meter and if nitrogen is flowing, the pressure should rise on the meter (around 20-25 on the left-hand side meter)

Press on the switches located on the side of the PP96 positive pressure device simultaneously to load down the elevator.

Set the low pressure knob to 5 and wait for the sample to load on the cartridges. In the meantime, you should be able to see the sample being washed off and collected on the washing plate. The procedure should take around 8 min. If necessary, increase the pressure up to 10 or 15. Release the plate by pressing the switches again. If there is any liquid remaining on any of the wells use the high pressure knob and set the pressure to 15 for 20 sec or 1 min and that should be enough. Release the pressure again and make sure that all the liquid has eluted.

Add 200 µL of HPLC water to wash the wells using multi-channel pipette.

Regulate the pressure with the low pressure knob and increase it accordingly.

Add 200 µL of 0.2% acetic acid to wash the wells (around 5min).

After this step, MAKE SURE YOU REMOVE THE WASHING PLATE AND REPLACE IT BY THE 96 WELL PLATE COLLECTION PLATE THAT WILL BE USED TO COLLECT THE SAMPLES AND PUT IN THE UPLC AUTOSAMPLER.

Add 30 µL of elution solution to each well and apply pressure using the low pressure knob (around 5min).

Add another 30 µL of elution to each well and apply pressure using the low pressure knob (around 5min).

Add another 30 µL of elution to each well and apply pressure using the low pressure knob (around 5min).

After the elution is complete, remove the 96 well plate from the PP96 and immediately add the adhesive seal to prevent evaporation.

Mix the plate for 30 sec using a plate mixer

DO NOT FORGET TO TURN OFF THE NITROGEN FAUCET AFTERWARDS!

Start worklist (can start it 3 hours before to run the standard mix already

Procedure SHIMADZU triple quadruple UPLC-MS

Mobile phase preparation

Mobile phase A:

Add 2.5mL of formic acid (HPLC grade) to a 2.5L HPLC grade water bottle. Write down date of opening and "+ 0.1% FA". Mix the bottle thoroughly. Clean (with HPLC water and acetone) the 1L glass bottle containing Mob phase A on auto-sampler and fill with new 0.1% FA water almost to the top.

Mobile phase B:

Add 2.5mL of formic acid (HPLC grade) to a 2.5L HPLC grade acetonitrile bottle. Write down date of opening and "+ 0.1% FA". Mix the bottle thoroughly. Clean (with HPLC water and acetone) the 1L glass bottle containing Mob phase B on auto-sampler and fill with new 0.1% FA acetonitrile almost to the top.

Column and guard column

Raptor Biphenly, 2.1 x 50 mm, 1.8 µm (Restek, 9309252) with compatible Raptor Biphenyl Guard Cartridges 5 X 2.1MM.

Keep track of pressure before each run when pumping 99% of A and 1 % of B (around 330-340 bars)

Sequence set-up:

Copy and paste the prepared sequence from Excel to run on the SHIMADZU LabSolution work station Batch Editor, and save the batch into the folder. (Under the user's folder please setup a new folder named after the running date and save batch into the folder. Copy the method into this folder as well. When you paste the data file name into the sequence they will be saved in the same folder).

Make sure all samples are set in correct positions, A1 position on 96 well plates should be placed on the bottom left on the tray. Tray 0 is the small one with 10 positions where you can put calibration curve vials and water/MeOH. Tray 1 is the 96 well plate position near the door, tray 2 is the one further inside.

Take the lid of water/MeOH off if you have more than 1 plate to run in one go to avoid blockage from the material of lid

Remember to put shut down after run in the batch file, right click-shut down.

Queue batch run.

REMEMBER TO CHANGE THE POSITION OF VIALS in tray 0 2-3h after the start of the calibration curve.

To edit the batch in the middle of a run: pause the queue 1 row before the row that you wish to edit, edit and choose the paused row and all the rows below (it is important that you choose all the rest rows) and start. Check the time for editing and try to finish editing before the stopped row start.

6. Health & Safety

Clean the equipment between runs

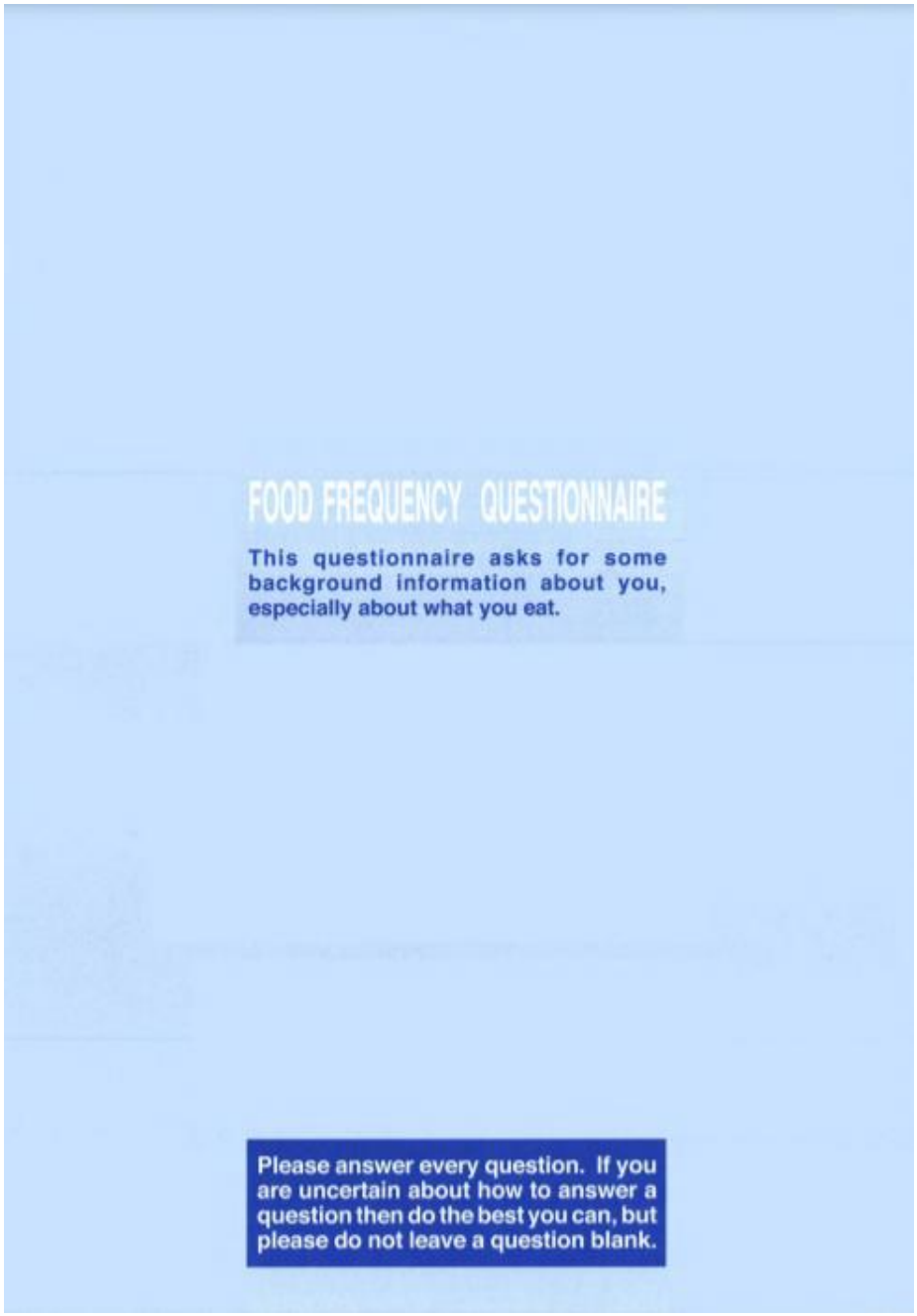
Wear gloves as protection against acids

Dispose of waste in appropriate bins (SPE waste: put 1% virkon and stay for over 30min, then wash it down through sink)

Turn all equipment on standby when not it use

Appendix L

L.1 EPIC NORFOL FFQ



1. **YOUR DIET LAST YEAR**

For each food there is an amount shown, either a "medium serving" or a common household unit such as a slice or teaspoon. Please put a tick (✓) in the box to indicate how often, **on average**, you have eaten the specified amount of each food **during the past year**.

EXAMPLES:

For white bread the amount is one slice, so if you ate 4 or 5 slices a day, you should put a tick in the column headed "4-5 per day".

FOODS AND AMOUNTS	AVERAGE USE LAST YEAR								
BREAD AND SAVOURY BISCUITS (one slice or biscuit)	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
White bread and rolls								✓	

For chips, the amount is a "medium serving", so if you had a helping of chips twice a week you should put a tick in the column headed "2-4 per week".

FOODS AND AMOUNTS	AVERAGE USE LAST YEAR								
POTATOES, RICE AND PASTA (medium serving)	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Chips				✓					

For very seasonal fruits such as strawberries and raspberries you should estimate your average use when the fruits are in season, so if you ate strawberries or raspberries about once a week when they were in season you should put a tick in the column headed "once a week"

FOODS AND AMOUNTS	AVERAGE USE LAST YEAR								
FRUIT (1 fruit or medium serving)	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Strawberries, raspberries, kiwi fruit			✓						

Please estimate your average food use as best you can, and please answer every question - do not leave ANY lines blank. PLEASE PUT A TICK (✓) ON EVERY LINE

FOODS AND AMOUNTS	AVERAGE USE LAST YEAR								
MEAT AND FISH (medium serving)	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Beef: roast, steak, mince, stew or casserole									
Beefburgers									
Pork: roast, chops, stew or slices									
Lamb: roast, chops or stew									
Chicken or other poultry eg. turkey									
Bacon									
Ham									
Corned beef, Spam, luncheon meats									
Sausages									
Savoury pies, eg. meat pie, pork pie, pasties, steak & kidney pie, sausage rolls									
Liver, liver paté, liver sausage									
Fried fish in batter, as in fish and chips									
Fish fingers, fish cakes									
Other white fish, fresh or frozen, eg. cod, haddock, plaice, sole, halibut									
Oily fish, fresh or canned, eg. mackerel, kippers, tuna, salmon, sardines, herring									
Shellfish, eg. crab, prawns, mussels									
Fish roe, taramasalata									
	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Please check that you have a tick (✓) on EVERY line

PLEASE PUT A TICK (✓) ON EVERY LINE

FOODS AND AMOUNTS	AVERAGE USE LAST YEAR								
BREAD AND SAVOURY BISCUITS (one slice or biscuit)	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
White bread and rolls									
Brown bread and rolls									
Wholemeal bread and rolls									
Cream crackers, cheese biscuits									
Crispbread, eg. Ryvita									
CEREALS (one bowl)									
Porridge, Readybrek									
Breakfast cereal such as cornflakes, muesli etc.									
POTATOES, RICE AND PASTA (medium serving)									
Boiled, mashed, instant or jacket potatoes									
Chips									
Roast potatoes									
Potato salad									
White rice									
Brown rice									
White or green pasta, eg. spaghetti, macaroni, noodles									
Wholemeal pasta									
Lasagne, moussaka									
Pizza									
	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Please check that you have a tick (✓) on EVERY line

PLEASE PUT A TICK (✓) ON EVERY LINE

FOODS AND AMOUNTS	AVERAGE USE LAST YEAR								
DAIRY PRODUCTS AND FATS	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Single or sour cream (tablespoon)									
Double or clotted cream (tablespoon)									
Low fat yogurt, fromage frais (125g carton)									
Full fat or Greek yogurt (125g carton)									
Dairy desserts (125g carton)									
Cheese, eg. Cheddar, Brie, Edam (medium serving)									
Cottage cheese, low fat soft cheese (medium serving)									
Eggs as boiled, fried, scrambled, etc. (one)									
Quiche (medium serving)									
Low calorie, low fat salad cream(tablespoon)									
Salad cream, mayonnaise (tablespoon)									
French dressing (tablespoon)									
Other salad dressing (tablespoon)									
The following on bread or vegetables									
Butter (teaspoon)									
Block margarine, eg. Stork, Krona (teaspoon)									
Polyunsaturated margarine (tub), eg. Flora, sunflower (teaspoon)									
Other soft margarine,dairy spreads (tub), eg. Blue Band, Clover (teaspoon)									
Low fat spread (tub), eg. Outline, Gold (teaspoon)									
Very low fat spread (tub) (teaspoon)									
	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Please check that you have a tick (✓) on EVERY line

PLEASE PUT A TICK (✓) ON EVERY LINE

FOODS AND AMOUNTS	AVERAGE USE LAST YEAR								
SWEETS AND SNACKS (medium serving)	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Sweet biscuits, chocolate , eg. digestive (one)									
Sweet biscuits, plain, eg. Nice, ginger (one)									
Cakes eg. fruit, sponge, home baked									
Cakes eg. fruit, sponge, ready made									
Buns, pastries eg. scones, flapjacks, home baked									
Buns, pastries eg. croissants, doughnuts, ready made									
Fruit pies, tarts, crumbles, home baked									
Fruit pies, tarts, crumbles, ready made									
Sponge puddings, home baked									
Sponge puddings, ready made									
Milk puddings, eg. rice, custard, trifle									
Ice cream, choc ices									
Chocolates, single or squares									
Chocolate snack bars eg. Mars, Crunchie									
Sweets, toffees, mints									
Sugar added to tea, coffee, cereal (teaspoon)									
Crisps or other packet snacks, eg. Wotsits									
Peanuts or other nuts									
SOUPS, SAUCES, AND SPREADS									
Vegetable soups (bowl)									
Meat soups (bowl)									
Sauces, eg. white sauce, cheese sauce, gravy (tablespoon)									
Tomato ketchup (tablespoon)									
Pickles, chutney (tablespoon)									
Marmite, Bovril (teaspoon)									
Jam, marmalade, honey (teaspoon)									
Peanut butter (teaspoon)									
	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Please check that you have a tick (✓) on EVERY line

PLEASE PUT A TICK (✓) ON EVERY LINE

FOODS AND AMOUNTS	AVERAGE USE LAST YEAR								
DRINKS	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Tea (cup)									
Coffee, instant or ground (cup)									
Coffee, decaffeinated (cup)									
Coffee whitener, eg. Coffee-mate (teaspoon)									
Cocoa, hot chocolate (cup)									
Horlicks, Ovaltine (cup)									
Wine (glass)									
Beer, lager or cider (half pint)									
Port, sherry, vermouth, liqueurs (glass)									
Spirits, eg. gin, brandy, whisky, vodka (single)									
Low calorie or diet fizzy soft drinks (glass)									
Fizzy soft drinks, eg. Coca cola, lemonade (glass)									
Pure fruit juice (100%) eg. orange, apple juice (glass)									
Fruit squash or cordial (glass)									
FRUIT **For seasonal fruits marked *, please estimate your average use when the fruit is in season**									
Apples (1 fruit)									
Pears (1 fruit)									
Oranges, satsumas, mandarins (1 fruit)									
Grapefruit (half)									
Bananas (1 fruit)									
Grapes (medium serving)									
Melon (1 slice)									
* Peaches, plums, apricots (1 fruit)									
* Strawberries, raspberries, kiwi fruit (medium serving)									
Tinned fruit (medium serving)									
Dried fruit, eg. raisins, prunes (medium serving)									
	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Please check that you have a tick (✓) on EVERY line

PLEASE PUT A TICK (✓) ON EVERY LINE

FOODS AND AMOUNTS	AVERAGE USE LAST YEAR								
VEGETABLES Fresh, frozen or tinned (medium serving)	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Carrots									
Spinach									
Broccoli, spring greens, kale									
Brussels sprouts									
Cabbage									
Peas									
Green beans, broad beans, runner beans									
Marrow, courgettes									
Cauliflower									
Parsnips, turnips, swedes									
Leeks									
Onions									
Garlic									
Mushrooms									
Sweet peppers									
Beansprouts									
Green salad, lettuce, cucumber, celery									
Watercress									
Tomatoes									
Sweetcorn									
Beetroot									
Coleslaw									
Avocado									
Baked beans									
Dried lentils, beans, peas									
Tofu , soya meat, TVP, Vegeburger									
	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Please check that you have a tick (✓) on EVERY line

YOUR DIET LAST YEAR, continued

2. Are there any **OTHER** foods which you ate more than once a week? Yes ☐ No ☐

If yes, please list below

Food	Usual serving size	Number of times eaten each week

3. What type of milk did you most often use?

Select one only

Full cream, silver ☐
Skimmed/blue ☐
Dried milk ☐
Other, *specify* ☐
Semi-skimmed, red/white ☐
Channel Islands, gold ☐
Soya ☐
None ☐

4. How much milk did you drink each day, including milk with tea, coffee, cereals etc?

None ☐
Quarter of a pint ☐
Half a pint ☐
Three quarters of a pint ☐
One pint ☐
More than one pint ☐

5. Did you usually eat breakfast cereal (excluding porridge and Ready Brek mentioned earlier)? Yes ☐ No ☐

If yes, which brand and type of breakfast cereal, including muesli, did you usually eat?
List the one or two types most often used

Brand *e.g. Kellogg's*	Type *e.g. cornflakes*

6. What kind of fat did you most often use for frying, roasting, grilling etc?

Select one only

Butter ☐
Lard/dripping ☐
Vegetable oil ☐
Solid vegetable fat ☐
Margarine ☐
None ☐

If you used vegetable oil, please give type eg. corn, sunflower ☐

7. What kind of fat did you most often use for baking cakes etc?

Select one only

Butter ☐
Lard/dripping ☐
Vegetable oil ☐
Solid vegetable fat ☐
Margarine ☐
None ☐

If you used margarine, please give name or type eg. Flora, Stork ☐

8. How often did you eat food that was fried at home?

Daily ☐ 1-3 times a week ☐ 4-6 times a week ☐
Less than once a week ☐ Never ☐

9. How often did you eat fried food away from home?

Daily ☐ 1-3 times a week ☐ 4-6 times a week ☐
Less than once a week ☐ Never ☐

10. What did you do with the visible fat on your meat?

Ate most of the fat ☐ Ate as little as possible ☐
Ate some of the fat ☐ Did not eat meat ☐

11. How often did you eat grilled or roast meat? ☐☐ times a week

12. How well cooked did you usually have grilled or roast meat?

Well done /dark brown ☐ Lightly cooked/rare ☐
Medium ☐ Did not eat meat ☐

13. How often did you add salt to food while cooking?

Always ☐ Rarely ☐
Usually ☐ Never ☐
Sometimes ☐

14. How often did you add salt to any food at the table?

Always ☐ Rarely ☐
Usually ☐ Never ☐
Sometimes ☐

15. Did you regularly use a salt substitute (eg LoSalt)? Yes ☐ No ☐

If yes, which brand? ☐

16. During the course of last year, on average, how many times a week did you eat the following foods?

Food type	**Times/week**	**Portion size**
Vegetables (not including potatoes)	☐☐	medium serving
Salads	☐☐	medium serving
Fruit and fruit products (not including fruit juice)	☐☐	medium serving or 1 fruit
Fish and fish products	☐☐	medium serving
Meat, meat products and meat dishes (including bacon, ham and chicken)	☐☐	medium serving

17. Have you taken any vitamins, minerals, fish oils, fibre or other food supplements during the past year? Yes ☐ No ☐ Don't know ☐

If yes, please complete the table below. If you have taken more than 5 types of supplement please put the most frequently consumed brands first.

Vitamin supplements	**Average frequency** Tick **one** box per line to show how often on average you consumed supplements									
Name and brand Please list full name, **brand** and strength	**Dose** Please state number of pills, capsules or teaspoons consumed	Never or less than once a month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Thank you for your help

Appendix M

M.1 IPAQ Questionnaire

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE (October 2002)

LONG LAST 7 DAYS SELF-ADMINISTERED FORMAT

FOR USE WITH YOUNG AND MIDDLE-AGED ADULTS (15-69 years)

The International Physical Activity Questionnaires (IPAQ) comprises a set of 4 questionnaires. Long (5 activity domains asked independently) and short (4 generic items) versions for use by either telephone or self-administered methods are available. The purpose of the questionnaires is to provide common instruments that can be used to obtain internationally comparable data on health–related physical activity.

Background on IPAQ

The development of an international measure for physical activity commenced in Geneva in 1998 and was followed by extensive reliability and validity testing undertaken across 12 countries (14 sites) during 2000. The final results suggest that these measures have acceptable measurement properties for use in many settings and in different languages, and are suitable for national population-based prevalence studies of participation in physical activity.

Using IPAQ

Use of the IPAQ instruments for monitoring and research purposes is encouraged. It is recommended that no changes be made to the order or wording of the questions as this will affect the psychometric properties of the instruments.

Translation from English and Cultural Adaptation

Translation from English is encouraged to facilitate worldwide use of IPAQ. Information on the availability of IPAQ in different languages can be obtained at www.ipaq.ki.se. If a new translation is undertaken we highly recommend using the prescribed back translation methods available on the IPAQ website. If possible please consider making your translated version of IPAQ available to others by contributing it to the IPAQ website. Further details on translation and cultural adaptation can be downloaded from the website.

Further Developments of IPAQ

International collaboration on IPAQ is on-going and an ***International Physical Activity Prevalence Study*** is in progress. For further information see the IPAQ website.

More Information

More detailed information on the IPAQ process and the research methods used in the development of IPAQ instruments is available at www.ipaq.ki.se and Booth, M.L. (2000). *Assessment of Physical Activity: An International Perspective*. Research Quarterly for Exercise and Sport, 71 (2): s114-20. Other scientific publications and presentations on the use of IPAQ are summarized on the website.

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

We are interested in finding out about the kinds of physical activities that people do as part of their everyday lives. The questions will ask you about the time you spent being physically active in the **last 7 days**. Please answer each question even if you do not consider yourself to be an active person. Please think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport.

Think about all the **vigorous** and **moderate** activities that you did in the **last 7 days**. **Vigorous** physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. **Moderate** activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal.

PART 1: JOB-RELATED PHYSICAL ACTIVITY

The first section is about your work. This includes paid jobs, farming, volunteer work, course work, and any other unpaid work that you did outside your home. Do not include unpaid work you might do around your home, like housework, yard work, general maintenance, and caring for your family. These are asked in Part 3.

1. Do you currently have a job or do any unpaid work outside your home?

   ☐ Yes

   ☐ No → ***Skip to PART 2: TRANSPORTATION***

The next questions are about all the physical activity you did in the **last 7 days** as part of your paid or unpaid work. This does not include traveling to and from work.

2. During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, digging, heavy construction, or climbing up stairs **as part of your work**? Think about only those physical activities that you did for at least 10 minutes at a time.

   _____ **days per week**

   ☐ No vigorous job-related physical activity → ***Skip to question 4***

3. How much time did you usually spend on one of those days doing **vigorous** physical activities as part of your work?

   hours per day
   _____ **minutes per day**

4. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** physical activities like carrying light loads **as part of your work**? Please do not include walking.

   _____ **days per week**

   ☐ No moderate job-related physical activity → ***Skip to question 6***

5. How much time did you usually spend on one of those days doing **moderate** physical activities as part of your work?

_____ **hours per day**
_____ **minutes per day**

6. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time **as part of your work**? Please do not count any walking you did to travel to or from work.

_____ **days per week**

☐ No job-related walking → ***Skip to PART 2: TRANSPORTATION***

7. How much time did you usually spend on one of those days **walking** as part of your work?

_____ **hours per day**
minutes per day

PART 2: TRANSPORTATION PHYSICAL ACTIVITY

These questions are about how you traveled from place to place, including to places like work, stores, movies, and so on.

8. During the **last 7 days**, on how many days did you **travel in a motor vehicle** like a train, bus, car, or tram?

_____ **days per week**

☐ No traveling in a motor vehicle → ***Skip to question 10***

9. How much time did you usually spend on one of those days **traveling** in a train, bus, car, tram, or other kind of motor vehicle?

_____ **hours per day**
_____ **minutes per day**

Now think only about the **bicycling** and **walking** you might have done to travel to and from work, to do errands, or to go from place to place.

10. During the **last 7 days**, on how many days did you **bicycle** for at least 10 minutes at a time to go **from place to place**?

_____ **days per week**

☐ No bicycling from place to place → ***Skip to question 12***

11. How much time did you usually spend on one of those days to **bicycle** from place to place?

hours per day
_____ **minutes per day**

12. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time to go **from place to place**?

_____ **days per week**

☐ No walking from place to place → ***Skip to PART 3: HOUSEWORK, HOUSE MAINTENANCE, AND CARING FOR FAMILY***

13. How much time did you usually spend on one of those days **walking** from place to place?

_____ **hours per day**
_____ **minutes per day**

PART 3: HOUSEWORK, HOUSE MAINTENANCE, AND CARING FOR FAMILY

This section is about some of the physical activities you might have done in the **last 7 days** in and around your home, like housework, gardening, yard work, general maintenance work, and caring for your family.

14. Think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, chopping wood, shoveling snow, or digging **in the garden or yard**?

_____ **days per week**

☐ No vigorous activity in garden or yard → ***Skip to question 16***

15. How much time did you usually spend on one of those days doing **vigorous** physical activities in the garden or yard?

_____ **hours per day**
_____ **minutes per day**

16. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** activities like carrying light loads, sweeping, washing windows, and raking **in the garden or yard**?

days per week

☐ No moderate activity in garden or yard → ***Skip to question 18***

17. How much time did you usually spend on one of those days doing **moderate** physical activities in the garden or yard?

hours per day
_____ **minutes per day**

18. Once again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** activities like carrying light loads, washing windows, scrubbing floors and sweeping **inside your home**?

_____ **days per week**

☐ No moderate activity inside home → ***Skip to PART 4: RECREATION, SPORT AND LEISURE-TIME PHYSICAL ACTIVITY***

19. How much time did you usually spend on one of those days doing **moderate** physical activities inside your home?

_____ **hours per day**
_____ **minutes per day**

PART 4: RECREATION, SPORT, AND LEISURE-TIME PHYSICAL ACTIVITY

This section is about all the physical activities that you did in the **last 7 days** solely for recreation, sport, exercise or leisure. Please do not include any activities you have already mentioned.

20. Not counting any walking you have already mentioned, during the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time **in your leisure time**?

_____ **days per week**

☐ No walking in leisure time → ***Skip to question 22***

21. How much time did you usually spend on one of those days **walking** in your leisure time?

_____ **hours per day**
_____ **minutes per day**

22. Think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **vigorous** physical activities like aerobics, running, fast bicycling, or fast swimming **in your leisure time**?

days per week

☐ No vigorous activity in leisure time → ***Skip to question 24***

23. How much time did you usually spend on one of those days doing **vigorous** physical activities in your leisure time?

_____ **hours per day**
_____ **minutes per day**

24. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** physical activities like bicycling at a regular pace, swimming at a regular pace, and doubles tennis **in your leisure time**?

_____ **days per week**

☐ No moderate activity in leisure time → ***Skip to PART 5: TIME SPENT SITTING***

25. How much time did you usually spend on one of those days doing **moderate** physical activities in your leisure time?

_____ **hours per day**
_____ **minutes per day**

PART 5: TIME SPENT SITTING

The last questions are about the time you spend sitting while at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading or sitting or lying down to watch television. Do not include any time spent sitting in a motor vehicle that you have already told me about.

26. During the **last 7 days**, how much time did you usually spend **sitting** on a **weekday**?

_____ **hours per day**
_____ **minutes per day**

27. During the **last 7 days**, how much time did you usually spend **sitting** on a **weekend day**?

_____ **hours per day**
_____ **minutes per day**

This is the end of the questionnaire, thank you for participating.

Appendix N

N.1 24 hr Dietary Recall Questions

24h dietary recall

Participant number:

Date:

Visit number:

Using this dietary recall, we would like you to tell us what you ate and drank the last 24h. Please be as specific as possible by telling what food/drink and how much you had.

Yesterday Morning

Yesterday noon/afternoon

Yesterday evening/night

Appendix O

O.1 BluLife study key stats for each main effect, interaction, and post-hoc test.

Table O.1 Total Acquisition-type III Tests of Fixed Effects

Total Acquisition				
Source	Numerator df	Denominator df	F	p-value
Treatment	1	67.980	6.285	0.015
AgeGroup	4	73.605	6.315	0.000
Treatment * AgeGroup	4	68.046	2.579	0.045

Table O.1.1 Total Acquisition- Pairwise comparison to show age group x treatment interactions.

AgeGroup			Mean Difference (I-J)	Std. Error	df	Sig.[c]	95% Confidence Interval for Difference[c]	
							Lower Bound	Upper Bound
8-10	T4739	T7910	-5.129*	2.480	69.163	0.042	-10.076	-0.182
	T7910	T4739	5.129*	2.480	69.163	0.042	0.182	10.076
14-18	T4739	T7910	-0.430	1.920	68.768	0.823	-4.260	3.400
	T7910	T4739	0.430	1.920	68.768	0.823	-3.400	4.260
22-28	T4739	T7910	2.194	1.782	65.034	0.223	-1.365	5.754
	T7910	T4739	-2.194	1.782	65.034	0.223	-5.754	1.365
40-50	T4739	T7910	-3.277	2.101	69.830	0.123	-7.469	0.914
	T7910	T4739	3.277	2.101	69.830	0.123	-0.914	7.469
65-80	T4739	T7910	-4.939*	1.999	67.084	0.016	-8.928	-0.950
	T7910	T4739	4.939*	1.999	67.084	0.016	0.950	8.928
[c.] Adjustment for multiple comparisons: Bonferroni.								

Table O.2 Immediate Recall-type III Tests of Fixed Effects

Immediate Recall				
Source	Numerator df	Denominator df	F	p-value
Treatment	1	80.764	1.133	0.290
AgeGroup	4	84.687	4.975	0.001
Treatment * AgeGroup	4	79.687	2.206	0.076

Table O.2.1 Immediate Recall- Pairwise comparison to show age group x treatment interactions

AgeGroup			Mean Difference (I-J)	Std. Error	df	Sig.[b]	95% Confidence Interval for Difference[b]	
							Lower Bound	Upper Bound
8-10	T4739	T7910	-0.006	0.663	87.581	0.993	-1.325	1.312
	T7910	T4739	0.006	0.663	87.581	0.993	-1.312	1.325
14-18	T4739	T7910	-0.826	0.458	76.262	0.075	-1.738	0.086
	T7910	T4739	0.826	0.458	76.262	0.075	-0.086	1.738
22-28	T4739	T7910	0.790	0.418	73.451	0.063	-0.043	1.623
	T7910	T4739	-0.790	0.418	73.451	0.063	-1.623	0.043
40-50	T4739	T7910	-0.547	0.501	80.122	0.279	-1.544	0.451
	T7910	T4739	0.547	0.501	80.122	0.279	-0.451	1.544
65-80	T4739	T7910	-0.621	0.469	80.045	0.189	-1.555	0.313
	T7910	T4739	0.621	0.469	80.045	0.189	-0.313	1.555
b. Adjustment for multiple comparisons: Bonferroni.								

Table O.3 Proactive Interference-type III Tests of Fixed Effects

Proactive Interference				
Source	Numerator df	Denominator df	F	p-value
Treatment	1	94.315	4.849	0.030
AgeGroup	4	93.372	1.807	0.134
Treatment * AgeGroup	4	94.562	3.137	0.018

Table O.3.1 Proactive Interference - Pairwise comparison to show age group x treatment interactions

AgeGroup			Mean Difference (I-J)	Std. Error	df	Sig.[c]	95% Confidence Interval for Difference[c]	
							Lower Bound	Upper Bound
8-10	T4739	T7910	-1.732*	0.815	95.110	0.036	-3.350	-0.114
	T7910	T4739	1.732*	0.815	95.110	0.036	0.114	3.350
14-18	T4739	T7910	-0.899	0.623	92.411	0.152	-2.135	0.338
	T7910	T4739	0.899	0.623	92.411	0.152	-0.338	2.135
22-28	T4739	T7910	1.138	0.575	90.211	0.051	-0.003	2.280
	T7910	T4739	-1.138	0.575	90.211	0.051	-2.280	0.003
40-50	T4739	T7910	-0.481	0.672	97.416	0.476	-1.815	0.854
	T7910	T4739	0.481	0.672	97.416	0.476	-0.854	1.815
65-80	T4739	T7910	-1.314*	0.636	96.970	0.042	-2.576	-0.051
	T7910	T4739	1.314*	0.636	96.970	0.042	0.051	2.576
c. Adjustment for multiple comparisons: Bonferroni.								

Table O.4 Retroactive Interference-type III Tests of Fixed Effects

Retroactive Interference				
Source	Numerator df	Denominator df	F	p-value
Treatment	1	76.456	1.012	0.318
AgeGroup	4	80.118	5.053	0.001
Treatment * AgeGroup	4	75.928	0.367	0.831

Table O.4.1 Retroactive Interference - Pairwise comparison to show age group x treatment interactions.

AgeGroup			Mean Difference (I-J)	Std. Error	df	Sig.[b]	95% Confidence Interval for Difference[b]	
							Lower Bound	Upper Bound
8-10	T4739	T7910	-0.840	0.755	77.990	0.269	-2.343	0.663
	T7910	T4739	0.840	0.755	77.990	0.269	-0.663	2.343
14-18	T4739	T7910	0.250	0.559	71.933	0.656	-0.865	1.365
	T7910	T4739	-0.250	0.559	71.933	0.656	-1.365	0.865
22-28	T4739	T7910	-0.196	0.514	70.116	0.705	-1.221	0.830
	T7910	T4739	0.196	0.514	70.116	0.705	-0.830	1.221
40-50	T4739	T7910	-0.208	0.627	80.813	0.742	-1.456	1.041
	T7910	T4739	0.208	0.627	80.813	0.742	-1.041	1.456
65-80	T4739	T7910	-0.386	0.586	79.349	0.511	-1.552	0.780
	T7910	T4739	0.386	0.586	79.349	0.511	-0.780	1.552
b. Adjustment for multiple comparisons: Bonferroni.								

Table O.5 Delayed Recall-type III Tests of Fixed Effects

Delayed Recall				
Source	Numerator df	Denominator df	F	p-value
Treatment	1	78.798	2.176	0.144
AgeGroup	4	81.005	3.348	0.014
Treatment * AgeGroup	4	78.381	0.327	0.859

Table O.5.1 Delayed Recall - Pairwise comparison to show age group x treatment interactions

AgeGroup			Mean Difference (I-J)	Std. Error	df	Sig.[b]	95% Confidence Interval for Difference[b]	
							Lower Bound	Upper Bound
8-10	T4739	T7910	-1.200	1.430	79.876	0.404	-4.045	1.645
	T7910	T4739	1.200	1.430	79.876	0.404	-1.645	4.045
14-18	T4739	T7910	-0.393	0.937	76.828	0.676	-2.259	1.473
	T7910	T4739	0.393	0.937	76.828	0.676	-1.473	2.259
22-28	T4739	T7910	0.080	0.849	71.487	0.926	-1.613	1.772
	T7910	T4739	-0.080	0.849	71.487	0.926	-1.772	1.613
40-50	T4739	T7910	-1.296	1.056	84.204	0.223	-3.397	0.804
	T7910	T4739	1.296	1.056	84.204	0.223	-0.804	3.397
65-80	T4739	T7910	-0.695	0.948	78.661	0.465	-2.582	1.191
	T7910	T4739	0.695	0.948	78.661	0.465	-1.191	2.582
b. Adjustment for multiple comparisons: Bonferroni.								

Table O.6 Word Recognition List A- type III Tests of Fixed Effects

Source	Numerator df	Denominator df	F	p-value
Treatment	1	58.914	0.128	0.722
AgeGroup	4	68.046	10.862	0.000
Treatment * AgeGroup	4	59.269	0.655	0.626

Table O.6.1 Word Recognition List A- Pairwise comparison to show age group x treatment interactions.

AgeGroup			Mean Difference (I-J)	Std. Error	df	Sig.[b]	95% Confidence Interval for Differenceb	
							Lower Bound	Upper Bound
1	T4739	T7910	0.003	0.929	58.237	0.997	-1.857	1.864
	T7910	T4739	-0.003	0.929	58.237	0.997	-1.864	1.857
2	T4739	T7910	-0.270	0.716	57.383	0.707	-1.703	1.163
	T7910	T4739	0.270	0.716	57.383	0.707	-1.163	1.703
3	T4739	T7910	0.793	0.680	60.420	0.248	-0.566	2.152
	T7910	T4739	-0.793	0.680	60.420	0.248	-2.152	0.566
4	T4739	T7910	-0.553	0.732	61.189	0.453	-2.017	0.911
	T7910	T4739	0.553	0.732	61.189	0.453	-0.911	2.017
5	T4739	T7910	-0.582	0.723	59.102	0.424	-2.030	0.865
	T7910	T4739	0.582	0.723	59.102	0.424	-0.865	2.030

b **Adjustment for multiple comparisons using Bonferroni correction.**

Table O.7 Word Recognition List B- type III Tests of Fixed Effects

Word Recognition List B				
Source	Numerator df	Denominator df	F	p-value
Treatment	1	48.177	0.037	0.848
AgeGroup	4	60.725	1.579	0.191
Treatment * AgeGroup	4	47.492	0.913	0.464

Table O.7.1 Word Recognition List B- Pairwise comparison to show age group x treatment interactions.

AgeGroup			Mean Difference (I-J)	Std. Error	df	Sig.[b]	95% Confidence Interval for Difference[b]	
							Lower Bound	Upper Bound
8-10	T4739	T7910	0.118	2.013	48.248	0.954	-3.928	4.164
	T7910	T4739	-0.118	2.013	48.248	0.954	-4.164	3.928
14-18	T4739	T7910	-0.761	1.540	45.661	0.623	-3.861	2.339
	T7910	T4739	0.761	1.540	45.661	0.623	-2.339	3.861
22-28	T4739	T7910	1.918	1.456	48.619	0.194	-1.008	4.844
	T7910	T4739	-1.918	1.456	48.619	0.194	-4.844	1.008
40-50	T4739	T7910	-1.702	1.559	48.353	0.280	-4.836	1.432
	T7910	T4739	1.702	1.559	48.353	0.280	-1.432	4.836
65-80	T4739	T7910	1.136	1.551	47.200	0.467	-1.983	4.256
	T7910	T4739	-1.136	1.551	47.200	0.467	-4.256	1.983
b. Adjustment for multiple comparisons: Bonferroni.								

Table O.8 Corsi Blocks (correct number of sequences)- type III Tests of Fixed Effects

Corsi Blocks (correct number of sequences)				
Source	Numerator df	Denominator df	F	p-value
Treatment	1	82.932	6.149	0.015
Agegroup	4	87.927	10.300	0.000
Treatment * Agegroup	4	84.479	0.674	0.612

Table O.8.1 Corsi Blocks (correct number of sequences)-Pairwise comparison to show age group x treatment interactions.

Agegroup			Mean Difference (I-J)	Std. Error	df	Sig.[c]	95% Confidence Interval for Difference[c]	
							Lower Bound	Upper Bound
8-10	T4739	T7910	-0.342	2.349	79.087	0.885	-5.017	4.334
	T7910	T4739	0.342	2.349	79.087	0.885	-4.334	5.017
14-18	T4739	T7910	-0.804	1.879	84.641	0.670	-4.540	2.931
	T7910	T4739	0.804	1.879	84.641	0.670	-2.931	4.540
22-28	T4739	T7910	-2.634	1.835	84.401	0.155	-6.283	1.014
	T7910	T4739	2.634	1.835	84.401	0.155	-1.014	6.283
40-50	T4739	T7910	-4.469*	1.905	87.264	0.021	-8.256	-0.682
	T7910	T4739	4.469*	1.905	87.264	0.021	0.682	8.256
65-80	T4739	T7910	-2.691	1.897	86.795	0.160	-6.462	1.080
	T7910	T4739	2.691	1.897	86.795	0.160	-1.080	6.462
c. Adjustment for multiple comparisons: Bonferroni.								

Table O.9 Corsi Blocks (correct number of blocks)- type III Tests of Fixed Effects

Corsi Blocks (correct number of blocks)				
Source	Numerator df	Denominator df	F	p-value
Treatment	1	89.428	4.639	0.034
Agegroup	4	90.645	6.865	0.000
Treatment * Agegroup	4	89.274	1.065	0.379

Table O.9.1 Corsi Blocks (correct number of blocks)-Pairwise comparison to show age group x treatment interactions.

Agegroup			Mean Difference (I-J)	Std. Error	df	Sig.[c]	95% Confidence Interval for Difference[c]	
							Lower Bound	Upper Bound
1	T4739	T7910	0.268	2.749	83.706	0.923	-5.199	5.736
	T7910	T4739	-0.268	2.749	83.706	0.923	-5.736	5.199
2	T4739	T7910	-2.388	2.209	90.594	0.283	-6.775	2.000
	T7910	T4739	2.388	2.209	90.594	0.283	-2.000	6.775
3	T4739	T7910	-1.009	2.069	91.432	0.627	-5.118	3.101
	T7910	T4739	1.009	2.069	91.432	0.627	-3.101	5.118
4	T4739	T7910	-6.093*	2.215	91.022	0.007	-10.493	-1.694
	T7910	T4739	6.093*	2.215	91.022	0.007	1.694	10.493
5	T4739	T7910	-1.879	2.211	91.095	0.398	-6.272	2.514
	T7910	T4739	1.879	2.211	91.095	0.398	-2.514	6.272
c. Adjustment for multiple comparisons: Bonferroni.								

Table O.10 Serial 3's Accuracy- type III Tests of Fixed Effects

Serial 3's Accuracy				
Source	Numerator df	Denominator df	F	p-value
Treatment	1	74.053	0.351	0.555
AgeGroup	4	79.845	5.929	0.000
Treatment * AgeGroup	4	75.128	0.945	0.443

Table O.10.1 Serial 3's Accuracy-Pairwise comparison to show age group x treatment interactions.

AgeGroup			Mean Difference (I-J)	Std. Error	df	Sig.[b]	95% Confidence Interval for Difference[b]	
							Lower Bound	Upper Bound
8-10	T4739	T7910	-3.052	2.526	71.474	0.231	-8.089	1.985
	T7910	T4739	3.052	2.526	71.474	0.231	-1.985	8.089
14-18	T4739	T7910	1.535	1.794	72.569	0.395	-2.040	5.110
	T7910	T4739	-1.535	1.794	72.569	0.395	-5.110	2.040
22-28	T4739	T7910	-1.368	1.671	73.218	0.416	-4.697	1.962
	T7910	T4739	1.368	1.671	73.218	0.416	-1.962	4.697
40-50	T4739	T7910	1.530	1.940	80.988	0.433	-2.331	5.390
	T7910	T4739	-1.530	1.940	80.988	0.433	-5.390	2.331
65-80	T4739	T7910	-1.263	1.848	76.776	0.496	-4.943	2.416
	T7910	T4739	1.263	1.848	76.776	0.496	-2.416	4.943
b. Adjustment for multiple comparisons: Bonferroni.								

Table O.11 Serial 7's Accuracy- type III Tests of Fixed Effects

Serial 7's Accuracy				
Source	Numerator df	Denominator df	F	p-value
Treatment	1	88.748	0.831	0.365
AgeGroup	4	92.820	2.623	0.040
Treatment * AgeGroup	4	88.234	0.199	0.939

Table O.11.1 Serial 7's Accuracy-Pairwise comparison to show age group x treatment interactions.

Pairwise Comparisons[a]								
AgeGroup			Mean Difference (I-J)	Std. Error	df	Sig.[b]	95% Confidence Interval for Difference[b]	
							Lower Bound	Upper Bound
8-10	T4739	T7910	-0.399	1.674	91.696	0.812	-3.723	2.926
	T7910	T4739	0.399	1.674	91.696	0.812	-2.926	3.723
14-18	T4739	T7910	-0.371	1.189	84.878	0.756	-2.736	1.994
	T7910	T4739	0.371	1.189	84.878	0.756	-1.994	2.736
22-28	T4739	T7910	-0.897	1.087	82.337	0.412	-3.059	1.266
	T7910	T4739	0.897	1.087	82.337	0.412	-1.266	3.059
40-50	T4739	T7910	-1.191	1.227	90.599	0.335	-3.629	1.247
	T7910	T4739	1.191	1.227	90.599	0.335	-1.247	3.629
65-80	T4739	T7910	0.215	1.228	90.718	0.861	-2.224	2.654
	T7910	T4739	-0.215	1.228	90.718	0.861	-2.654	2.224
b. Adjustment for multiple comparisons: Bonferroni.								

Table O.12 TST Accuracy- type III Tests of Fixed Effects

TST Accuracy				
Source	Numerator df	Denominator df	F	P-value
Treatment	1	758.639	1.345	0.247
SwitchTrial	3	562.579	11.204	0.000
TaskType	1	545.648	0.014	0.906
AgeGroup	4	95.490	13.003	0.000
Treatment * SwitchTrial	3	763.736	0.114	0.952
Treatment * TaskType	1	764.732	0.001	0.972
Treatment * SwitchTrial * TaskType	6	597.155	0.448	0.846
Treatment * AgeGroup	4	759.773	16.105	0.000
Treatment * SwitchTrial * AgeGroup	24	601.826	0.979	0.492
Treatment * TaskType * AgeGroup	8	599.540	1.093	0.366
Treatment * SwitchTrial * TaskType * AgeGroup	24	617.770	0.471	0.986

Table O.12.1 TST Accuracy-Pairwise comparison to show switch trial x treatment interactions.

SwitchTrial			Mean Difference (I-J)	Std. Error	df	Sig.[b]	95% Confidence Interval for Difference[b]	
							Lower Bound	Upper Bound
1	T4739	T7910	0.003	0.008	766.879	0.698	-0.012	0.018
	T7910	T4739	-0.003	0.008	766.879	0.698	-0.018	0.012
2	T4739	T7910	0.002	0.008	765.921	0.785	-0.013	0.017
	T7910	T4739	-0.002	0.008	765.921	0.785	-0.017	0.013
3	T4739	T7910	0.005	0.008	759.274	0.484	-0.010	0.021
	T7910	T4739	-0.005	0.008	759.274	0.484	-0.021	0.010
4	T4739	T7910	0.008	0.008	759.507	0.307	-0.007	0.023
	T7910	T4739	-0.008	0.008	759.507	0.307	-0.023	0.007
b. Adjustment for multiple comparisons: Bonferroni.								

Table O.12.2 TST Accuracy-Pairwise comparison to show Task type x treatment interactions.

Task Type			Mean Difference (I-J)	Std. Error	df	Sig.[b]	95% Confidence Interval for Difference[b]	
							Lower Bound	Upper Bound
1	T4739	T7910	0.004	0.006	765.050	0.420	-0.006	0.015
	T7910	T4739	-0.004	0.006	765.050	0.420	-0.015	0.006
2	T4739	T7910	0.005	0.006	759.229	0.390	-0.006	0.016
	T7910	T4739	-0.005	0.006	759.229	0.390	-0.016	0.006
b. Adjustment for multiple comparisons: Bonferroni.								

Table O.12.3 TST Accuracy-Pairwise comparison to show Treatment x age group interactions.

AgeGroup			Mean Difference (I-J)	Std. Error	df	Sig.[c]	95% Confidence Interval for Difference[c]	
							Lower Bound	Upper Bound
8-10	T4739	T7910	.068*	0.010	733.872	0.000	0.048	0.089
	T7910	T4739	-.068*	0.010	733.872	0.000	-0.089	-0.048
14-18	T4739	T7910	-.033*	0.008	727.877	0.000	-0.049	-0.018
	T7910	T4739	.033*	0.008	727.877	0.000	0.018	0.049
22-28	T4739	T7910	-0.003	0.008	764.015	0.734	-0.018	0.013
	T7910	T4739	0.003	0.008	764.015	0.734	-0.013	0.018
40-50	T4739	T7910	0.007	0.009	768.763	0.435	-0.010	0.023
	T7910	T4739	-0.007	0.009	768.763	0.435	-0.023	0.010
65-80	T4739	T7910	-0.016	0.009	801.406	0.090	-0.034	0.002
	T7910	T4739	0.016	0.009	801.406	0.090	-0.002	0.034
c. Adjustment for multiple comparisons: Bonferroni.								

Table O.12.4 TST Accuracy-Pairwise comparison to show treatment x task type x switch trial interactions.

Treatment				Mean Difference (I-J)	Std. Error	df	Sig.[b]	95% Confidence Interval for Difference[b]	
								Lower Bound	Upper Bound
T4739	1	1	2	0.002	0.011	511.491	0.822	-0.019	0.024
		2	1	-0.002	0.011	511.491	0.822	-0.024	0.019
	2	1	2	5.157E-05	0.011	510.837	0.996	-0.021	0.022
		2	1	-5.157E-05	0.011	510.837	0.996	-0.022	0.021
	3	1	2	-0.001	0.011	510.694	0.947	-0.022	0.021
		2	1	0.001	0.011	510.694	0.947	-0.021	0.022
	4	1	2	-0.001	0.011	511.211	0.926	-0.022	0.020
		2	1	0.001	0.011	511.211	0.926	-0.020	0.022
T7910	1	1	2	-0.007	0.008	441.673	0.341	-0.023	0.008
		2	1	0.007	0.008	441.673	0.341	-0.008	0.023
	2	1	2	0.008	0.008	442.108	0.317	-0.008	0.023
		2	1	-0.008	0.008	442.108	0.317	-0.023	0.008
	3	1	2	-0.004	0.008	442.505	0.650	-0.019	0.012
		2	1	0.004	0.008	442.505	0.650	-0.012	0.019
	4	1	2	0.005	0.008	441.501	0.520	-0.010	0.020
		2	1	-0.005	0.008	441.501	0.520	-0.020	0.010
b. Adjustment for multiple comparisons: Bonferroni.									

Table O.12.5 TST Accuracy-Pairwise comparison to show treatment x age group x switch trial interactions.

SwitchTrial				Mean Difference (I-J)	Std. Error	df	Sig.[c]	95% Confidence Interval for Difference[c]	
								Lower Bound	Upper Bound
1	8-10	T4739	T7910	.047*	0.021	730.474	0.025	0.006	0.087
		T7910	T4739	-.047*	0.021	730.474	0.025	-0.087	-0.006
	14-18	T4739	T7910	-0.021	0.016	727.657	0.179	-0.053	0.010
		T7910	T4739	0.021	0.016	727.657	0.179	-0.010	0.053
	22-28	T4739	T7910	-0.004	0.016	779.830	0.810	-0.034	0.027
		T7910	T4739	0.004	0.016	779.830	0.810	-0.027	0.034
	40-50	T4739	T7910	0.022	0.017	787.684	0.190	-0.011	0.054
		T7910	T4739	-0.022	0.017	787.684	0.190	-0.054	0.011

	65-80	T4739	T7910	-0.028	0.017	829.663	0.110	-0.062	0.006
		T7910	T4739	0.028	0.017	829.663	0.110	-0.006	0.062
2	8-10	T4739	T7910	.056*	0.021	728.079	0.007	0.015	0.097
		T7910	T4739	-.056*	0.021	728.079	0.007	-0.097	-0.015
	14-18	T4739	T7910	-0.027	0.016	727.618	0.091	-0.058	0.004
		T7910	T4739	0.027	0.016	727.618	0.091	-0.004	0.058
	22-28	T4739	T7910	-0.006	0.015	761.779	0.709	-0.036	0.024
		T7910	T4739	0.006	0.015	761.779	0.709	-0.024	0.036
	40-50	T4739	T7910	0.005	0.016	785.376	0.745	-0.027	0.038
		T7910	T4739	-0.005	0.016	785.376	0.745	-0.038	0.027
	65-80	T4739	T7910	-0.018	0.018	841.751	0.305	-0.052	0.016
		T7910	T4739	0.018	0.018	841.751	0.305	-0.016	0.052
3	8-10	T4739	T7910	.081*	0.021	728.402	0.000	0.041	0.122
		T7910	T4739	-.081*	0.021	728.402	0.000	-0.122	-0.041
	14-18	T4739	T7910	-.040*	0.016	727.710	0.013	-0.071	-0.008
		T7910	T4739	.040*	0.016	727.710	0.013	0.008	0.071
	22-28	T4739	T7910	0.000	0.015	761.765	0.985	-0.030	0.030
		T7910	T4739	0.000	0.015	761.765	0.985	-0.030	0.030
	40-50	T4739	T7910	-0.002	0.016	776.832	0.910	-0.034	0.030
		T7910	T4739	0.002	0.016	776.832	0.910	-0.030	0.034
	65-80	T4739	T7910	-0.012	0.017	816.732	0.485	-0.046	0.022
		T7910	T4739	0.012	0.017	816.732	0.485	-0.022	0.046
4	8-10	T4739	T7910	.090*	0.021	730.596	0.000	0.049	0.130
		T7910	T4739	-.090*	0.021	730.596	0.000	-0.130	-0.049
	14-18	T4739	T7910	-.046*	0.016	727.716	0.004	-0.077	-0.014
		T7910	T4739	.046*	0.016	727.716	0.004	0.014	0.077
	22-28	T4739	T7910	-0.001	0.015	777.264	0.946	-0.031	0.029
		T7910	T4739	0.001	0.015	777.264	0.946	-0.029	0.031
	40-50	T4739	T7910	0.002	0.016	776.829	0.925	-0.031	0.034
		T7910	T4739	-0.002	0.016	776.829	0.925	-0.034	0.031
	65-80	T4739	T7910	-0.005	0.017	805.817	0.770	-0.039	0.029
		T7910	T4739	0.005	0.017	805.817	0.770	-0.029	0.039
c. Adjustment for multiple comparisons: Bonferroni.									

Table O.12.6 TST Accuracy-Pairwise comparison to show treatment x age group x task type interactions.

AgeGroup				Mean Difference (I-J)	Std. Error	df	Sig.[c]	95% Confidence Interval for Difference[c]	
								Lower Bound	Upper Bound
8-10	1	T4739	T7910	.073*	0.015	728.341	0.000	0.044	0.102
		T7910	T4739	-.073*	0.015	728.341	0.000	-0.102	-0.044
	2	T4739	T7910	.063*	0.015	734.788	0.000	0.035	0.092
		T7910	T4739	-.063*	0.015	734.788	0.000	-0.092	-0.035
14-18	1	T4739	T7910	-.048*	0.011	727.654	0.000	-0.070	-0.026
		T7910	T4739	.048*	0.011	727.654	0.000	0.026	0.070
	2	T4739	T7910	-0.019	0.011	727.859	0.096	-0.041	0.003
		T7910	T4739	0.019	0.011	727.859	0.096	-0.003	0.041
22-28	1	T4739	T7910	0.002	0.011	768.393	0.847	-0.020	0.024
		T7910	T4739	-0.002	0.011	768.393	0.847	-0.024	0.020
	2	T4739	T7910	-0.008	0.011	768.442	0.492	-0.029	0.014
		T7910	T4739	0.008	0.011	768.442	0.492	-0.014	0.029
40-50	1	T4739	T7910	0.007	0.012	790.794	0.558	-0.016	0.030
		T7910	T4739	-0.007	0.012	790.794	0.558	-0.030	0.016
	2	T4739	T7910	0.006	0.012	766.140	0.587	-0.017	0.030
		T7910	T4739	-0.006	0.012	766.140	0.587	-0.030	0.017
65-80	1	T4739	T7910	-0.012	0.013	820.329	0.359	-0.037	0.013
		T7910	T4739	0.012	0.013	820.329	0.359	-0.013	0.037
	2	T4739	T7910	-0.020	0.012	814.985	0.112	-0.044	0.005
		T7910	T4739	0.020	0.012	814.985	0.112	-0.005	0.044
c. Adjustment for multiple comparisons: Bonferroni.									

Table O.12.7 TST Accuracy-Pairwise comparison to Treatment x age group x task type x switch trial interactions.

Treatment				Mean	Std. Error	df	95% Confidence Interval	
							Lower Bound	Upper Bound
T4739	8-10	1	1	.787[b]	0.026	578.269	0.736	0.837
			2	.850[b]	0.025	559.023	0.800	0.899
			3	.855[b]	0.025	559.871	0.806	0.905
			4	.849[b]	0.025	553.469	0.800	0.899
		2	1	.789[b]	0.025	560.733	0.739	0.838
			2	.807[b]	0.025	560.907	0.757	0.856

			3	.837[b]	0.025	559.361	0.788	0.887
			4	.838[b]	0.025	551.155	0.789	0.888
	14-18	1	1	.849[b]	0.019	535.432	0.811	0.887
			2	.868[b]	0.019	534.185	0.830	0.905
			3	.872[b]	0.019	534.373	0.834	0.909
			4	.869[b]	0.019	534.197	0.831	0.906
		2	1	.866[b]	0.019	538.019	0.829	0.904
			2	.905[b]	0.019	534.197	0.867	0.943
			3	.887[b]	0.019	534.291	0.849	0.924
			4	.882[b]	0.019	534.186	0.844	0.920
	22-28	1	1	.905[b]	0.018	546.746	0.870	0.941
			2	.919[b]	0.018	538.313	0.884	0.955
			3	.909[b]	0.018	538.064	0.874	0.944
			4	.918[b]	0.018	538.414	0.883	0.953
		2	1	.896[b]	0.018	548.226	0.861	0.932
			2	.917[b]	0.018	538.262	0.882	0.953
			3	.909[b]	0.018	538.015	0.874	0.944
			4	.913[b]	0.018	539.441	0.878	0.948
	40-50	1	1	.920[b]	0.019	561.550	0.881	0.958
			2	.911[b]	0.019	550.329	0.874	0.949
			3	.909[b]	0.019	550.765	0.872	0.947
			4	.908[b]	0.019	550.977	0.870	0.946
		2	1	.909[b]	0.019	560.833	0.871	0.948
			2	.927[b]	0.020	562.610	0.889	0.966
			3	.919[b]	0.020	563.719	0.880	0.957
			4	.919[b]	0.020	563.000	0.881	0.958
	65-80	1	1	.897[b]	0.020	579.113	0.857	0.937
			2	.908[b]	0.020	580.396	0.867	0.948
			3	.907[b]	0.020	566.881	0.868	0.946
			4	.908[b]	0.020	565.706	0.868	0.947
		2	1	.885[b]	0.020	564.611	0.845	0.924
			2	.899[b]	0.020	567.028	0.859	0.938
			3	.904[b]	0.020	566.185	0.865	0.944
			4	.904[b]	0.020	550.740	0.865	0.942
T7910	8-10	1	1	.733[b]	0.021	314.052	0.692	0.774
			2	.791[b]	0.020	297.752	0.750	0.831
			3	.772[b]	0.020	294.077	0.732	0.812
			4	.753[b]	0.020	295.219	0.713	0.793
		2	1	.750[b]	0.021	318.674	0.708	0.791
			2	.754[b]	0.020	298.974	0.714	0.794
			3	.758[b]	0.021	305.458	0.718	0.799
			4	.755[b]	0.020	299.862	0.715	0.796
	14-18	1	1	.886[b]	0.015	270.957	0.856	0.916
			2	.919[b]	0.015	269.710	0.889	0.949
			3	.911[b]	0.015	270.103	0.881	0.941
			4	.933[b]	0.015	269.777	0.903	0.963

		2	1	.872[b]	0.015	271.926	0.842	0.902
			2	.908[b]	0.015	269.703	0.878	0.938
			3	.927[b]	0.015	269.706	0.897	0.957
			4	.909[b]	0.015	269.825	0.879	0.938
	22-28	1	1	.900[b]	0.015	324.852	0.870	0.930
			2	.924[b]	0.015	305.477	0.894	0.953
			3	.904[b]	0.015	306.020	0.875	0.934
			4	.915[b]	0.015	316.691	0.885	0.945
		2	1	.909[b]	0.015	302.982	0.880	0.938
			2	.925[b]	0.015	304.502	0.895	0.954
			3	.914[b]	0.015	303.405	0.885	0.944
			4	.918[b]	0.015	316.807	0.888	0.948
	40-50	1	1	.884[b]	0.016	305.077	0.853	0.915
			2	.913[b]	0.016	317.913	0.882	0.945
			3	.914[b]	0.016	307.007	0.883	0.945
			4	.910[b]	0.016	307.104	0.879	0.940
		2	1	.902[b]	0.016	305.868	0.871	0.933
			2	.915[b]	0.016	307.897	0.884	0.946
			3	.918[b]	0.016	307.159	0.887	0.948
			4	.915[b]	0.016	307.032	0.884	0.946
	65-80	1	1	.915[b]	0.017	338.013	0.882	0.948
			2	.918[b]	0.017	354.965	0.885	0.952
			3	.916[b]	0.017	339.363	0.883	0.949
			4	.917[b]	0.017	338.712	0.883	0.950
		2	1	.923[b]	0.016	323.471	0.890	0.955
			2	.924[b]	0.017	325.383	0.891	0.956
			3	.919[b]	0.017	326.248	0.887	0.952
			4	.905[b]	0.017	326.404	0.873	0.938

a. Dependent Variable: AccuracyScore.

b. Covariates appearing in the model are evaluated at the following values: AccuracyScoreBL = .8848.

Table O.13 TST RT- type III Tests of Fixed Effects

TST RT				
Source	Numerator df	Denominator df	F	Sig.
Treatment	1	677.764	2.618	0.106
SwitchTrial	3	717.771	79.128	0.000
TaskType	1	628.367	0.155	0.694
AgeGroup	4	86.509	6.349	0.000
Treatment * SwitchTrial	3	701.599	0.814	0.486
Treatment * TaskType	1	701.384	0.002	0.961
Treatment * SwitchTrial * TaskType	6	662.280	0.271	0.951
Treatment * AgeGroup	4	679.256	6.556	0.000
Treatment * SwitchTrial * AgeGroup	24	667.701	1.210	0.225
Treatment * TaskType * AgeGroup	8	668.140	1.249	0.267
Treatment * SwitchTrial * TaskType * AgeGroup	24	667.231	0.454	0.989

Table O.13.1 TST RT-Pairwise comparison to show switch trial x treatment interactions.

Pairwise Comparisons[a]								
SwitchTrial			Mean Difference (I-J)	Std. Error	df	Sig.[b]	95% Confidence Interval for Difference[b]	
							Lower Bound	Upper Bound
1	T4739	T7910	22.565	12.279	708.537	0.067	-1.541	46.672
	T7910	T4739	-22.565	12.279	708.537	0.067	-46.672	1.541
2	T4739	T7910	15.270	12.112	691.416	0.208	-8.512	39.051
	T7910	T4739	-15.270	12.112	691.416	0.208	-39.051	8.512
3	T4739	T7910	-1.749	12.109	691.049	0.885	-25.524	22.026
	T7910	T4739	1.749	12.109	691.049	0.885	-22.026	25.524
4	T4739	T7910	4.348	12.108	690.798	0.720	-19.424	28.120
	T7910	T4739	-4.348	12.108	690.798	0.720	-28.120	19.424
b. Adjustment for multiple comparisons: Bonferroni.								

Table O.13.2 TST RT-Pairwise comparison to show Task type x treatment interactions.

Treatment			Mean Difference (I-J)	Std. Error	df	Sig.[b]	95% Confidence Interval for Difference[b]	
							Lower Bound	Upper Bound
T4739	1	2	2.115	7.595	405.700	0.781	-12.815	17.046
	2	1	-2.115	7.595	405.700	0.781	-17.046	12.815
T7910	1	2	1.520	7.570	369.109	0.841	-13.365	16.406
	2	1	-1.520	7.570	369.109	0.841	-16.406	13.365
b. Adjustment for multiple comparisons: Bonferroni.								

Table O.13.3 TST RT-Pairwise comparison to show Treatment x age group interactions.

AgeGroup			Mean Difference (I-J)	Std. Error	df	Sig.[c]	95% Confidence Interval for Difference[c]	
							Lower Bound	Upper Bound
8-10	T4739	T7910	.068*	0.010	733.872	0.000	0.048	0.089
	T7910	T4739	-.068*	0.010	733.872	0.000	-0.089	-0.048
14-18	T4739	T7910	-.033*	0.008	727.877	0.000	-0.049	-0.018
	T7910	T4739	.033*	0.008	727.877	0.000	0.018	0.049
22-28	T4739	T7910	-0.003	0.008	764.015	0.734	-0.018	0.013
	T7910	T4739	0.003	0.008	764.015	0.734	-0.013	0.018
40-50	T4739	T7910	0.007	0.009	768.763	0.435	-0.010	0.023
	T7910	T4739	-0.007	0.009	768.763	0.435	-0.023	0.010
65-80	T4739	T7910	-0.016	0.009	801.406	0.090	-0.034	0.002
	T7910	T4739	0.016	0.009	801.406	0.090	-0.002	0.034
c. Adjustment for multiple comparisons: Bonferroni.								

Table O.13.4 TST RT-Pairwise comparison to show treatment x task type x switch trial interactions.

				Mean Difference (I-J)	Std. Error	df	Sig.[b]	95% Confidence Interval for Difference[b]	
								Lower Bound	Upper Bound
T4739	1	1	2	8.312	15.413	406.414	0.590	-21.986	38.611
		2	1	-8.312	15.413	406.414	0.590	-38.611	21.986
	2	1	2	10.238	15.115	405.505	0.499	-19.476	39.951
		2	1	-10.238	15.115	405.505	0.499	-39.951	19.476
	3	1	2	-12.470	15.115	405.515	0.410	-42.184	17.244
		2	1	12.470	15.115	405.515	0.410	-17.244	42.184
	4	1	2	2.381	15.112	405.246	0.875	-27.326	32.089
		2	1	-2.381	15.112	405.246	0.875	-32.089	27.326
T7910	1	1	2	5.562	15.275	369.631	0.716	-24.474	35.598
		2	1	-5.562	15.275	369.631	0.716	-35.598	24.474
	2	1	2	-5.081	15.098	369.192	0.737	-34.770	24.609
		2	1	5.081	15.098	369.192	0.737	-24.609	34.770
	3	1	2	4.514	15.097	369.088	0.765	-25.173	34.200
		2	1	-4.514	15.097	369.088	0.765	-34.200	25.173
	4	1	2	1.086	15.095	368.957	0.943	-28.597	30.769
		2	1	-1.086	15.095	368.957	0.943	-30.769	28.597

b. Adjustment for multiple comparisons: Bonferroni.

Table O.13.5 TST RT-Pairwise comparison to show treatment x age group x switch trial interactions.

SwitchTrial				Mean Difference (I-J)	Std. Error	df	Sig.[c]	95% Confidence Interval for Difference[c]	
								Lower Bound	Upper Bound
1	8-10	T4739	T7910	165.188*	32.872	689.567	0.000	100.648	229.729
		T7910	T4739	-165.188*	32.872	689.567	0.000	-229.729	-100.648
	14-18	T4739	T7910	-75.175*	25.226	677.265	0.003	-124.705	-25.645
		T7910	T4739	75.175*	25.226	677.265	0.003	25.645	124.705
	22-28	T4739	T7910	7.545	24.970	714.817	0.763	-41.478	56.567
		T7910	T4739	-7.545	24.970	714.817	0.763	-56.567	41.478
	40-50	T4739	T7910	4.166	26.209	710.639	0.874	-47.290	55.622
		T7910	T4739	-4.166	26.209	710.639	0.874	-55.622	47.290
	65-80	T4739	T7910	11.102	27.248	753.320	0.684	-42.388	64.593
		T7910	T4739	-11.102	27.248	753.320	0.684	-64.593	42.388
2	8-10	T4739	T7910	44.848	32.273	661.416	0.165	-18.521	108.217
		T7910	T4739	-44.848	32.273	661.416	0.165	-108.217	18.521
	14-18	T4739	T7910	-5.689	24.981	660.219	0.820	-54.742	43.363
		T7910	T4739	5.689	24.981	660.219	0.820	-43.363	54.742
	22-28	T4739	T7910	23.614	24.130	694.476	0.328	-23.763	70.990
		T7910	T4739	-23.614	24.130	694.476	0.328	-70.990	23.763
	40-50	T4739	T7910	-2.488	26.036	700.248	0.924	-53.606	48.629
		T7910	T4739	2.488	26.036	700.248	0.924	-48.629	53.606
	65-80	T4739	T7910	16.064	27.257	753.640	0.556	-37.445	69.573
		T7910	T4739	-16.064	27.257	753.640	0.556	-69.573	37.445
3	8-10	T4739	T7910	12.860	32.259	660.664	0.690	-50.483	76.202
		T7910	T4739	-12.860	32.259	660.664	0.690	-76.202	50.483
	14-18	T4739	T7910	-13.563	24.975	659.769	0.587	-62.603	35.477
		T7910	T4739	13.563	24.975	659.769	0.587	-35.477	62.603
	22-28	T4739	T7910	-0.393	24.131	694.568	0.987	-47.771	46.986
		T7910	T4739	0.393	24.131	694.568	0.987	-46.986	47.771
	40-50	T4739	T7910	-20.756	26.034	700.168	0.426	-71.871	30.359
		T7910	T4739	20.756	26.034	700.168	0.426	-30.359	71.871
	65-80	T4739	T7910	13.107	27.267	754.118	0.631	-40.421	66.635
		T7910	T4739	-13.107	27.267	754.118	0.631	-66.635	40.421

4	8-10	T4739	T7910	60.072	32.247	659.995	0.063	-3.246	123.391
		T7910	T4739	-60.072	32.247	659.995	0.063	-123.391	3.246
	14-18	T4739	T7910	-38.780	24.972	659.565	0.121	-87.814	10.254
		T7910	T4739	38.780	24.972	659.565	0.121	-10.254	87.814
	22-28	T4739	T7910	5.736	24.130	694.486	0.812	-41.641	53.112
		T7910	T4739	-5.736	24.130	694.486	0.812	-53.112	41.641
	40-50	T4739	T7910	10.223	26.039	700.406	0.695	-40.901	61.347
		T7910	T4739	-10.223	26.039	700.406	0.695	-61.347	40.901
	65-80	T4739	T7910	-15.512	27.265	754.154	0.570	-69.036	38.012
		T7910	T4739	15.512	27.265	754.154	0.570	-38.012	69.036
c. Adjustment for multiple comparisons: Bonferroni.									

Table O.13.6 TST RT-Pairwise comparison to show treatment x age group x task type interactions.

AgeGroup				Mean Difference (I-J)	Std. Error	df	Sig.[c]	95% Confidence Interval for Difference[c]	
								Lower Bound	Upper Bound
8-10	1	T4739	T7910	80.812*	22.932	669.616	0.000	35.784	125.840
		T7910	T4739	-80.812*	22.932	669.616	0.000	-125.840	-35.784
	2	T4739	T7910	60.672*	22.907	667.663	0.008	15.693	105.652
		T7910	T4739	-60.672*	22.907	667.663	0.008	-105.652	-15.693
14-18	1	T4739	T7910	-31.314	17.658	659.607	0.077	-65.987	3.359
		T7910	T4739	31.314	17.658	659.607	0.077	-3.359	65.987
	2	T4739	T7910	-35.289*	17.747	668.492	0.047	-70.137	-0.442
		T7910	T4739	35.289*	17.747	668.492	0.047	0.442	70.137
22-28	1	T4739	T7910	8.729	17.386	692.488	0.616	-25.406	42.865
		T7910	T4739	-8.729	17.386	692.488	0.616	-42.865	25.406
	2	T4739	T7910	9.521	17.393	691.903	0.584	-24.627	43.670
		T7910	T4739	-9.521	17.393	691.903	0.584	-43.670	24.627
40-50	1	T4739	T7910	-11.633	18.631	692.065	0.533	-48.213	24.947
		T7910	T4739	11.633	18.631	692.065	0.533	-24.947	48.213
	2	T4739	T7910	7.206	18.688	696.759	0.700	-29.487	43.898
		T7910	T4739	-7.206	18.688	696.759	0.700	-43.898	29.487
65-80	1	T4739	T7910	5.436	19.816	735.474	0.784	-33.466	44.338
		T7910	T4739	-5.436	19.816	735.474	0.784	-44.338	33.466
	2	T4739	T7910	6.945	19.825	736.200	0.726	-31.976	45.866
		T7910	T4739	-6.945	19.825	736.200	0.726	-45.866	31.976
c. Adjustment for multiple comparisons: Bonferroni.									

Table O.13.7 TST RT-Pairwise comparison to Treatment x age group x task type x switch trial interactions.

Treatment				Mean	Std. Error	df	95% Confidence Interval	
							Lower Bound	Upper Bound
T4739	8-10	1	1	1192.173[b]	43.019	283.948	1107.496	1276.849
			2	1014.663[b]	41.978	264.672	932.009	1097.316
			3	956.827[b]	41.917	263.471	874.292	1039.362
			4	1003.492[b]	41.874	262.626	921.041	1085.944
		2	1	1164.873[b]	43.154	286.650	1079.934	1249.812
			2	1009.902[b]	41.992	264.937	927.222	1092.582
			3	955.067[b]	41.891	262.967	872.582	1037.552
			4	996.750[b]	41.912	263.365	914.226	1079.274
	14-18	1	1	940.317[b]	32.658	268.277	876.019	1004.615
			2	847.030[b]	32.390	261.493	783.250	910.809
			3	811.751[b]	32.386	261.391	747.979	875.522
			4	839.558[b]	32.391	261.494	775.779	903.338
		2	1	931.185[b]	33.161	280.475	865.909	996.461
			2	859.874[b]	32.398	261.690	796.080	923.669
			3	874.491[b]	32.394	261.579	810.705	938.276
			4	841.353[b]	32.383	261.293	777.589	905.117
	22-28	1	1	906.649[b]	30.966	281.000	845.694	967.604
			2	810.156[b]	30.416	267.261	750.270	870.043
			3	790.855[b]	30.337	265.096	731.123	850.587
			4	813.685[b]	30.357	265.650	753.913	873.457
		2	1	891.757[b]	30.559	270.609	831.593	951.921
			2	789.069[b]	30.416	267.241	729.184	848.954
			3	782.303[b]	30.366	265.876	722.516	842.091
			4	789.730[b]	30.350	265.436	729.973	849.486
	40-50	1	1	894.298[b]	32.592	266.588	830.128	958.468
			2	817.074[b]	32.416	262.131	753.246	880.903
			3	816.236[b]	32.442	262.795	752.356	880.115
			4	826.591[b]	32.405	261.870	762.783	890.400
		2	1	941.443[b]	32.624	267.408	877.210	1005.675
			2	817.813[b]	32.472	263.557	753.875	881.751
			3	807.442[b]	32.449	262.965	743.550	871.335
			4	833.654[b]	32.413	262.052	769.831	897.476
	65-80	1	1	1012.486[b]	33.730	295.416	946.104	1078.868
			2	882.062[b]	33.356	284.937	816.406	947.718
			3	854.767[b]	33.372	285.293	789.081	920.452
			4	858.222[b]	33.347	284.717	792.584	923.860
		2	1	975.104[b]	33.584	291.439	909.006	1041.202
			2	843.139[b]	33.391	285.768	777.415	908.863

			3	873.480[b]	33.413	286.294	807.714	939.246
			4	868.156[b]	33.357	284.938	802.499	933.813
T7910	8-10	1	1	1018.501[b]	41.324	238.098	937.093	1099.909
			2	953.593[b]	41.157	235.146	872.509	1034.676
			3	929.449[b]	41.103	234.191	848.471	1010.427
			4	942.365[b]	41.123	234.550	861.348	1023.383
		2	1	1008.168[b]	41.649	243.854	926.131	1090.205
			2	981.276[b]	41.150	235.026	900.206	1062.346
			3	956.726[b]	41.148	234.991	875.660	1037.792
			4	937.732[b]	41.132	234.701	856.698	1018.766
	14-18	1	1	1002.285[b]	32.215	242.796	938.828	1065.741
			2	848.719[b]	31.896	235.507	785.882	911.557
			3	844.022[b]	31.861	234.721	781.252	906.793
			4	868.885[b]	31.840	234.248	806.155	931.615
		2	1	1019.567[b]	32.651	251.833	955.264	1083.870
			2	869.563[b]	31.862	234.726	806.791	932.334
			3	869.345[b]	31.855	234.588	806.586	932.104
			4	889.586[b]	31.849	234.450	826.838	952.333
	22-28	1	1	910.772[b]	31.332	271.887	849.088	972.456
			2	778.758[b]	30.995	264.575	717.728	839.787
			3	804.296[b]	31.001	264.726	743.256	865.336
			4	792.602[b]	30.963	263.768	731.635	853.568
		2	1	872.545[b]	31.699	279.458	810.145	934.944
			2	773.241[b]	31.072	266.529	712.062	834.420
			3	769.648[b]	30.981	264.221	708.648	830.648
			4	799.342[b]	30.963	263.783	738.376	860.308
	40-50	1	1	908.516[b]	33.387	267.829	842.781	974.250
			2	818.593[b]	33.307	265.847	753.013	884.172
			3	848.599[b]	33.324	266.233	782.988	914.211
			4	825.025[b]	33.309	265.864	759.442	890.607
		2	1	918.893[b]	33.928	278.781	852.104	985.681
			2	821.271[b]	33.368	267.252	755.573	886.969
			3	816.591[b]	33.342	266.646	750.943	882.238
			4	814.775[b]	33.306	265.818	749.197	880.352
	65-80	1	1	986.148[b]	34.346	289.171	918.548	1053.748
			2	856.678[b]	33.848	277.328	790.046	923.311
			3	855.272[b]	33.854	277.484	788.628	921.915
			4	887.694[b]	33.846	277.247	821.068	954.321
		2	1	979.238[b]	34.189	285.359	911.944	1046.531
			2	836.394[b]	33.861	277.655	769.738	903.051
			3	846.761[b]	33.857	277.554	780.112	913.410
			4	869.707[b]	33.846	277.264	803.080	936.335

Table O.14 PANAS Positive Score (pre-tasks)- type III Tests of Fixed Effects

PANAS Positive Score (pre-tasks)				
Source	Numerator df	Denominator df	F	Sig.
Treatment	1	81.151	0.491	0.485
AgeGroup	4	87.958	1.512	0.205
Treatment * AgeGroup	4	80.937	1.188	0.322

Table O.14.1 PANAS Positive Score (pre-tasks)- Pairwise comparison to show age group x treatment interactions

AgeGroup			Mean Difference (I-J)	Std. Error	df	Sig.[c]	95% Confidence Interval for Difference[c]	
							Lower Bound	Upper Bound
1	T4739	T7910	2.597	2.824	81.274	0.361	-3.022	8.216
	T7910	T4739	-2.597	2.824	81.274	0.361	-8.216	3.022
2	T4739	T7910	-0.550	2.028	76.335	0.787	-4.588	3.488
	T7910	T4739	0.550	2.028	76.335	0.787	-3.488	4.588
3	T4739	T7910	0.027	1.949	81.071	0.989	-3.851	3.905
	T7910	T4739	-0.027	1.949	81.071	0.989	-3.905	3.851
4	T4739	T7910	-4.718*	2.218	82.018	0.036	-9.131	-0.305
	T7910	T4739	4.718*	2.218	82.018	0.036	0.305	9.131
5	T4739	T7910	-0.896	2.173	83.908	0.681	-5.216	3.425
	T7910	T4739	0.896	2.173	83.908	0.681	-3.425	5.216
c. Adjustment for multiple comparisons: Bonferroni.								

Table O.14.2 PANAS Positive Score (Post-tasks)- type III Tests of Fixed Effects

PANAS Positive Score (Post-tasks)				
Source	Numerator df	Denominator df	F	Sig.
Treatment	1	72.489	0.796	0.375
AgeGroup	4	75.094	1.756	0.147
Treatment * AgeGroup	4	73.218	1.882	0.123

Table O.14.3 PANAS Positive Score (post-tasks)- Pairwise comparison to show age group x treatment interactions

AgeGroup			Mean Difference (I-J)	Std. Error	df	Sig.[c]	95% Confidence Interval for Difference[c]	
							Lower Bound	Upper Bound
1	T4739	T7910	0.391	2.541	73.295	0.878	-4.672	5.454
	T7910	T4739	-0.391	2.541	73.295	0.878	-5.454	4.672
2	T4739	T7910	-1.722	1.815	69.404	0.346	-5.343	1.899
	T7910	T4739	1.722	1.815	69.404	0.346	-1.899	5.343
3	T4739	T7910	-0.191	1.715	71.958	0.912	-3.610	3.228
	T7910	T4739	0.191	1.715	71.958	0.912	-3.228	3.610
4	T4739	T7910	-4.800*	1.907	73.648	0.014	-8.600	-1.000
	T7910	T4739	4.800*	1.907	73.648	0.014	1.000	8.600
5	T4739	T7910	2.328	1.951	77.146	0.237	-1.558	6.213
	T7910	T4739	-2.328	1.951	77.146	0.237	-6.213	1.558
c. Adjustment for multiple comparisons: Bonferroni.								

Table O.14.4 PANAS Negative Score (Pre-tasks)- type III Tests of Fixed Effects

PANAS Negative Score (Pre-tasks)				
Source	Numerator df	Denominator df	F	Sig.
Treatment	1	81.831	5.818	0.018
AgeGroup	4	83.350	1.323	0.268
Treatment * AgeGroup	4	81.215	2.716	0.035

Table O.14.5 PANAS Negative Score (pre-tasks)- Pairwise comparison to show age group x treatment interactions

AgeGroup			Mean Difference (I-J)	Std. Error	df	Sig.[c]	95% Confidence Interval for Difference[c]	
							Lower Bound	Upper Bound
1	T4739	T7910	3.422*	1.145	80.677	0.004	1.144	5.700
	T7910	T4739	-3.422*	1.145	80.677	0.004	-5.700	-1.144
2	T4739	T7910	1.305	0.819	76.159	0.115	-0.327	2.937
	T7910	T4739	-1.305	0.819	76.159	0.115	-2.937	0.327
3	T4739	T7910	1.304	0.807	82.383	0.110	-0.301	2.908
	T7910	T4739	-1.304	0.807	82.383	0.110	-2.908	0.301
4	T4739	T7910	-0.967	0.905	82.891	0.288	-2.767	0.832
	T7910	T4739	0.967	0.905	82.891	0.288	-0.832	2.767
5	T4739	T7910	-0.094	0.886	84.509	0.916	-1.857	1.669
	T7910	T4739	0.094	0.886	84.509	0.916	-1.669	1.857
c. Adjustment for multiple comparisons: Bonferroni.								

Table O.14.6 PANAS Negative Score (Post-tasks)- type III Tests of Fixed Effects

PANAS Negative Score (Post-tasks)				
Source	Numerator df	Denominator df	F	Sig.
Treatment	1	79.562	0.030	0.864
AgeGroup	4	81.884	1.117	0.354
Treatment * AgeGroup	4	78.336	0.729	0.575

Table O.14.7 PANAS Negative Score (post-tasks)- Pairwise comparison to show age group x treatment interactions

AgeGroup			Mean Difference (I-J)	Std. Error	df	Sig.[b]	95% Confidence Interval for Difference[b]	
							Lower Bound	Upper Bound
1	T4739	T7910	0.100	1.351	79.024	0.941	-2.589	2.789
	T7910	T4739	-0.100	1.351	79.024	0.941	-2.789	2.589
2	T4739	T7910	-1.509	0.958	74.755	0.120	-3.418	0.400
	T7910	T4739	1.509	0.958	74.755	0.120	-0.400	3.418
3	T4739	T7910	0.522	0.902	76.790	0.565	-1.275	2.319
	T7910	T4739	-0.522	0.902	76.790	0.565	-2.319	1.275
4	T4739	T7910	0.111	1.005	78.989	0.912	-1.888	2.111
	T7910	T4739	-0.111	1.005	78.989	0.912	-2.111	1.888
5	T4739	T7910	0.367	1.020	83.057	0.720	-1.662	2.397
	T7910	T4739	-0.367	1.020	83.057	0.720	-2.397	1.662
b. Adjustment for multiple comparisons: Bonferroni.								

Appendix P

P.1 BluLife Study Plasma metabolite concentration for all quantified metabolites

Plasma Concentration (nM)

(Poly)phenol Metabolite	Placebo Mean (±SD)		WBB Mean (±SD)	
	0hr	2hr	0hr	2hr
Benzoic Acid Derivatives				
3,5-Dihydroxybenzoic acid	321±319	366±557	389±324	320±318
2,5-Dihydroxybenzoic acid	602±565	426±967	674±490	406±335
2-Hydroxybenzoic acid	1878±1786	1425±4402	2440±1468	1699±2162
2,6-Dihydroxybenzoic acid	794±1095	609±1316	995±630	749±1034
Vanillic acid	909±754	887±762	829±760	869±726
3-Hydroxybenzoic acid	620±562	512±469	611±464	526±484
2,4-Dihydroxybenzoic acid†	736±980	753±1760	955±910	775±1251
4-Hydroxybenzoic acid	668±761	673±668	549±737	663±900
Benzoic acid	7701±7270	7715±6196	6315±6788	8128±6738
2-Hydroxy-4-methoxybenzoic acid[b]*	166±198	164±191	140±198	136±190
2,3,4-Trihydroxybenzoic acid	508±290	517±281	509±288	493±293
Vanillic acid-4-O-sulfate	220±996	28±120	87±42	32±29
Protocatechuic acid sulfate mix [a]*	43±130	13±13	15±12	85±72
Isovanillic acid 3-O-sulfate [a]* †	363±389	240±302	293±238	289±305

Gallic acid	20±37	24±13	12±56	12±7
Syringic acid [a]*	162±127	153±91	147±118	175±99
4-Methylgallic-3-O-sulfate [a]*	276±1094	83±739	152±365	121±225
<u>Phenylacetic acid derivatives</u>				
Homovanillic acid sulfate sodium salt	70±216	36±38	43±26	41±26
3,4-dihidroxyphenylacetic acid	205±376	120±79	121±88	144±86
DL-p-Hydroxyphenyllactic acid	6484±1839	5485±1811	6579±1543	5620±1264
<u>Propionic acid derivatives</u>				
3-(2,4-Dihydroxyphenyl)propionic acid	158±300	74±88	75±52	96±208
(R)-(+)-2-(4-hydroxyphenoxy)-propionic acid	655±821	616±787	613±833	647±813
3-(2,3-Dihydroxyphenyl) propionic Acid	421±312	435±309	405±311	404±317
3-(4-hydroxy-3-methoxyphenyl)propionic acid	1121±1179	1035±1126	1070±1048	1133±1118
3-(2-hydroxyphenyl) propionic acid[a]*†	283±287	261±274	250±293	522±704
3-(3-hydroxyphenyl)propanoic acid	1482±1083	1473±1161	1338±1307	1515±1345
<u>Benzaldehyde derivatives</u>				
4-Hydroxybenzaldehyde	255±163	194±152	242±165	172±119
Vanillin	791±1015	857±1000	764±1042	718±1002
<u>Pyrogallol derivatives</u>				
Pyrogallol-O-sulfate mixture [a]#	375±332	253±296	346±219	288±263
Pyrogallol-2'-O-Sulfate[b]*†	909±3071	772±614	272±2603	227±395

2-Methylpyrogallol-O-sulfate†	950±1577	919±2317	1108±1625	921±1755
1-Methylpyrogallol-O-sulfate [a]*	1167±2693	607±829	501±1576	406±626
Benzene diols and triols				
4-Methylcatechol-O-sulfate	7546±11822	5011±6010	5252±5315	4638±5387
4-Methylcatechol	862±1060	788±1023	825±865	789±963
Catechol-O-1-glucuronide	62±131	24±83	49±39	35±39
Hippuric acid derivatives				
4-Hydroxyhippuric acid	744±2960	283±416	242±453	305±522
3-hydroxyhippuric acid	1720±5315	854±1315	954±1291	695±942
Hippuric acid	30008±81929	13944±19858	17848±19003	14690±16524
2-hydroxyhippuric acid	744±1781	387±608	559±276	422±329
Cinnamic acid derivatives				
Cinnamic acid [a#]	253±108	248±111	235±98	254±113
3,4-dihydroxycinnamic acid	103±97	75±167	125±113	124±288
3,5-Dihydroxyhydrocinnamic acid	129±212	94±120	131±71	111±155
Caffeic acid-4-glucuronide	31±27	32±24	31±39	28±19
Caffeic acid-3-glucuronide [a]*	12±11	10±8	10±9	14±10
Dihydro Caffeic acid 3-O-β-d-glucuronide	51±49	46±26	47±20	53±33
Dihydro Caffeic Acid 3-O-Sulfate Sodium Salt [a]*	791±920	718±794	748±833	724±770

Dihydro ferulic Acid 4-O-Sulfate	72±175	41±28	37±65	65±148
Ferulic Acid 4-O-Sulfate Disodium Salt [a]*	435±1638	72±142	155±36	463±294
Trans-ferulic acid	591±1577	157±300	344±135	546±535
Dihydro Isoferulic acid 3-O-Sulfate [a]#	251±424	227±558	285±279	283±511
Dihydroferulic acid 4-O-β-d-glucuronide [a]#	245±253	198±202	198±190	234±216
Isoferulic Acid 3-O-Sulfate [a]*†	605±462	745±449	658±404	591±465
Dihydro isoferulic acid 3-O-β-d-glucuronide [a]*	84±76	76±65	72±69	85±68
Isoferulic acid 3-O-β-d-glucuronide	576±649	552±636	499±657	500±606
Ferulic Acid 4-O- β -D-Glucuronide[a]#	608±782	644±795	634±802	614±727
Isoferulic acid	4172±4224	4056±4325	4346±4192	4202±4279
p-coumaric	16±21	14±8	10±13	21±16
m-coumaric acid	1541±1977	1887±1852	1469±2469	1863±2536
o-coumaric acid	401±179	399±181	407±179	395±180
Sinapic acid	109±126	105±122	102±125	105±120
Chlorogenic acid [a]*	4±5	4±1	3±1	50±43
4-Feruloylquinic acid	268±301	307±285	294±240	255±246
3-Feruloylquinic acid	2362±2833	2739±3001	2655±3084	1900±2834
<u>Flavanol derivatives</u>				
(-)-Epicatechin-3-O-sulfate [a]*	498±566	544±559	482±586	777±777
Epicatechin	343±276	343±267	364±269	370±276

(-)-Epicatechin-3-O-methylether	287±312	288±306	270±311	282±310
Flavonol derivatives				
Quercetin	144±100	145±95	141±102	144±98
Quercetin-7-O-ß-D-glucuronide	38±0	38±0	38±0	48±43
Quercetin 3-sulfate potassium salt†	25±16	24±17	25±16	25±16
Kaempferol-3-glucuronide	1312±1010	1262±995	1381±1022	1323±1009

Data is represented as (nM) ±SD
*Significance at the p<0.05 level for Treatment
#Trend towards significance at p< 0.10 for Treatment
†Significance at the p<0.05 level for Age × Drink interaction
[a]Higher metabolite concentration post WBB consumption
[b]Higher metabolite concentration post placebo consumption

P.2 BluLife Study Urinary Metabolite Concentration for All Quantified Metabolites

(Poly)phenol Metabolite	*Total 24 h Urinary Excretion (µg)*	
	Placebo Mean (±SD)	**WBB Mean (±SD)**
<u>Benzoic Acid Derivatives</u>		
3,5-Dihydroxybenzoic acid	5008±5905	5208±7880
2,5-dihydroxybenzoic acid	10093±26735	9373±10608
2-hydroxybenzoic acid	2767±4552	3248±5093
2,6-Dihydroxybenzoic acid	5170±8374	4889±4919
Vanillic acid	6581±4326	6691±4233
3-hydroxybenzoic acid [a]*	3976±6211	4065±9516
2,4-dihydroxybenzoic acid [b#†]	21199±51076	17806±24751
4-hydroxybenzoic acid	40002±49170	39724±35085
Benzoic acid	42408±31733	42854±29278
2-Hydroxy-4-methoxybenzoic acid[†]	1195±750	1165±717
2,3,4-Trihydroxybenzoic acid [a#]	2682±1710	2635±1634
Vanillic acid-4-O-sulfate	13855±31843	12097±11805

protocatechuic acid sulfate mix[a]*[†]	1167±1195	1478±1392
isovanillic acid 3-O-sulfate[a]*	3714±3774	5326±4805
Gallic acid	192±290	201±249
Syringic acid[a]*	1736±1296	2246±1604
4-Methylgallic-3-O-sulfate[†]	1710±2767	1608±2900
2,3-dihydroxybenzoic acid	4250±4418	6744±15065
protocatechuic acid-2-sulfate mix[a]*	134±141	209±211
Protocatechuic acid 3-O-ß-D-glucuronide[a]*	68±48	78±54
Protocatechuic acid[a]*	590±1187	690±808
Phenylacetic acid derivatives		
Homovanillic acid sulfate sodium salt	9330±11096	9862±8603
3,4-dihydroxyphenylacetic acid[a]*[†]	6271±5784	8312±6789
DL-p-Hydroxyphenyllactic acid[†]	3291±5178	3019±3886
Propionic acid derivatives		
3-(2,4-Dihydroxyphenyl)propionic acid	4868±4800	5232±4384
(R)-(+)-2-(4-hydroxyphenoxy)-propionic acid[a#]	8009±5288	8969±6202

3-(2,3-Dihydroxyphenyl)Propionic Acid[a]*	2743±1595	2802±1586
3-(4-hydroxy-3-methoxyphenyl)propionic acid[a#]	75793±121429	101154±165478
3-(2-hydroxyphenyl) propionic acid	2317±1826	2288±2356
3-(3-hydroxyphenyl)propanoic acid	13392±38162	19095±75502
Benzaldehyde derivatives		
4-Hydroxybenzaldehyde[†]	216±198	184±197
Vanillin	7531±4650	7256±4501
Benzene diols and triols		
4-Methylcatechol-O-sulfate[a*†]	15566±16782	20335±23219
4-Methylcatechol[a*†]	11644±10848	18737±19659
Catechol-O-1-glucuronide[a*†]	1049±1198	1604±1585
Hippuric acid derivatives		
4-Hydroxyhippuric acid	62563±69294	60784±53477
3-hydroxyhippuric acid[a*]	122781±151961	151630±143564
Hippuric acid[a*†]	2092649±1427211	3304277±2335141
2-hydroxyhippuric acid	19964±33608	25942±41842

Cinnamic acid derivatives		
Cinnamic acid	1189±745	1147±762
3,4-dihydroxycinnamic acid	2872±4130	3864±4618
caffeic acid-4-glucuronide [a#]	480±394	582±386
caffeic acid-3-glucuronide [a*]	258±276	361±319
Dihydro Caffeic acid 3-O-β-d-glucuronide	1868±5669	2207±4658
Dihydro Caffeic Acid 3-O-Sulfate Sodium Salt [a*]	7362±5654	8003±5437
Dihydro ferulic Acid 4-O-Sulfate [†]	5046±5689	5419±5059
Ferulic Acid 4-O-Sulfate Disodium Salt [a*]	50775±42788	59822±41453
trans-ferulic acid	82984±92259	101681±78021
Dihydro Isoferulic acid 3-O-Sulfate	8627±13096	9335±17265
Dihydroferulic acid 4-O-β-d-glucuronide	7377±8072	9030±9955
Isoferulic Acid 3-O-Sulfate	3839±3927	3564±2114
Dihydro isoferulic acid 3-O-β-d-glucuronide	4468±6004	4499±6015
Isoferulic acid 3-O-β-d-glucuronide	18334±28551	21515±17540
Ferulic Acid 4-O- β -D-Glucuronide [a*]	18718±17640	28232±22338

Isoferulic acid[a]†*	104601±205022	598448±616133
*p-coumaric[a]**	923±867	1444±1651
o-coumaric acid	1069±675	1042±644
*Sinapic acid[a]**	1210±817	1346±970
*Chlorogenic acid[a]**	1303±1842	2237±2006
4-Feruloylquinic acid	2817±4386	3633±4600
3-Feruloylquinic acid[a]†*	2080±1988	5561±5275
*caffeic acid sulfate mix[a]**	179±214	223±222
caffeic acid-2-sulfate mix	3067±3633	4322±4186
p-coumaric acid 4-O-sulfate dissodium salt	673±813	832±950
p-coumaric acid-4-O-ß-D-glucuronide	379±469	388±441
*Caffeic acid[a]**	1255±746	1622±967
4-Caffeoylquinic acid	533±365	567±339
3,5-Dihydroxyhydrocinnamic acid	7114±7384	8178±7307
Flavanol derivatives		
(-)-Epicatechin-3-O-sulfate	7167±17984	7813±16067

Epicatechin	2446±1676	2369±1579
(-)-Epicatechin-3-O-methylether	2376±1498	2319±1418
Flavonol derivatives		
Quercetin	828±520	813±510
Quercetin-7-O-ß-D-glucuronide[a]*[†]	123±75	122±86
quercetin 3-sulfate potassium salt[b#]	635±1041	447±575
Kaempferol-3-glucuronide	356±225	347±202
Valerolactones		
(4R)-5-(3'-hydroxyphenyl)-γ-valerolactone-4'-sulfate	9956±10893	11746±12880

Data is represented as (nM) ±SD
*Significance at the p<0.05 level for Treatment
[#]Trend towards significance at p< 0.10 for Treatment
[†]Significance at the p<0.05 level for Age × Drink interaction
[a]Higher metabolite concentration post WBB consumption
[b]Higher metabolite concentration post placebo consumption

Appendix Q

Q.1 BluFlow study Key stats for each main effect, interaction, and post-hoc test

Table Q.1 Total Acquisition-type III Tests of Fixed Effects

Total Acquisition				
Source	Numerator df	Denominator df	F	p-value
Treatment	1	46	0.036	0.851

Table Q.1.1 Total Acquisition- Pairwise comparisons

(I) Treatment		Mean Difference (I-J)	Std. Error	df	Sig.[b]	95% Confidence Interval for Difference[b]	
						Lower Bound	Upper Bound
AB	YZ	0.338	1.795	46	0.851	-3.274	3.950
YZ	AB	-0.338	1.795	46	0.851	-3.950	3.274
b. Adjustment for multiple comparisons: Bonferroni.							

Table Q.2 Immediate Recall-type III Tests of Fixed Effects

Immediate Recall				
Source	Numerator df	Denominator df	F	Sig.
Treatment	1	46	4.321	0.043

Table Q.2.1 Immediate Recall- Pairwise comparisons

(I) Treatment		Mean Difference (I-J)	Std. Error	df	Sig.[c]	95% Confidence Interval for Difference[c]	
						Lower Bound	Upper Bound
AB	YZ	-.854*	0.411	46	0.043	-1.682	-0.027
YZ	AB	.854*	0.411	46	0.043	0.027	1.682
c. Adjustment for multiple comparisons: Bonferroni.							

Table Q.3 Proactive Interference-type III Tests of Fixed Effects

Proactive Interference				
Source	Numerator df	Denominator df	F	Sig.
Treatment	1	47	1.607	0.211

Table Q.3.1 Proactive Interference - Pairwise comparisons

(I) Treatment		Mean Difference (I-J)	Std. Error	df	Sig.[b]	95% Confidence Interval for Difference[b]	
						Lower Bound	Upper Bound
AB	YZ	-0.618	0.488	47	0.211	-1.599	0.363
YZ	AB	0.618	0.488	47	0.211	-0.363	1.599
b. Adjustment for multiple comparisons: Bonferroni.							

Table Q.4 Retroactive Interference-type III Tests of Fixed Effects

Retroactive Interference				
Source	Numerator df	Denominator df	F	Sig.
Treatment	1	49	0.854	0.360

Table Q.4.1 Retroactive Interference - Pairwise comparisons

(I) Treatment		Mean Difference (I-J)	Std. Error	df	Sig.[b]	95% Confidence Interval for Difference[b]	
						Lower Bound	Upper Bound
AB	YZ	-0.449	0.485	49	0.360	-1.424	0.527
YZ	AB	0.449	0.485	49	0.360	-0.527	1.424
b. Adjustment for multiple comparisons: Bonferroni.							

Table Q.5 Delay Recall-type III Tests of Fixed Effects

Delay Recall				
Source	Numerator df	Denominator df	F	Sig.
Treatment	1	47	5.042	0.029

Table Q.5.1 Delay Recall - Pairwise comparisons

(I) Treatment		Mean Difference (I-J)	Std. Error	df	Sig.[c]	95% Confidence Interval for Difference[c]	
						Lower Bound	Upper Bound
AB	YZ	1.507*	0.671	47	0.029	0.157	2.857
YZ	AB	-1.507*	0.671	47	0.029	-2.857	-0.157

Table Q.6 Word Recognition List A- type III Tests of Fixed Effects

Word Recognition List A				
Source	Numerator df	Denominator df	F	Sig.
Treatment	1	49	0.029	0.866

Table Q.6.1 Word Recognition List A-- Pairwise comparisons

(I) Treatment		Mean Difference (I-J)	Std. Error	df	Sig.[b]	95% Confidence Interval for Difference[b]	
						Lower Bound	Upper Bound
AB	YZ	0.087	0.510	49	0.866	-0.939	1.112
YZ	AB	-0.087	0.510	49	0.866	-1.112	0.939
b. Adjustment for multiple comparisons: Bonferroni.							

Table Q.7 Word Recognition List B- type III Tests of Fixed Effects

Word Recognition List B				
Source	Numerator df	Denominator df	F	Sig.
Treatment	1	51	0.742	0.393

Table Q.7.1 Word Recognition List A-- Pairwise comparisons

(I) Treatment		Mean Difference (I-J)	Std. Error	df	Sig.[b]	95% Confidence Interval for Difference[b]	
						Lower Bound	Upper Bound
AB	YZ	0.969	1.125	51	0.393	-1.289	3.227
YZ	AB	-0.969	1.125	51	0.393	-3.227	1.289
b. Adjustment for multiple comparisons: Bonferroni.							

Table Q.8 Corsi Blocks (correct number of sequences)- type III Tests of Fixed Effects

Corsi Blocks (correct number of sequences)				
Source	Numerator df	Denominator df	F	Sig.
Treatment	1	48	0.085	0.772

Table Q.8.1 Corsi Blocks (correct number of sequences)- Pairwise comparisons

(I) Treatment		Mean Difference (I-J)	Std. Error	df	Sig.[b]	95% Confidence Interval for Difference[b]	
						Lower Bound	Upper Bound
AB	YZ	-0.253	0.867	48	0.772	-1.996	1.491
YZ	AB	0.253	0.867	48	0.772	-1.491	1.996
b. Adjustment for multiple comparisons: Bonferroni.							

Table Q.9 Corsi Blocks (correct number of blocks)- type III Tests of Fixed Effects

Corsi Blocks (correct number of blocks)				
Source	Numerator df	Denominator df	F	Sig.
Treatment	1	48	0.189	0.666

Table Q.9.1 Corsi Blocks (correct number of blocks)- Pairwise comparisons

(I) Treatment		Mean Difference (I-J)	Std. Error	df	Sig.[b]	95% Confidence Interval for Difference[b]	
						Lower Bound	Upper Bound
AB	YZ	-0.482	1.110	48	0.666	-2.713	1.749
YZ	AB	0.482	1.110	48	0.666	-1.749	2.713
b. Adjustment for multiple comparisons: Bonferroni.							

Table Q.10 Serial 3's Accuracy- type III Tests of Fixed Effects

Serial 3's Accuracy				
Source	Numerator df	Denominator df	F	Sig.
Treatment	1	38	0.748	0.393

Table Q.10.1 Serial 3's Accuracy -Pairwise comparisons

(I) Treatment		Mean Difference (I-J)	Std. Error	df	Sig.[b]	95% Confidence Interval for Difference[b]	
						Lower Bound	Upper Bound
AB	YZ	1.700	1.967	38	0.393	-2.281	5.682
YZ	AB	-1.700	1.967	38	0.393	-5.682	2.281
b. Adjustment for multiple comparisons: Bonferroni.							

Table Q.11 Serial 7's Accuracy- type III Tests of Fixed Effects

Source	Numerator df	Denominator df	F	Sig.
Treatment	1	43	0.002	0.968

Table Q.11.1 Serial 7's Accuracy -Pairwise comparisons

(I) Treatment		Mean Difference (I-J)	Std. Error	df	Sig.[b]	95% Confidence Interval for Difference[b]	
						Lower Bound	Upper Bound
AB	YZ	0.049	1.221	43	0.968	-2.414	2.512
YZ	AB	-0.049	1.221	43	0.968	-2.512	2.414
b. Adjustment for multiple comparisons: Bonferroni.							

Table Q.12 TST Accuracy- type III Tests of Fixed Effects

Source	Numerator df	Denominator df	F	Sig.
Treatment	1	90.119	5.129	0.026
SwitchTrial	3	94.234	12.572	0.000
TaskType	1	90.285	1.428	0.235

Treatment * SwitchTrial	3	90.922	0.601	0.616
Treatment * TaskType	1	89.927	0.316	0.575
Treatment * SwitchTrial * TaskType	6	90.857	1.212	0.308

Table Q.12.1 TST Accuracy- Pairwise comparisons

(I) Treatment		Mean Difference (I-J)	Std. Error	df	Sig.[c]	95% Confidence Interval for Difference[c]	
						Lower Bound	Upper Bound
AB	YZ	-.096*	0.043	90.119	0.026	-0.181	-0.012
YZ	AB	.096*	0.043	90.119	0.026	0.012	0.181
c. Adjustment for multiple comparisons: Bonferroni.							

Table Q.12.2 TST Accuracy-Pairwise comparison to show switch trial x treatment interactions.

SwitchTrial			Mean Difference (I-J)	Std. Error	df	Sig.[c]	95% Confidence Interval for Difference[c]	
							Lower Bound	Upper Bound
1	AB	YZ	-.095*	0.043	88.437	0.029	-0.180	-0.010
	YZ	AB	.095*	0.043	88.437	0.029	0.010	0.180
2	AB	YZ	-.092*	0.043	90.033	0.037	-0.178	-0.006
	YZ	AB	.092*	0.043	90.033	0.037	0.006	0.178
3	AB	YZ	-.100*	0.043	90.594	0.022	-0.185	-0.015
	YZ	AB	.100*	0.043	90.594	0.022	0.015	0.185
4	AB	YZ	-.099*	0.043	90.433	0.025	-0.185	-0.013
	YZ	AB	.099*	0.043	90.433	0.025	0.013	0.185
c. Adjustment for multiple comparisons: Bonferroni.								

Table O.12.3 TST Accuracy-Pairwise comparison to show Task type x treatment interactions.

TaskType			Mean Difference (I-J)	Std. Error	df	Sig.[b]	95% Confidence Interval for Difference[b]	
							Lower Bound	Upper Bound
1	AB	YZ	-0.120	0.061	90.056	0.051	-0.241	0.000
	YZ	AB	0.120	0.061	90.056	0.051	0.000	0.241
2	AB	YZ	-0.072	0.060	89.989	0.227	-0.191	0.046
	YZ	AB	0.072	0.060	89.989	0.227	-0.046	0.191
b. Adjustment for multiple comparisons: Bonferroni.								

Table O.12.4 TST Accuracy-Pairwise comparison to show Treatment x Switch Trial x Task type interactions.

SwitchTrial				Mean Difference (I-J)	Std. Error	df	Sig.[c]	95% Confidence Interval for Difference[c]	
								Lower Bound	Upper Bound
1	1	AB	YZ	-.131*	0.061	88.509	0.034	-0.252	-0.010
		YZ	AB	.131*	0.061	88.509	0.034	0.010	0.252
	2	AB	YZ	-0.058	0.060	88.118	0.331	-0.177	0.060
		YZ	AB	0.058	0.060	88.118	0.331	-0.060	0.177
2	1	AB	YZ	-0.110	0.062	89.995	0.078	-0.233	0.013
		YZ	AB	0.110	0.062	89.995	0.078	-0.013	0.233
	2	AB	YZ	-0.073	0.061	89.873	0.230	-0.194	0.047
		YZ	AB	0.073	0.061	89.873	0.230	-0.047	0.194
3	1	AB	YZ	-.124*	0.061	90.513	0.046	-0.245	-0.002
		YZ	AB	.124*	0.061	90.513	0.046	0.002	0.245
	2	AB	YZ	-0.076	0.060	90.550	0.207	-0.195	0.043
		YZ	AB	0.076	0.060	90.550	0.207	-0.043	0.195
4	1	AB	YZ	-0.116	0.062	90.382	0.064	-0.239	0.007
		YZ	AB	0.116	0.062	90.382	0.064	-0.007	0.239
	2	AB	YZ	-0.082	0.061	90.306	0.179	-0.203	0.038
		YZ	AB	0.082	0.061	90.306	0.179	-0.038	0.203
Based on estimated marginal means									

*. The mean difference is significant at the .05 level.
a. Dependent Variable: Accuracy Post intervention.
c. Adjustment for multiple comparisons: Bonferroni.

Table Q.13 TST RT- type III Tests of Fixed Effects

Source	Numerator df	Denominator df	F	Sig.
Treatment	1	89.532	0.345	0.559
SwitchTrial	3	102.581	16.981	0.000
TaskType	1	87.730	0.143	0.706
Treatment * SwitchTrial	3	90.015	1.055	0.372
Treatment * TaskType	1	87.661	0.124	0.725
Treatment * SwitchTrial * TaskType	6	90.390	0.495	0.811

Table Q.13.1 TST RT- Pairwise comparisons

(I) Treatment		Mean Difference (I-J)	Std. Error	df	Sig.[b]	95% Confidence Interval for Difference[b]	
						Lower Bound	Upper Bound
AB	YZ	24.967	42.530	89.532	0.559	-59.532	109.465
YZ	AB	-24.967	42.530	89.532	0.559	-109.465	59.532
Based on estimated marginal means							
a. Dependent Variable: Reaction time Post Intervention.							
b. Adjustment for multiple comparisons: Bonferroni.							

Table Q.13.2 TST RT-Pairwise comparison to show switch trial x treatment interactions.

SwitchTrial			Mean Difference (I-J)	Std. Error	df	Sig.[b]	95% Confidence Interval for Difference[b]	
							Lower Bound	Upper Bound
1	AB	YZ	39.473	70.573	87.980	0.577	-100.778	179.723
	YZ	AB	-39.473	70.573	87.980	0.577	-179.723	100.778
2	AB	YZ	9.599	37.430	87.993	0.798	-64.786	83.983
	YZ	AB	-9.599	37.430	87.993	0.798	-83.983	64.786
3	AB	YZ	42.232	47.369	96.498	0.375	-51.789	136.253
	YZ	AB	-42.232	47.369	96.498	0.375	-136.253	51.789
4	AB	YZ	8.563	43.374	87.675	0.844	-77.638	94.764
	YZ	AB	-8.563	43.374	87.675	0.844	-94.764	77.638
b. Adjustment for multiple comparisons: Bonferroni.								

Table Q.13.3 TST RT-Pairwise comparison to show Task type x treatment interactions.

TaskType			Mean Difference (I-J)	Std. Error	df	Sig.[b]	95% Confidence Interval for Difference[b]	
							Lower Bound	Upper Bound
1	AB	YZ	39.830	60.058	89.242	0.509	-79.499	159.159
	YZ	AB	-39.830	60.058	89.242	0.509	-159.159	79.499
2	AB	YZ	10.103	59.755	87.950	0.866	-108.648	128.855
	YZ	AB	-10.103	59.755	87.950	0.866	-128.855	108.648
b. Adjustment for multiple comparisons: Bonferroni.								

Table Q.13.4 TST RT-Pairwise comparison to show Treatment x Switch Trial x Task type interactions.

SwitchTrial				Mean Difference (I-J)	Std. Error	df	Sig.[b]	95% Confidence Interval for Difference[b]	
								Lower Bound	Upper Bound
1	1	AB	YZ	80.602	100.742	88.673	0.426	-119.579	280.784
		YZ	AB	-80.602	100.742	88.673	0.426	-280.784	119.579
	2	AB	YZ	-1.657	98.568	86.908	0.987	-197.574	194.261
		YZ	AB	1.657	98.568	86.908	0.987	-194.261	197.574
2	1	AB	YZ	1.251	52.736	87.197	0.981	-103.563	106.066
		YZ	AB	-1.251	52.736	87.197	0.981	-106.066	103.563
	2	AB	YZ	17.946	52.531	86.231	0.733	-86.479	122.370
		YZ	AB	-17.946	52.531	86.231	0.733	-122.370	86.479
3	1	AB	YZ	50.243	66.889	96.031	0.454	-82.531	183.017
		YZ	AB	-50.243	66.889	96.031	0.454	-183.017	82.531
	2	AB	YZ	34.221	66.705	95.183	0.609	-98.202	166.645
		YZ	AB	-34.221	66.705	95.183	0.609	-166.645	98.202
4	1	AB	YZ	27.222	61.181	87.099	0.657	-94.380	148.825
		YZ	AB	-27.222	61.181	87.099	0.657	-148.825	94.380
	2	AB	YZ	-10.096	61.066	86.486	0.869	-131.482	111.289
		YZ	AB	10.096	61.066	86.486	0.869	-111.289	131.482
b. Adjustment for multiple comparisons: Bonferroni.									

Table Q.14 PANAS Positive Score (pre-tasks)- type III Tests of Fixed Effects

PANAS Positive Score (pre-tasks)				
Source	Numerator df	Denominator df	F	Sig.
Treatment	1	50	0.297	0.588

Table Q.14.1 PANAS Positive Score (pre-tasks)- Pairwise comparison to show age group x treatment interactions

(I) Treatment		Mean Difference (I-J)	Std. Error	df	Sig.[b]	95% Confidence Interval for Difference[b]	
						Lower Bound	Upper Bound
AB	YZ	1.013	1.858	50	0.588	-2.718	4.744
YZ	AB	-1.013	1.858	50	0.588	-4.744	2.718
b. Adjustment for multiple comparisons: Bonferroni.							

Table Q.14.2 PANAS Positive Score (Post-tasks)- type III Tests of Fixed Effects

PANAS Positive Score (Post-tasks)				
Source	Numerator df	Denominator df	F	Sig.
Treatment	1	50	0.721	0.400

Table Q.14.3 PANAS Positive Score (post-tasks)- Pairwise comparison to show age group x treatment interactions

(I) Treatment	Mean Difference (I-J)	Std. Error	df	Sig.[b]	95% Confidence Interval for Difference[b]	
					Lower Bound	Upper Bound

AB	YZ	2.014	2.372	50	0.400	-2.751	6.779
YZ	AB	-2.014	2.372	50	0.400	-6.779	2.751
b. Adjustment for multiple comparisons: Bonferroni.							

Table Q.14.4 PANAS Negative Score (Pre-tasks)- type III Tests of Fixed Effects

PANAS Negative Score (Pre-tasks)				
Source	Numerator df	Denominator df	F	Sig.
Treatment	1	51	0.251	0.619

Table Q.14.5 PANAS Negative Score (pre-tasks)- Pairwise comparison to show age group x treatment interactions

(I) Treatment		Mean Difference (I-J)	Std. Error	df	Sig.[b]	95% Confidence Interval for Difference[b]	
						Lower Bound	Upper Bound
AB	YZ	0.354	0.707	51	0.619	-1.065	1.772
YZ	AB	-0.354	0.707	51	0.619	-1.772	1.065
b. Adjustment for multiple comparisons: Bonferroni.							

Table Q.14.6 PANAS Negative Score (Post-tasks)- type III Tests of Fixed Effects

PANAS Negative Score (Post-tasks)				
Source	Numerator df	Denominator df	F	Sig.
Treatment	1	50	0.275	0.602

Table Q.14.7 PANAS Negative Score (post-tasks)- Pairwise comparison to show age group x treatment interactions

(I) Treatment		Mean Difference (I-J)	Std. Error	df	Sig.[b]	95% Confidence Interval for Difference[b]	
						Lower Bound	Upper Bound
AB	YZ	0.517	0.985	50	0.602	-1.462	2.495
YZ	AB	-0.517	0.985	50	0.602	-2.495	1.462
b. Adjustment for multiple comparisons: Bonferroni.							

Appendix R

R.1 BluFlow Study Plasma metabolite concentration for all quantified metabolites

Plasma Concentration (nM)

(Poly)phenol Metabolite	**Placebo Mean (±SD)**		**WBB Mean (±SD)**	
	Baseline	3 months	Baseline	3 months
Benzoic Acid Derivatives				
3,5-Dihydroxybenzoic acid	1030±1184	901±1157	1302±1294	1039±1414
2,5-Dihydroxybenzoic acid	1924±2801	1611±1077	1382±752	1291±827
2-Hydroxybenzoic acid	2484±1839	6370±18734	2833±2720	2558±3050
2,6-Dihydroxybenzoic acid	2373±2187	2602±2009	3097±2684	2003±1605
Vanillic acid[b]*	1036±498	884±314	859±35	873±45
3-Hydroxybenzoic acid	1467±1606	1385±1327	1292±1378	1054±1064
2,4-Dihydroxybenzoic acid[b#]	3534±4338	3887±3789	4844±5169	2486±1975
4-Hydroxybenzoic acid	686±1028	561±382	713±1102	461±294
Benzoic acid	5431±1160	5446±1614	5920±2947	5637±1417

2-Hydroxy-4-methoxybenzoic acid	-	-	-	-
2,3,4-Trihydroxybenzoic acid	114±4	114±5	113±4	115±5
Vanillic acid-4-O-sulfate	694±2558	59±90	41±35	29±16
Protocatechuic acid sulfate mix	174±448	16±32	18±14	6±2
Protocatechuic acid-2-sulfate mix	108±349	42±101	22±27	14±12
Isovanillic acid 3-O-sulfate	2303±3207	2123±4389	1294±1210	1394±1260
Gallic acid	42±90	19±43	22±45	27±61
Syringic acid	228±168	210±163	277±215	285±186
4-Methylgallic-3-O-sulfate	711±1192	571±1204	882±2644	813±2740
2,3-dihydroxybenzoic acid	-	-	-	-
Protocatechuic acid 3-O-ß-D-glucuronide	29±9	20±8	22±3	20±2
Protocatechuic acid	-	-	-	-
Phenylacetic acid derivatives				
Homovanillic acid sulfate sodium salt	247±592	135±88	131±78	118±43
3,4-dihidroxyphenylacetic acid	631±991	398±182	353±181	363±263
DL-p-Hydroxyphenyllactic acid	8207±2394	8173±2070	7894±1476	7551±1838
3-hydroxyphenylacetic acid	-	-	-	-
Phenylacetic acid [b]*	3355±5650	5242±2075	2898±2661	3171±2942
Propionic acid derivatives				
3-(2,4-Dihydroxyphenyl)propionic acid	507±544	401±356	461±502	454±335

(R)-(+)-2-(4-hydroxyphenoxy)-propionic acid	426±1678	99±85	97±65	80±43
3-(2,3-Dihydroxyphenyl) propionic Acid	46±147	13±11	16±9	16±9
3-(4-hydroxy-3-methoxyphenyl)propionic acid	2370±2270	2519±1851	1734±1108	1868±931
3-(2-hydroxyphenyl) propionic acid	190±148	151±145	138±100	163±128
3-(3-hydroxyphenyl)propanoic acid	5541±7928	4100±4109	4590±4445	3606±3322
Benzaldehyde derivatives				
4-Hydroxybenzaldehyde	344±122	366±168	335±135	315±124
Vanillin	244±66	231±65	218±64	232±65
3,4-Dihydroxybenzaldehyde	-	-	-	-
Pyrogallol derivatives				
*Pyrogallol-O-sulfate [a]**	343±284	172±239	166±176	250±345
Pyrogallol-2'-O-Sulfate	2595±4225	978±1902	1489±4400	2105±6036
*2-Methylpyrogallol-O-sulfate [a]**	441±541	266±323	607±1712	688±1620
1-Methylpyrogallol-O-sulfate	2444±2732	993±1432	2424±7090	2718±8453
Benzene diols and triols				
*4-Methylcatechol-O-sulfate [a]**	9769±6959	9196±7134	14164±19928	16764±17137
*4-Methylcatechol [a]**	1226±1001	1266±1044	1750±2002	2124±1736
Catechol-O-1-glucuronide	222±519	98±138	72±65	79±61
Hippuric acid derivatives				
4-Hydroxyhippuric acid	2055±3779	1012±927	1629±2156	1234±1743

3-hydroxyhippuric acid	9411±18449	5945±8233	7174±10463	5075±4982
Hippuric acid	164672±289660	137999±244230	106140±118658	112389±85871
2-hydroxyhippuric acid	612±1050	1613±5809	448±454	400±487
Alfa-hydroxyhippuric acid	-	-	-	-
Cinnamic acid derivatives				
Cinnamic acid	336±34	354±54	342±31	346±37
3,4-dihydroxycinnamic acid	178±523	52±49	74±48	65±49
3,5-Dihydroxyhydrocinnamic acid	445±555	314±283	433±524	402±420
Caffeic acid-4-glucuronide	137±154	93±51	123±66	100±40
Caffeic acid-3-glucuronide	38±62	23±13	24±25	23±14
Dihydro Caffeic acid 3-O-β-d-glucuronide	176±398	48±99	12±13	34±40
Dihydro Caffeic Acid 3-O-Sulfate Sodium Salt	720±1317	416±493	335±558	253±398
Dihydro ferulic Acid 4-O-Sulfate [b#]	510±1535	101±104	102±119	64±50
Ferulic Acid 4-O-Sulfate Disodium Salt	6309±17865	383±962	198±162	176±316
Trans-ferulic acid	25116±57148	876±1820	293±288	728±0
Dihydro Isoferulic acid 3-O-Sulfate	365±480	622±1673	382±733	352±710
Dihydroferulic acid 4-O-β-d-glucuronide	678±1150	371±465	388±269	280±277
Isoferulic Acid 3-O-Sulfate	118±342	25±47	30±62	27±44
Dihydro isoferulic acid 3-O-β-d-glucuronide	350±704	250±505	175±182	108±123
Isoferulic acid 3-O-β-d-glucuronide	678±1879	195±372	52±50	166±246

Ferulic Acid 4-O- β -D-Glucuronide	1008±2196	277±653	132±120	108±77
*Isoferulic acid[a]**	847±2215	398±99	404±76	576±115
p-coumaric	34±89	20±21	16±10	16±9
m-coumaric acid	5946±4785	6281±5849	4982±3433	4911±2609
o-coumaric acid	182±3	182±6	182±3	183±3
Sinapic acid	68±126	43±28	48±18	46±23
Chlorogenic acid	-	-	-	-
4-Feruloylquinic acid	147±204	94±39	92±2	92±0
3-Feruloylquinic acid	-	-	-	-
caffeic acid sulfate mix	-	-	-	-
caffeic acid sulfate mix 2	-	-	-	-
p-coumaric acid 4-O-sulfate dissodium salt	259±407	27±26	14±10	13±0
p-coumaric acid-4-O-ß-D-glucuronide	44±59	8±9	23±15	24±16
Caffeic acid	47±93	30±60	17±12	11±15
4-Caffeoylquinic acid	-	-	-	-
Flavanol derivatives				
(-)-Epicatechin-3-O-sulfate	161±315	111±68	126±79	345±1077
Epicatechin	73±55	48±52	56±46	34±24
(-)-Epicatechin-3-O-methylether	156±3	159±8	155±2	156±3
Flavonol derivatives				
Quercetin	44±19	43±13	39±12	43±14
Quercetin-7-O-ß-D-glucuronide	39±1	39±1	39±2	39±2

Quercetin 3-sulfate potassium salt	17±13	16±7	16±9	16±8
Kaempferol-3-glucuronide	13±18	12±16	11±16	12±24
Myricetin	-	-	-	-
Quercetin 3-O-?-D-glucuronide	-	-	-	-
Valeractones				
(4R)-5-(3',4'-Dihydroxyphenyl)-gamma- valerolactone-4'-O-sulfate sodium salt	431±415	456±645	163±0	898±0

Data is represented as (nM) ±SD
*Significance at the p<0.05 level for Treatment
#Trend towards significance at p< 0.10 for Treatment
[a]Higher metabolite concentration post WBB consumption
[b]Higher metabolite concentration post placebo consumption

R.2 BluFlow Study urine metabolite concentration for all quantified metabolites

Total 24 h Urinary Excretion (µg)

(Poly)phenol Metabolite	**Placebo Mean (±SD)**		**WBB Mean (±SD)**	
	Baseline	3 months	Baseline	3 months
Benzoic Acid Derivatives				
3,5-Dihydroxybenzoic acid	5291±6239	5119±6362	5474±3397	6286±6822
2,5-Dihydroxybenzoic acid	10579±18499	11460±11528	1128±666	9124±6598
2-Hydroxybenzoic acid	2881±3241	3150±6570	179523±142348	1694±1184
2,6-Dihydroxybenzoic acid	5922±4713	5849±5723	3193±6180	7083±8607

Vanillic acid	4307±1058	4097±954	8082±4816	3930±316
3-Hydroxybenzoic acid	2412±2783	2428±2608	3660±2557	2833±1744
2,4-Dihydroxybenzoic acid	28375±30809	28332±34141	601±541	36603±50917
4-Hydroxybenzoic acid	27115±18541	27759±23025	3990±2862	23420±8914
Benzoic acid	26686±18736	25133±7721	1324±142	26371±8948
2-Hydroxy-4-methoxybenzoic acid	736±5	735±1	289±144	735±0
2,3,4-Trihydroxybenzoic acid	1662±56	1651±31	0±0	1661±45
Vanillic acid-4-O-sulfate	5500±3247	5105±2926	35942±19136	4598±2339
Protocatechuic acid sulfate mix	984±669	963±588	127±71	1084±528
protocatechuic acid-2-sulfate mix	163±119	146±99	6653±8658	154±80
Isovanillic acid 3-O-sulfate	6425±5040	5251±3803	243±128	4970±3236
*Gallic acid[a]**	254±301	119±90	940±528	242±197
Syringic acid	1966±3133	1213±657	6033±5497	1330±568
4-Methylgallic-3-O-sulfate	3877±2718	2633±2447	6748±6525	3074±2386
2,3-dihydroxybenzoic acid	5960±6533	4819±5175	3115±2591	12325±21209
Protocatechuic acid 3-O-ß-D-glucuronide	54±20	49±10	3978±2014	51±14
Protocatechuic acid	520±582	469±487	9325±6482	292±283
<u>Phenylacetic acid derivatives</u>				
Homovanillic acid sulfate sodium salt	5602±3488	6119±3001	1053±448	7302±5515
3,4-dihidroxyphenylacetic acid	4338±2506	4708±3193	10714±12154	5635±3437

DL-p-Hydroxyphenyllactic acid	1546±1237	1527±928	1796±961	1171±648
Propionic acid derivatives				
3-(2,4-Dihydroxyphenyl)propionic acid	4496±3119	4796±3312	859±527	6494±3426
(R)-(+)-2-(4-hydroxyphenoxy)-propionic acid	6055±3203	6309±2876	7979±4369	7181±2614
3-(2,3-Dihydroxyphenyl) propionic Acid	1979±645	1953±440	2689±574	2042±464
3-(4-hydroxy-3-methoxyphenyl)propionic acid	51407±38042	66956±92069	6866±6607	65951±63103
3-(2-hydroxyphenyl) propionic acid	1364±170	1290±112	102406±61690	1311±185
3-(3-hydroxyphenyl)propanoic acid	11322±11103	11935±10845	1225±771	13658±11485
Benzaldehyde derivatives				
4-Hydroxybenzaldehyde	240±208	217±187	1795±958	192±201
Vanillin	4713±191	4663±215	78332±80411	4716±283
3,4-Dihydroxybenzaldehyde	-	-	-	-
Pyrogallol derivatives				
Pyrogallol-O-sulfate mixture	-	-	-	-
Pyrogallol-2'-O-Sulfate	-	-	-	-
2-Methylpyrogallol-O-sulfate	-	-	-	-
1-Methylpyrogallol-O-sulfate	-	-	-	-
Benzene diols and triols				
4-Methylcatechol-O-sulfate [a#]	6575±4261	7383±4499	8448±10090	14612±12842
4-Methylcatechol	5889±3459	6520±3591	3857±4080	11202±9005

*Catechol-O-1-glucuronide [a]**	1217±1120	1156±886	358±327	1844±1300
Hippuric acid derivatives				
4-Hydroxyhippuric acid	33848±22302	34850±16872	49±14	32857±17059
3-hydroxyhippuric acid	137271±142678	152687±179202	2762±1737	184355±109395
*Hippuric acid [a]**	2922288±936296	3002033±1294064	231±179	3729241±1102230
2-hydroxyhippuric acid	21239±39817	28782±62632	1728±1978	20320±10489
Alfa-hydroxyhippuric acid	-	-	-	-
Cinnamic acid derivatives				
Cinnamic acid	1196±2229	795±193	200±175	807±411
3,4-dihydroxycinnamic acid	3644±4200	3614±3551	440±296	4033±2695
3,5-Dihydroxyhydrocinnamic acid	7231±5618	7429±5754	5324±3878	10487±7005
Caffeic acid-4-glucuronide	832±463	862±457	1584±818	973±450
*Caffeic acid-3-glucuronide [a]**	417±405	350±245	6519±5559	495±218
Dihydro Caffeic acid 3-O-β-d-glucuronide	725±843	776±774	3031317±1324534	976±936
Dihydro Caffeic Acid 3-O-Sulfate Sodium Salt	5593±2291	5725±1590	18456±11867	6728±4154
Dihydro ferulic Acid 4-O-Sulfate [a#]	5215±5896	4277±2076	82130±48174	5855±3912
Ferulic Acid 4-O-Sulfate Disodium Salt	72058±57999	67239±37406	148602±106192	77525±38069
Trans-ferulic acid	119046±113548	112074±75203	1177±1049	135193±72933
Dihydro Isoferulic acid 3-O-Sulfate	3525±3262	10015±24571	618±293	6170±11586

Dihydroferulic acid 4-O-β-d-glucuronide	8625±13068	7888±9751	2306±690	12823±15743
*Isoferulic Acid 3-O-Sulfate [a]**	2773±1322	2432±389	6481±2836	2673±426
Dihydro isoferulic acid 3-O-β-d-glucuronide	7085±8936	6682±10444	27619±14791	5612±4847
Isoferulic acid 3-O-β-d-glucuronide	23501±19378	23924±18803	4785±4499	28581±14932
*Ferulic Acid 4-O- β -D-Glucuronide [a]**	32778±54139	30468±25617	4762±265	43554±25669
*Isoferulic acid [a]**	93539±176406	101535±125850	522±644	556146±308779
*p-coumaric [a]**	1644±1714	1249±992	40909±31237	1405±741
m-coumaric acid	-	-	-	-
o-coumaric acid	648±13	650±8	82±11	653±7
Sinapic acid	1041±499	972±391	126±179	894±232
Chlorogenic acid	1862±2009	2339±3796	4251±657	2490±1681
*4-Feruloylquinic acid [b]**	3072±3298	4578±5570	26453±14071	4040±4759
3-Feruloylquinic acid	3132±2872	3422±3619	89145±335304	3934±2291
*Caffeic acid 4-O-sulfate[a]**	295±315	230±182	8529±4919	298±199
*Caffeic acid 3-O-sulfate [a]**	3303±2760	3171±2135	44946±59166	4311±2505
p-coumaric acid 4-O-sulfate dissodium salt	815±793	709±557	417±406	486±359
p-coumaric acid-4-O-ß-D-glucuronide	585±984	467±478	4977±5302	379±403
Caffeic acid	1374±731	1442±705	2442±2093	1820±859
4-Caffeoylquinic acid	502±314	573±613	3093±530	505±215
Flavanol derivatives				

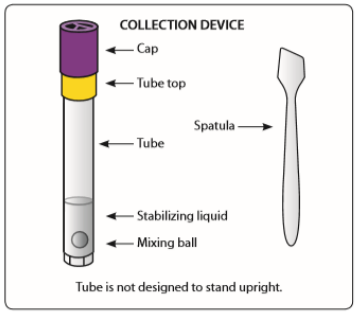
(-)-Epicatechin-3-O-sulfate	5963±11043	3591±2556	13480±14530	5147±10812
Epicatechin	1451±94	1453±85	13868±11697	1445±89
(-)-Epicatechin-3-O-methylether	1484±68	1465±33	1029±560	1470±33
Flavonol derivatives				
Quercetin	553±127	537±110	1641±22	526±55
Quercetin-7-O-ß-D-glucuronide [a#]	97±74	79±12	735±0	85±21
Quercetin 3-sulfate potassium salt	422±902	342±628	1467±27	453±747
Kaempferol-3-glucuronide	306±114	309±132	623±626	284±80
Myricetin	-	-	-	-
Quercetin 3-O- ß--D-glucuronide	78±66	89±33	654±5	182±166
Valeractone				
(4R)-5-(3',4'-Dihydroxyphenyl)-gamma-valerolactone-4'-O-sulfate sodium salt [a#]	5289±3330	4319±3458	1445±91	4929±3078

Appendix S

S.1 Faecal Collection Kit Instructions

OMNIgene•GUT

For microbiome



Summary and explanation of the kit:
OMNIgene•GUT provides the materials and instructions for collecting and stabilizing microbial DNA from a fecal sample.

Warnings and precautions:
- FOR EXTERNAL USE ONLY.
- Do NOT remove the yellow tube top from the tube.
- Do NOT spill the stabilizing liquid in the tube.
- Wash with water if liquid comes in contact with eyes or skin. Do NOT ingest. See MSDS at www.dnagenotek.com
- If collecting a liquid fecal sample, see separately provided user instructions.
- Small items may pose a choking hazard.

Storage: Room temperature storage pre- and post-collection 15°C to 25°C (60°F to 78°F).

Ship in accordance to applicable regulations covering transport of biological specimens.

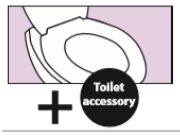
Label legend:

	Collect sample by (Use by)
REF	Catalog number
	Manufacturer
	Caution, consult instructions for use
LOT	Lot number
	Authorized Representative
15°C 25°C / 60°F 78°F	Storage instructions

USER INSTRUCTIONS

Read all instructions prior to collection
Other languages are available at www.dnagenotek.com

Procedure:

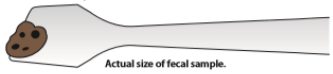


1 **IMPORTANT PREPARATIONS:**
- Empty your bladder before beginning the collection.
- Collect fecal sample free of urine or toilet water.
- Utilize any toilet accessory which may be supplied with the collection device.
- Toilet paper or tissues may be required.

2 While holding the yellow tube top, unscrew ONLY the purple cap and set aside for later use.

IMPORTANT:
Do NOT remove the yellow tube top.
Do NOT spill the stabilizing liquid in the tube.

3 Use the spatula to collect a small amount of fecal sample.

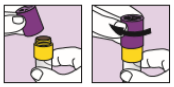


4 Transfer the fecal sample into the top portion of the yellow tube top. Repeat until the sample fills the yellow tube top.

IMPORTANT: Do NOT push sample into the tube.

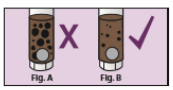
5 Scrape horizontally across the tube top to level the sample and remove any excess.

Wipe exterior of tube and top with toilet paper or tissue as needed.



6 Pick up the purple cap and screw onto the yellow tube top until tightly closed.

7 For **a minimum of 30 seconds**, shake the sealed tube as hard and fast as possible in a back and forth motion.



8 The fecal sample will be mixed with the stabilizing liquid in the tube; not all particles will dissolve.

IMPORTANT: Continue shaking if **large** particles remain as shown in Figure A.

9 Place spatula in original packaging or wrap in toilet paper and discard in garbage.

IMPORTANT: Send the sample for processing following the delivery instructions supplied separately by the kit provider.

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