University of Reading

The acute effects of wild blueberry extract on cognition in healthy older adults

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School of Psychology and Clinical Language Sciences

Nancy Cheng

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Declaration

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

Nancy Cheng

Abstract

Dietary Flavonoids and Human Cognition: Meta-analyses

Background:

Dietary flavonoids have been examined for their cognitive benefits with mixed findings. General conclusions over flavonoid efficacy have therefore not been possible.

Objectives: to quantitatively examine the effects of flavonoid consumption on cognition.

Methods:

Flavonoid effects on cognitive performance and associated moderating factors were assessed by meta-analysis of human intervention studies (k=80).

Results:

Flavonoid consumption was shown to have a significant positive effect on cognition, determined by cognitive health status. Positive effects were found following berry, cocoa or GB supplementation, low and medium doses, in middle-aged and older adults, chronic studies and particularly in long-term memory, processing speed and mood.

Conclusions:

Flavonoid supplementation may support positive cognitive functioning.

ROAB and BEAT trials

Background:

Significant flavonoid effects were found in older adults and following berry supplementation by meta-analysis. In particular, chronic treatment with wild blueberry extract (WBE) has been shown to improve EM and reduce SBP in older adults.

Objectives:

ROAB trial: to determine acute WBE effects on cognitive function and whether these are dose and time-dependent. BEAT trial: to replicate alleviation of cognitive decline during a predicted post-lunch dip following WBE 222.

Methods:

Both studies employed a randomised, double-blind, placebo-controlled, within-subject design to investigate the cognitive and cardiovascular effects of WBE in older adults (68-75 years). In ROAB, 28 participants received WBE doses of 111mg, 222mg, 444mg, 888mg and placebo, with measurements taken at 2 hours, 4 hours and 6 hours post-intervention. In BEAT (n=45), measurements were taken 1 hour after lunch at 14:00 to coincide with the post-lunch dip in cognitive performance.

Results:

ROAB trial: Benefits to EM and EF speed in more cognitively challenging tasks, and alleviation of a post-lunch decline in EF were found. SBP and DBP were significantly reduced following WBE 222. BEAT trial: This showed improved EF speed only. A post-lunch dip was not observed.

Conclusions:

These studies indicate that acute WBE supplementation may attenuate cognitive decline associated with the post-lunch dip and in keeping with proposed vasodilatory mechanisms, may also reduce BP.

Summary:

Dietary flavonoids were found to have positive effects on human cognition. Acute WBE showed benefits to EF speed, however EM speed and BP reductions were only apparent under conditions of increased cognitive fatigue, such as the post-lunch dip.

Abbreviations:

BEAT - Blueberry Extract Acute Trial

BP – blood pressure

DBP – diastolic blood pressure

EF – executive function

EM – episodic memory

GB – Ginkgo biloba

ROAB - Randomised Older Adult Blueberry Trial

SBP – systolic blood pressure

WBE – wild blueberry extract

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Chapter 1: Introduction

1.1 General Introduction

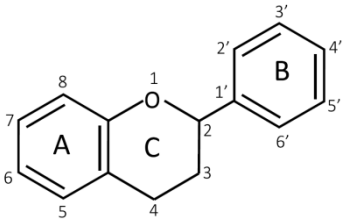
The UK population is continually expanding and simultaneously ageing (ONS, 2022). As a consequence, prevalence of age-related neurodegenerative conditions, namely Alzheimer's Disease (AD) and Parkinson's Disease (PD) has increased. There are currently an estimated 850,00 people living with dementia in the United Kingdom and this number has been forecast to rise to 2 million by 2050 (Public Health England, 2021). The economic cost of dementia in the UK is currently an estimated £34.7 billion a year and predicted to triple over the next two decades (Alzheimer's Society. 2019). However, there are limited treatment options for dementia. Recent development of monoclonal antibody therapies for the treatment of early AD have yet to become readily accessible to patients. For example, at the time of writing aducanumab and lecanemab have not obtained marketing authorisation approval by the European Medicines Agency (European Medicines Agency, 2022). The identification of viable preventative measures for dementia therefore poses a relevant and significant public health challenge. To this end, research efforts have turned to diet and lifestyle factors as routes of protection against the onset and progression of dementia. Indeed, diet and lifestyle modifications have been shown to not only extend life expectancy but also the number of years older adults can expect to live dementia-free (Dhana et al., 2022).

1.2 Flavonoids

Flavonoids are one such group of dietary components purported to confer protection from the development of dementia and improvements to cognition. Flavonoids are a key group of dietary polyphenols found abundantly in fruits, vegetables and grains with over 8,000 identified (Alseekh, de

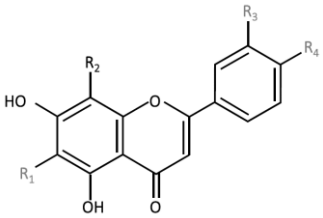
Souza, Benina, & Fernie, 2020). This family of phytochemicals share a common diphenylpropane (C6–C3–C6) backbone in which two aromatic rings are linked via a three-carbon chain (Saito et al., 2013; Tohge & R Fernie, 2017). This consists of a benzene ring (A) linked to a pyrone ring (C), with a phenyl ring (B) substituted at the C2 or C3 position (see figure 1.1a). Based on the level of unsaturation and oxidation of the C ring, flavonoids can be divided into the following main subclasses: anthocyanins, flavanols, flavonols, flavones, flavanones and isoflavones (See figure 1.1b). The impact of the structural differences between flavonoid subclasses are discussed later in Section 1.5 with respect to metabolism and bioavailability.

A



B

Flavone



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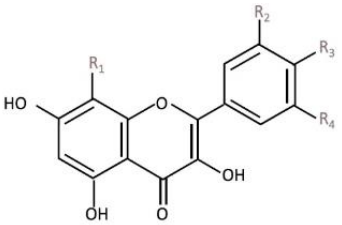
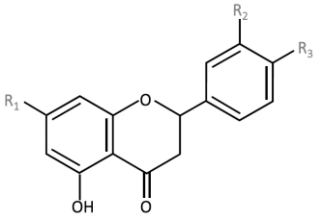
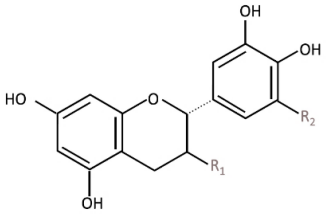


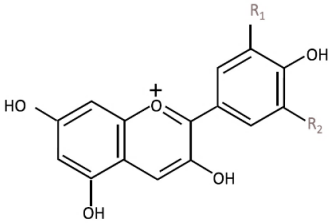
Flavanones



Flavanols



Anthocyanins



Isoflavones

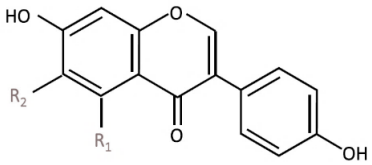


Figure 1.1: Structural configuration of:
A) flavonoid diphenylpropane (C6-C3-C6) backbone (adapted from Alseekh et al., 2020) and B) major flavonoid subclasses Flavones, Flavonols, Flavanones, Flavanols, Anthocyanins and Isoflavones. Flavones have a double bond between C2 and C3 and a ketone at C4, with hydroxyl groups at position 5 and 7 of the A ring. Flavonols contain a hydroxyl group at C3. Flavanones contain a saturated C ring. Flavanols are hydroxy derivatives of flavanones. Anthocyanins exist in several forms, of which the flavylium cation is the most stable. In isoflavones, the phenyl ring (B) is substituted at position 3 of the C ring

1.3 Dietary Sources of Flavonoids

Flavonoids are found in a wide variety of fruits, vegetables and plant-based products. Flavan-3-ols are the most commonly consumed flavonoid subclass across the globe (Bai, Wang, & Ren, 2014; Otaki et al., 2009; Sebastian, Goldman, Enns, & Moshfegh, 2017; Vogiatzoglou et al., 2014; Wang et al., 2014). Major sources of flavanols include tea, cocoa and red wine. Anthocyanins are pigments responsible for colours in plants and fruits. They are predominantly found in the skin of various fruits such as blueberries, cranberries, black currants, red grapes, raspberries, strawberries, bilberries and blackberries (Kozłowska & Szostak-Węgierek, 2019). Flavonols are found in vegetables such as onions and leeks, as well as fresh capers and dried herbs including parsley. They can also be found in smaller quantities in tea (Bai et al., 2014; Chun, Chung, & Song, 2007). Flavones are present in parsley and celery with flavanones predominantly found in citrus fruits. Isoflavones are mostly consumed as soybeans or soybean products such as tofu and soy milk (Kozłowska & Szostak-Węgierek, 2019). Cultural differences and food preferences contribute to the profiles of flavonoids ingested and the food sources from which they are obtained (Beecher, 2003; Zamora-Ros et al., 2013). For example, in known tea-drinking nations such as Japan, Australia and the United Kingdom, tea is the main source of flavon-3-ols (Otaki et al., 2009; Somerset & Johannot, 2008; Vogiatzoglou et al., 2015). Further, a diet that includes a variety of fruits, vegetables and plant-based foods is likely to provide a range of flavonoids.

1.4 Flavonoids and Cognition

Over the past two decades, the body of evidence for the benefits of dietary flavonoids to cognitive performance and prevention of neurodegenerative diseases has expanded rapidly. The existing literature reporting the effects of dietary flavonoids on cognition from human studies will be reviewed in this section.

1.4.1 Epidemiological Evidence

A number of large-scale epidemiological studies have investigated the effects of flavonoids and flavonoid-rich foods in the diet on the incidence of dementia, as well as age-related cognitive decline in otherwise healthy adults or improved cognitive performance in healthy populations. Cognitive changes in both healthy and pathological contexts, develop gradually which are unlikely to be detected under controlled trial conditions. Epidemiological data is therefore key to identifying the longer term impact of flavonoid consumption on these outcomes. However, in general, epidemiological studies do not inform on mechanisms of action and may be limited by residual confounders.

1.4.1.1 Risk of Dementia

In terms of human research, several epidemiological studies have shown higher intake of flavonoids to reduce the risk of dementia. The relationship between total flavonoid intake and incidence of dementia have been examined both at national and regional levels. In a population study, national dietary intake data obtained from FAO Food Balance Sheets and national dementia incidence data collated from WHO Burden of Disease Statistics, were used to examine the relationship between consumption of flavonoids (mg per capita per day) and disability-adjusted life years (DALY) resulting from Alzheimer's disease and related dementias (rate per 100,000) for each of 23 developed countries (Beking & Vieira, 2010). Intake of total flavonoids was found to be inversely correlated with dementia incidence. Overall fruit and vegetable intake was also assessed in this study. The correlation between flavonoid intake and dementia risk was found to be greater than between overall fruit and vegetable consumption and incidence of dementia. This suggests that flavonoids may be the dietary component responsible for the benefits to dementia risk rather than simply

consumption of a healthy diet indicated by fruit and vegetable intake. The generalisability of these findings may be limited in that only developed countries were included. Dietary intake data from developing countries were deemed insufficiently reliable.

Further support for the role of dietary flavonoids in reducing the risk of dementia were provided by the Paquid Study, a population-based cohort study of cognitive ageing in subjects aged 65 years and older (mean 76 years at dietary assessment) in south-west France (Commenges et al., 2000). There were however inconsistencies in the methodology within this study in that flavonoid intake was estimated using a 3-day food diary or food frequency questionnaire (FFQ) depending on study site. The incidence of dementia was assessed every two to three years during the course of a relatively short 5 year follow-up period. The age-adjusted relative risk of dementia (diagnosed according to DSMIII-R criteria) was found to be 0.55 for the two highest tertiles compared to the lowest tertile of flavonoid intake.

In addition to overall flavonoid consumption, inclusion of specific flavonoid-rich foods in the diet have also shown to reduce dementia risk. The 3C study is a cohort study including subjects aged 65 and over (mean 75.8 years at baseline), residing in the French cities of Bordeaux, Dijon and Montpellier (Lefèvre-Arbogast et al., 2018). Nutrient and polyphenol intake was derived from a 24-hour dietary recall in conjunction with the Phenol Explorer polyphenol database. Specifically, the intake of 26 polyphenol subclasses were assessed at baseline. Although an extensive polyphenol database was employed, the polyphenol intake during a single 24 hour period may inadequately represent an individual's habitual diet. During follow-up of 12 years, cognitive assessment including diagnosis of dementia according to DSM-IV was completed every 2 to 3 years. Results revealed a specific pattern of polyphenol intake, which included flavonoids, was inversely associated with the risk of dementia onset. This dietary pattern includes nuts, citrus, berries, leafy vegetables, soy, cereals, olive oil, red wine and tea. The highest quintile of pattern score showed 50% lower risk of

dementia compared to the lowest quintile. The benefits to longer-term risk of dementia as a result of specific polyphenol- and flavonoid-rich food intake suggests that flavonoid subclasses confer benefits to dementia risk differentially. However, the dietary pattern which was identified also included several other components which may contribute to reducing dementia risk, namely polyunsaturated and mono-unsaturated fats. Interestingly both the Paquid Study and 3C study examined the diets of populations in France and featured red wine, which limits the applicability of these findings to populations with very different dietary habits, in particular in cultures where alcohol is prohibited.

Identification of particular flavonoid subclasses which may reduce dementia risk were found in data from the Rush Memory and Aging Project (MAP), a population-based cohort study of older adults in the Chicago area (mean 81.2 years at baseline). This study showed a reduced incidence of AD was associated with increased frequency of strawberry consumption over 6.5 years of follow-up (Agarwal, Holland, Wang, Bennett, & Morris, 2019). Specifically, consumption of one or more servings per week was associated with a 34% lower risk of developing AD when compared to subjects consuming none or less than one serving per month. Although strawberries were investigated in particular, analysis of the key bioactives found in strawberries showed total flavonoids, total anthocyanidins and pelargonidin (an individual flavonoid) were also associated with lower risk of AD, indicating the beneficial effects of strawberries may be attributed to these components, which may be more informative with respect to the health messages that can be taken from this study. Further, data from the same cohort showed that subjects in the highest quintile of flavonol intake had a 48% lower rate of developing AD than in lowest quintile (Holland et al., 2020). Here, flavonol intake was determined by FFQ and the University of Minnesota's Flavonoid and Proanthocyanidin database, which is based on the USDA Database for the Flavonoid Content of Selected Foods. Annual neurological assessments were completed over a mean follow-up period of 6.1 years, whereby a diagnosis of AD was based on criteria of the National Institute of Neurologic

and Communicative Disorders and Stroke (NINCDS) and the Alzheimer's Disease and Related Disorders Association (ADRDA) working group.

These cohort studies suggest a relationship between total flavonoid intake and/or the flavonol and anthocyanidin subclasses and the risk of dementia in older adults. Two prospective cohort studies have examined the effects of flavonoid consumption during mid-life on subsequent dementia incidence. Subjects over the age of 50 years (mean 59.1 years) were included in the Framingham Offspring Cohort Study (Shishtar, Rogers, Blumberg, Au, & Jacques, 2020b). Cumulative intake across five follow-up examinations to account for changes in dietary patterns, of total flavonoids and six subclasses; flavonols, flavones, flavanones, flavan-3-ols, anthocyanins and flavonoid polymers, were derived from FFQs and the USDA database. Intake of flavonoids was categorised into percentiles; less than or equal to 15th, 15th to 30th, 30th to 60th, and more than 60th. Neurological assessments were conducted every 3 to 7 years over a 20 year follow-up period (mean 19.7 years). Dementia was diagnosed according to DSM-IV criteria and classified according to NINCDS-ADRDA. The highest (>60th percentile) intakes of flavonols, anthocyanins, and flavonoid polymers were associated with lower risk of Alzheimer's disease and related dementias (ADRD) compared to the lowest intakes (≤15th percentile). This non-linear inverse relationship was only evident for flavonols and anthocyanins when considering AD specifically. Similarly, consumption of flavonols and anthocyanins were shown to be associated with reduced incident dementia in a Danish prospective cohort study of 50 to 65 year olds (median 56 years) (Bondonno et al., 2021). Additionally, intake of flavanol oligomers and polymers, flavanones and flavones were also shown to be related to reduced dementia risk. Specifically, a non-linear dose response was demonstrated, whereby lower risk of dementia was associated with quintiles 3 and 4 compared with quintile 1 of intake of these flavonoid subclasses assessed at baseline using FFQ and Phenol Explorer polyphenol database. National patient and prescription registers were used to identify cases of dementia during 23 years of follow-up (median follow-up 21 years). Interestingly, these two studies showed that total flavonoid intake

during mid-life may be less important to dementia risk in later life. In contrast, flavonols and anthocyanins were consistently found to be associated with reduced dementia risk in both studies, which suggests that the reductions to incident dementia may be attributed to these subclasses in particular. Importantly, these studies indicate that the dose relationship between flavonoid intake and dementia risk is non-linear, with the exact shape of the dose-response curve yet to be elucidated.

Flavonoid consumption has not been universally associated with reduced dementia risk. For example, The Rotterdam Study, a Dutch population-based cohort study, examined the relationship between flavonoid consumption and risk of dementia as part of a wider investigation into the effects of anti-oxidants on dementia incidence in adults over the age of 55 years (mean 67.7 years at baseline) (Engelhart et al., 2002). Habitual flavonoid intake was estimated using FFQ and the Dutch Food composition table. Cognitive status was assessed according to DSM-III at baseline and at follow-up (mean follow-up 6 years). Data showed despite an association between higher flavonoid intake and higher mini-mental state examination (MMSE) score at baseline, there was no association between flavonoid intake and AD incidence. Data stratified by smoking status revealed that a benefit of flavonoids to AD risk was only apparent in current smokers (HR 0.54 95% CI 0.21 – 0.96). This was attributed to the elevated levels of free radicals in smokers and the anti-oxidant effect of flavonoids and the associated capacity to scavenge free radicals. Data from the 10 year follow-up cycle (mean 9.6 years) also showed that flavonoids were not associated with dementia risk (Devore et al., 2010). The relationship between habitual flavonoid intake during mid-life (45 -68 years, mean 52.4 years) and dementia risk was also examined in the Honolulu-Asia Aging Study, a prospective community-based study of Japanese-American men (Laurin, Masaki, Foley, White, & Launer, 2004). Flavonoid intake was estimated according to consumption of 11 types of tea infusions reported in two separate 24-hour dietary recall interviews and calculated according to the USDA database for flavonoids, the Canadian nutrition data bank, the Composition of Malaysian Foods and Standard

Tables of Food Composition in Japan. Initial screening of dementia was completed after 23 to 28 years of follow-up, and twice every 3 to 5 years thereafter, with a mean follow-up period of 30.2 years. Diagnosis of dementia was made according to DSM-III. Results showed flavonoid intake was not associated with the risk of dementia or its subtypes. The null effects observed in these three studies could be due to inaccurate estimates of flavonoid intake from using a limited flavonoid database (Devore et al., 2010; Engelhart et al., 2002) and basing flavonoid consumption on tea only (Laurin et al., 2004). In these studies, flavonoid intake was likely underestimated. Further, the Honolulu-Asia Aging Study used a 24-hour dietary recall, which may not have been representative of the habitual diet (Laurin et al., 2004) such that the true relationship between flavonoid intake and dementia risk may have been understated. The mixed findings may also result from inconsistent methodologies adopted across the epidemiological research. Follow-up periods range from 5 to 30 years in older adults (Commenges et al., 2000; Lefèvre-Arbogast et al., 2018). Considering the protracted prodromal phase of most types of dementia, the shorter studies may not have detected subsequent dementia cases.

1.4.1.2 Age-related Cognitive Decline and Improved Cognitive Performance in Healthy Populations

In addition to the relationship between flavonoid consumption and the incidence of pathological dementia, a number of epidemiological studies have demonstrated higher intake of flavonoids or flavonoid-rich foods to be associated with slower rate of age-related cognitive decline or improved cognitive function in healthy populations. The Paquid Study, a longitudinal prospective cohort study, investigated the association between flavonoid intake and cognitive decline in adults over 65 years (mean 77 years) (Letenneur, Proust-Lima, Le Gouge, Dartigues, & Barberger-Gateau, 2007). Flavonoid intake was estimated based on 5 major flavonoids reported by FFQ and food composition tables. Cognitive decline was measured by change in MMSE scores and global cognitive performance, assessed by combined scores for MMSE, Benton's visual retention test (BVRT) and

Isaac's Set Test (IST), every 2 to 3 years during a 10 year follow-up period. Results showed that quartiles 3 and 4 of flavonoid intake were associated with slower rate of decline in MMSE and global cognitive performance compared to quartile 1. This study indicates higher levels of flavonoid consumption may ameliorate cognitive decline in older age. However, it should be noted that flavonoid intake was estimated based on a very limited number of individual flavonoid compounds and in light of the vast array of flavonoids found in the diet, this is unlikely to represent the true quantities consumed.

Outside of older adults, studies into flavonoid intake at mid-life have also shown similar associations. Data from the Su.VI.MAX Cohort Study, revealed positive associations between total polyphenols, total flavonoids, and a number of subclasses including flavonols measured at baseline, and language and verbal memory score (RI-48, semantic fluency and phonemic fluency) recorded at follow-up of 13 years (mean age 66 years) (Kesse-Guyot et al., 2012). Habitual polyphenol and flavonoid intake during mid-life (45 -60 years) was estimated using a series of 24 hour dietary records and the Phenol Explorer database. Data from two cohort studies each with over 20 years' duration, the Nurses' Health Study which recruited female nurses aged between 30 and 55 years (mean 48 years) and the Health Professionals Follow-Up Study which included male health professionals aged between 40 and 75 years (mean 51 years), were examined for the relationship between long-term flavonoid intake and subjective cognitive decline (SCD) (Yeh et al., 2021). Estimates of intake of total flavonoids and 6 sub-classes (flavonols, flavones, flavanones, flavan-3-ols, anthocyanins and flavonoid polymers) were derived from FFQs completed every 4 years in conjunction with the USDA and EuroFIR eBASIS databases. Assessment of SCD was completed every 2 years between 2012 and 2014 in the Nurses' Health Study and between 2008 and 2012 in the Health Professionals Follow-Up Study, by online or postal questionnaire which assessed general memory, executive function, attention and visuospatial skills. SCD was considered a 3-point decline in assessment score. After controlling for age, total energy and major non-dietary factors, significant inverse associations

between total flavonoids and flavonoid subclasses were found in each cohort study. Pooled results across the studies revealed the strongest associations were for flavones and flavonones.

Data from the Framingham Offspring Study was used to examine the association between habitual flavonoid intake and annual change in cognitive performance across 15 years (Shishtar, Rogers, Blumberg, Au, & Jacques, 2020a). Quartiles of cumulative flavonoid intake across three follow-up examinations of total flavonoids and six subclasses; flavonols, flavones, flavanones, flavan-3-ols, anthocyanins and flavonoid polymers were derived from FFQs and the USDA database. In subjects aged 45 years and over at baseline, the cognitive domains of verbal and visual memory, verbal learning, attention, abstract reasoning, language, visuoperceptual organisation and global function were assessed three times over 15 years' follow-up (median follow-up 11.8 years, mean age at follow-up 60.8 years). Following correction for multiple comparisons, results only showed significantly slower decline between highest and lowest quartiles of intake for flavonols and flavan-3-ols for verbal and visual memory.

Together these studies suggest that long-term intake from mid-life onwards of flavonoids, particularly flavonols, flavan-3-ols, flavones and flavonones are important for favourable cognitive ageing and conserving cognitive function in later life in a dose-dependent manner. These benefits may also be specific to verbal and visual memory. These findings are discussed in terms of the bioavailability of the individual flavonoid subclasses in Section 1.5. Evidence for the benefits of whole flavonoid-rich foods to cognitive function and cognitive decline, with chocolate receiving particular attention, has also emerged. In a cross-sectional substudy of the HUSK Study in Norway, dietary and cognitive data was analysed from adults aged 70 to 74 years (Nurk et al., 2009). Intake of three key flavonoid sources (wine, tea and chocolate) was derived from FFQs and the University of Oslo's flavonoid database. Cognitive performance was assessed by the Kendrick object learning test (KOLT), TMT-A, modified Digit Symbol test (mDST), Block design, MMSE and the Controlled word

association test (S-task). Improved performance across the test battery was associated with increased consumption of each of the flavonoid-rich foods (except KOLT and chocolate intake) in a dose-dependent manner. Similarly, cognitive data from subjects aged between 23 and 98 years in the Maine-Syracuse Study, showed chocolate intake was positively associated with visuospatial memory and organisation, working memory (WM), scanning and tracking, similarities test, global composite score and MMSE (Crichton, Elias, & Alkerwi, 2016). Dietary intake was assessed by FFQ and cognitive assessment was carried out using the MSLS (Maine-Syracuse Longitudinal Study) neuropsychological test battery. In a longitudinal prospective cohort study of adults aged over 65 years (mean 70 years), chocolate intake was associated with a 41% lower risk of cognitive decline when compared with no chocolate intake (Moreira, Diógenes, de Mendonça, Lunet, & Barros, 2016). Analysis of the different levels of chocolate consumption, showed the reduced risk of cognitive decline was significant in subjects consuming moderate amounts of chocolate (less than 1 standard portion per week equivalent to three pieces of a chocolate bar or one tablespoon of cocoa powder). Here, cognitive decline was defined as a decrement of 2 MMSE points or more, across a follow-up period of between 2 and 9 years (median 48 months).

Data from the Nurses' Health Study also showed an inverse association between the rate of cognitive decline and levels of intake of blueberries and strawberries in older women (over 70 years) (Devore, Kang, Breteler, & Grodstein, 2012). Cognitive assessment was completed by telephone interview every 2 years between 1995 and 2001. Global composite and verbal memory composite scores were derived from Telephone Interview of Cognitive Status (TICS), MMSE, East Boston Memory test, category fluency, delayed recall of the telephone interview of cognitive status 10-word list and digit span backward. Comparison of highest and lowest intake groups of blueberries (1 or more servings of blueberries per week versus less than one serving per month) revealed significantly slower decline in global score, verbal score and TICs. For strawberries, the highest intake (2 or more servings per week) compared to the lowest intake level (less than 1 serving per week), showed

slower decline in global and verbal scores across follow-up. Higher intake of total flavonoids and greater intake of anthocyanidins were also associated with slower cognitive decline.

These studies show that the benefits of flavonoids can be achieved through the consumption of a range of flavonoid-rich foods and drinks. In contrast, in the Zutphen Elderly Study, a Dutch cohort study, flavonoid intake was estimated by mean average of two cross-check dietary history interviews 5 years apart (Kalmijn, Feskens, Launer, & Kromhout, 1997). MMSE was used to assess cognitive impairment at baseline and subsequent cognitive decline (defined as a drop of more than 2 points) in 342 very old men aged 69 to 89 years, after a follow-up period of 3 years. Flavonoid intake was not inversely associated with cognitive impairment at baseline nor with cognitive decline at follow-up. The absence of a significant association between flavonoid intake and cognitive decline may be due to the relatively short follow-up period and lack of sensitivity to change in cognitive status by MMSE.

Whilst most studies adjusted for covariates in their statistical models, residual confounders such as the effects of cognitive function in young age and depression have not been accounted for. Further, the effects of flavonoids cannot be isolated from the effects of consuming other nutrients found in the same foods. To this end the effects of flavonoids may be overstated. These epidemiological studies predominantly investigated the effects of flavonoid consumption in white subjects. This means that the conclusions may not necessarily apply to non-Caucasian populations and lower socio-economic groups, such that flavonoid intake may be a proxy indicator of an overall healthy diet and lifestyle.

In general, self-reported diet data as utilised by the above epidemiological studies, have been shown to contain reporting bias (Ottaviani, Sagi-Kiss, Schroeter, & Kuhnle, 2023). Participants wishing to appear to have healthier diets than that consumed due to social pressures contribute to this bias. As

a result, consumption of foods deemed healthy such as fruits and vegetables may be overestimated. Specifically when examining the effects of diet on cognitive decline, recall accuracy is likely to be reduced in participants affected by cognitive deficit. Flavonoid intake has been derived from either 24 hour recall, 3-day food diaries and FFQ by the epidemiological research reviewed above. The accuracy of 24 hour recall is limited by the ability of participants to remember the items consumed and to estimate the quantities in which they were consumed (Subar et al., 2015). FFQ have been deemed less reliable than 24 hour recall, in that these assess consumption of foods and beverages from fixed lists with details of methods of food preparation omitted. Validation of this tool has been demonstrated, albeit with varying degrees of correlation (Sierra-Ruelas et al. 2021). Subjects are required to complete 3 day food diaries at the time a food or beverage is consumed. However, intake is often documented retrospectively thus adding limitations as per 24 hour recall. Food diaries of between 3 and 14 days have shown validity (Subar et al., 2015). Further, this diet data was used in conjunction with food composition databases, most of which only provide details of the average flavonoid content of a food and do not account for the method of preparation (Ottaviani et al., 2023). Although estimates of absolute flavonoid intake in any population may be inaccurate due to the flaws in the methodology of self-reported diet data, conclusions can be drawn through interpretation of the patterns of consumption.

Collectively, these epidemiological studies support the hypothesis that a diet rich in flavonoids, from various sources, may support healthy cognitive ageing and reduce the risk of dementia. However, conclusions around causality and mechanisms of action cannot be determined. Intervention studies investigating the dose-response relationship of flavonoids and cognition in various populations are needed in order to understand the effects of dietary flavonoids on cognitive function more completely.

1.4.2 Human Intervention Studies

Evidence from human intervention studies for the benefits of dietary flavonoids on cognition across the lifespan has accumulated, from showing advantages to cognitive outcomes in healthy children to improved cognitive performance in dementia patients. In this section, a review of the existing literature investigating the acute effects (within 24 hours) of flavonoids on cognition will be followed by a review of studies which have examined longer-term flavonoid supplementation. A summary table of the key cognitive tests employed by flavonoid-cognition research can be found in Appendix A.

1.4.2.3 Acute Studies

Acute studies have typically assessed cognitive performance up to 6 hours post-intervention following a single dose of a flavonoid-rich food or preparation. Research from the past two decades is presented in order of increasing age from children to older adults to represent key developmental stages of cognition across the lifespan.

1.4.2.3.1 Acute Intervention in Children

A number of studies have investigated the effects of flavonoid interventions on the cognitive performance of children. These have revealed positive effects of blueberry supplementation over a period of up to 6 hours in subjects aged between 7 and 10 years. Specifically, improvements to episodic Memory (EM) and executive function (EF) have been shown following a single dose of a flavonoid-rich wild blueberry (WBB) drink (Barfoot et al., 2019; Whyte, Lamport, Schafer, & Williams, 2020; Whyte, Schafer, & Williams, 2016; Whyte, Schafer & Williams, 2017; Whyte & Williams, 2015).

In the first of these crossover studies, Whyte and Williams (2015) supplemented fourteen 8 to 10 year-old children with a WBB drink containing 143mg of anthocyanins, made up of 200g fresh Star variety blueberries, 100ml semi-skimmed milk and 8g sucrose, and a control drink which was matched for vitamin C and sugars, separated by a 7 day washout period. The test battery consisted of the Go-NoGo test, Rey's Auditory Verbal Learning Test (RAVLT), Word-colour Stroop, Visuospatial n-back task and Object Location Task, and was administered 2 hours post drink consumption. Significant improvements were seen in delayed auditory recall with the blueberry drink compared to placebo, indicating supplementation with blueberry can improve EM in school-aged children. This was a small study and may have been insufficiently powered to reveal positive effects on attention, inhibition and visuospatial memory. Further, although some research indicates that concomitant ingestion of dairy proteins do not alter the bioavailability of polyphenols (Draijer et al., 2016), some studies have suggested that anthocyanin absorption may be reduced by concurrent ingestion of milk (Xiao et al., 2017). In turn, this could have contributed to the null findings in relation to the cognitive domains of attention, inhibition and visuospatial memory assessed by Whyte and Williams (2015), in that these domains may require a higher bioavailable dose of anthocyanins to elicit a measurable change in cognitive performance.

In a larger study, Whyte et al. (2016) went on to examine the dose effects of WBB on cognition in children. In this double-blind cross-over study, twenty-one 7 to 10-year old children, were given blueberry drinks containing 30g WBB (253mg anthocyanins) and 15g WBB (127mg anthocyanins) prepared by mixing WBB powder with water and low flavonoid squash, and placebo (vehicle only). The three treatments were administered in a randomised order and were each separated by a 7-day washout period. A test battery consisting of an Auditory Verbal Learning Task (AVLT), a modified flanker task (MFT), the Go No-Go test and a Picture Matching Task (PMT), was administered throughout the day; at baseline, 1.15h, 3h and 6h post-intervention, to investigate time-course effects of the blueberry drinks. At 1.15h, dose-dependent effects were revealed for word

acquisition; improvements from baseline performance following 30g WBB were recorded in comparison to negative changes from baseline following vehicle and smaller negative changes following 15g WBB, with a significant difference between 30g WBB and placebo. For word recognition, a significantly larger decrease in accuracy following placebo than with 15g WBB was found at 6h. This drop in performance at 6h may indicate cognitive fatigue at a relatively late time in the day, such that benefits to word recognition were apparent in this study but not found by Whyte et al. (2015) where testing was conducted at 2 hours only. An overall positive linear trend indicated the effects of blueberry drink on word recognition were dose dependent. These findings provide further evidence for improvements to EM with WBB demonstrated by Whyte et al. (2015). For incongruent trials of the MFT, a dose-dependent effect of WBB was found at 3h, where the least accurate performance was recorded following placebo and most accurate results following 30g WBB. Not only does this exhibit improvements to EF following WBB supplementation, but also these effects are manifest in the more cognitively challenging aspects of a task.

Whyte et al. (2017) developed the Modified Attention Network task (MANT) to further investigate the relationship between the level of cognitive demand required to complete a task and flavonoid ingestion. The degree of cognitive demand was varied through levels of congruency of flanker items (neutral, congruent or incongruent), number of flanker items and speed of target presentation (faster 120ms considered more difficult trial than slower 500ms). The 30g WBB drink (253mg anthocyanins), for which EF effects were found by Whyte et al. (2016) was compared with a matched placebo, in a crossover design with a 7 day washout period. Twenty-one 7 to 10 year-olds were recruited to the study. Testing at 3 hours post-intervention, the timepoint at which WBB effects on EF were demonstrated by Whyte et al. (2016), revealed for trials where a visual cue was displayed prior to stimulus presentation, faster response times were recorded following 30g WBB than placebo irrespective of task difficulty. The benefits of WBB may have been through increased alerting effects provided by the cue. Importantly, faster response times were found in the more

cognitively challenging trials of the MANT (high load and incongruent trials when stimuli displayed for 500ms). These results further demonstrate the positive effects of WBB treatment on EF in school age children and these are apparent in versions of a task which exert higher cognitive demand on subjects. The earlier null findings in relation to EF by Whyte and Williams (2015) could therefore be due to insufficient cognitive load of the tasks employed for an effect of WBB to be elicited.

More recent studies in children have not replicated the effects of task difficulty manipulations on WBB cognitive effects. In a parallel groups design study (Barfoot et al., 2019) the effects of WBB (253mg anthocyanins) as per Whyte et al. (2016; 2017) were again compared with a sugar and vitamin C-matched placebo. The cognitive ability of fifty-four 7 to 10 year olds was assessed by AVLT, MANT and Test of Word Reading Efficiency-2 (TOWRE-2). Baseline readings were taken 1.15h after lunch (after which the intervention was consumed) and post-intervention testing took place at 2 hours to coincide with the time at which Whyte and Williams (2015) found effects for delayed auditory recall. A trend towards faster reaction times in the MANT on 120ms trials were seen in subjects who consumed WBB compared with subjects receiving placebo. While this finding does not demonstrate that WBB effects are most pronounced in more demanding trials of a cognitive task, it does further the evidence base for acute benefits of WBB to EF in children. With respect to AVLT measures, better accuracy in words recalled after a short delay and memory acquisition were found following WBB than placebo. This supports previous findings of improved EM following WBB in children. It is worth noting that the baseline readings were taken at 1.15h after lunch. Whilst the effects of taking lunch compared with not taking lunch have shown to be minimal in children (Schröder et al., 2016), post-lunch and pre-lunch cognitive performance has not been compared in this age group. The potential effects of a post-lunch dip on measured outcomes remains unclear.

In two separate experiments, Whyte et al. (2020) utilised an extensive task battery to further investigate the effects of 30g WBB drink on EF and memory in 7 to 10 year olds. In experiment 1, a

memory test battery consisting of AVLT (without the interference word list), Picture recognition Task, Visuo-Spatial Grid task (VSG) and the Brown-Peterson task (a measure of working memory) was administered at 75 minutes post-intervention to coincide with improved word acquisition detected by Whyte et al. (2016). In experiment 2, an EF test battery delivered at 3 hours post-intervention utilised the Stop Go Task (SGT), the Switching Task (ST) and the Attention Network Task (ANT). This replicates the timepoint at which Whyte et al. (2016) found improvements for incongruent trials of MFT. Both experiments employed a crossover design with a 7-day washout between interventions; WBB 30g (253mg anthocyanins) and a sugar, vitamin C and energy matched placebo. Faster reaction times were revealed on VSG and a trend towards faster reaction times for congruent trials of ANT. This is in keeping with previous findings of improved measures of EM and EF following 30g WBB drink. However, these were found to be irrespective of task difficulty.

Collectively these studies have demonstrated improvements in measures of EM following WBB drink (127mg, 143mg and 253mg anthocyanins) in healthy school age children. Importantly, improvements to measures of EF following 30g WBB (253mg anthocyanins) have been shown consistently in this age group. There is some evidence to suggest that these effects are most likely to be observed in more cognitively challenging aspects of a task to place sufficient cognitive load on the test subject, although data here is equivocal. No effects on EF were found following lower doses of WBB; 143mg and 127mg anthocyanins, which suggests that the threshold dose for acute EF changes lies somewhere between 143mg and 253mg anthocyanins. An optimum dose is yet to be established, as doses greater than 253mg anthocyanins were not examined in this limited set of studies and warrants further exploration. These findings are, however, confined to blueberry supplementation, insight into the effects of other flavonoid-rich foods with differing flavonoid profiles on cognition in children is severely lacking. Further research into broader flavonoid food sources is therefore required.

1.4.2.3.2 Acute Intervention in Young Adults (18 to 39 years)

The majority of acute flavonoid-cognition studies have been conducted in young adults and have shown evidence of association between flavonoid ingestion and short-term improvements in cognitive function. The findings from a wide range of flavonoid-rich foods are summarised here.

In the first of a series of acute studies to examine the effects of Ginkgo biloba (GB) extract on cognition in young adults, Kennedy, Scholey, and Wesnes (2000) supplemented eighteen 19 to 24 year olds (mean 19.9 years) with three different doses of GB extract and an inert control, in a crossover design study with a 7 day washout period. The three doses tested were 120mg, 240mg and 360mg of standardised GB extract containing 24% flavone glycosides and 6% terpene lactones in six capsules. A 20 minute cognitive test battery consisting of word presentation, immediate word recall, picture presentation, simple reaction time (SRT), digit vigilance task (DVT), Choice Reaction Time (CRT), spatial working memory (SWM), numeric working memory (NWM), word recall, delayed word recognition and delayed picture recognition was administered at baseline (immediately prior to ingestion of the intervention capsules), at 1h, 2.5h, 4h and 6h post-intervention. Composite scores for accuracy of attention, speed of attention, quality of memory and speed of memory were derived from the results of the test battery. Mood was also measured by Bond-Lader visual analogue scales (VAS). Change from baseline scores revealed faster speed of attention following 240mg and 360mg GB extract at 2.5h, 4h and 6h. This suggests that GB effects on attention are dose and time dependent, in that benefits were revealed for the higher doses and later during the test day. Evidence of positive effects on memory were seen as better performance in quality of memory score following 120mg GB extract at 1h and 4h and faster speed of memory following 360mg GB extract at 2.5h. No effects on mood were demonstrated following GB extract treatment.

In a subsequent study, Kennedy, Scholey, and Wesnes (2002) continued to investigate the effects of 360mg GB extract in young adults (mean 21.2 years) at the same timepoints during the day, using the same cognitive test battery plus Serial 3s and Serial 7s. Two additional composite scores were also measured; secondary memory score and WM score. A significant reduction from baseline in false alarms on DVT was shown 2.5h following 360mg GB extract and fewer errors for Serial 3s at 4h and more responses for Serial 7s at 4h and 6h. This further demonstrates acute 360mg GB extract supplementation is associated with positive effects on attention, although these results do not specifically replicate the speed of attention effects found by Kennedy et al. (2000). Better performance in the quality of memory score was found following 360mg GB extract at 1h and secondary memory score at 1h and 6h. Results of individual tasks revealed better immediate word recall at 6h and delayed word recall at 1h and 6h following 360mg GB extract. However, there was no evidence for GB effects on the WM score. In particular, there was no effect for a SWM task and significantly poorer performance was found at all tested timepoints for a NWM task. These results provide further evidence of the benefits of acute GB extract supplementation on memory performance and show that these benefits are particular to EM. However, the mood effects of GB extract were less compelling. Self-reported measures of alertness were shown to be higher following 360mg GB extract across all timepoints during the day. Although higher contentment scores at 1h, 4h and 6h were found following GB extract treatment, these results may have been confounded by higher baseline scores which were trending towards statistical significance.

An overall absence of positive effects following 120mg GB extract in studies by Kennedy, Jackson, Haskell, and Scholey (2007) and Elsabagh, Hartley, Ali, Williamson, and File (2005) support the suggestion that the effects of GB extract supplementation on memory and attention are dose dependent**.** In a crossover study with a 7 day washout period, twenty-eight young adults (mean 20.4 years) were supplemented with 120mg GB extract and an undefined inert placebo, contained in two capsules (Kennedy et al., 2007). The design of the test day with respect to testing timepoints,

cognitive test battery and mood measures matched those employed by Kennedy et al. (2002). Positive effects of 120mg GB extract on attention were limited to faster responses on DVT at 2.5h. Aside from this, poorer accuracy scores were found for CRT at 2.5h, 4h and 6h. Furthermore, there were no positive effects on memory outcomes following 120mg GB extract. Indeed, slower responses were recorded for a picture recognition task at 4h and for a spatial memory task at 6h. With respect to mood measures, participants reported higher calmness ratings following 120mg GB extract supplementation at 1h and 4h.

In experiment 1 of a parallel design study, Elsabagh, Hartley, Ali, et al. (2005) investigated the acute effects of 120mg GB extract in young adults aged between 18 and 26 years. Twenty-six subjects were supplemented with a standardised GB extract and 26 subjects were given an inert placebo (details not specified in paper), both interventions were provided in a single tablet. On acute test days, Bond-Lader VAS were used to measure mood followed by a cognitive test battery comprised of the Paced Auditory Serial Addition Task (PASAT), the pattern recognition memory (PRM), the spatial recognition memory (SRM) test, delayed word and picture recall, SWM test and the Intra Dimensional/Extra Dimensional set shifting task (IDED) was administered 4h post-intervention. Better attention performance was found for participants who received 120mg GB extract than those who received the placebo in PASAT during the faster (and therefore more challenging) trials. This suggests that a task may be required to exert sufficient cognitive demand in order for effects on sustained attention to manifest following low dose GB extract. Whyte et al. (2016, 2017) also reported effects of task difficulty, where positive effects of WBB were only evident in more cognitively challenging versions of the tasks used (the MFT and MANT respectively). With respect to EM and visual recognition response times in particular, no effects were detected for pattern recognition speed. This is in keeping with Kennedy et al. (2007) who also showed no benefits of 120mg GB extract to visual recognition response times in a picture recognition task at 4h post-intervention. Benefits to EM in the study by Elsabagh, Hartley, Ali, et al. (2005) were limited to more

accurate responses following 120mg GB extract compared to placebo for PRM. In contrast, Kennedy et al. (2007) found no effect on accuracy of picture recognition. No intervention effects were found for SRM, delayed word and picture recall. Together with the studies by Kennedy et al. (2000; 2007), there is a lack of evidence for marked improvements to EM following 120mg GB extract. With respect to measures of EF, the null findings for SWM by Elsabagh, Hartley, Ali, et al. (2005) echoed the results of Kennedy et al. (2002) for this measure. Other tests for EF (IDED and SoC) also yielded a lack of positive effects. Self-reported measures of mood did not reveal any significant effects of 120mg GB extract.

Taken together, this series of studies indicate that the cognitive effects of standardised GB extract are dose-dependent in that treatment with 240mg and 360mg GB extract elicited benefits to attention (particularly where performance is underpinned by psychomotor function) with further benefits to EM following the 360mg dose. In comparison, treatment with 120mg GB extract has not demonstrated clear benefits to cognitive performance. However, a more recent study by Ong et al. (2016), found positive effects of both 120mg and 240mg GB extract which were gender dependent. In this crossover study, twenty-four young adults (mean 20.2 years) were supplemented with two doses of GB extract (120mg and 240mg) and placebo, provided in the form of 2 capsules. Interestingly, rather than recording cognitive baseline measures on the day of testing, the authors used best test scores on the practice day as baselines. During the practice day, subjects repeatedly performed the cognitive tasks and continued to do so for as long as test scores improved. The best score was defined as the point at which improvement on the previous repeat did not exceed 5%. In doing so, Ong et al. (2016) aimed to eliminate practice effects on task outcomes, so that any changes in performance could be attributed to the interventions. However, this approach does not account for a day-to-day variation in cognitive performance which is likely to have confounded the results. On test days, blood pressure and heart rate were measured pre-intervention, at 60 minutes post-intervention and following each set of two cognitive tasks during the cognitive battery. The 30-

minute cognitive test battery was administered immediately after the 60-minute post-intervention BP measurements, was comprised of three sets of tasks where each set included two tasks as follows; set 1: visual search task, a psychomotor vigilance task, set 2: Stroop task, Bechara's gambling task and set 3: Berg's card sorting test and Tower of London. Improvements to EF, revealed as a reduced number of errors in the Stroop task and Berg's card sorting test, were associated with both doses of GB extract (120mg and 240mg) in female subjects only. Importantly, the GB effects on cognitive performance coincided with task-specific blood pressure effects. Significantly reduced change from baseline readings of SBP and DBP were recorded following 240mg GB compared to placebo after completion of the Stroop task and Bechara's gambling task (set 2), and significantly reduced change from baseline DBP in female participants after completion of Set 3: Berg's card sorting test and Tower of London following 240mg GB extract compared with placebo. BP measurements at 60 minutes post-intervention were used as baseline such that subsequent changes could be attributed to task completion. Blood pressure readings following placebo revealed differential changes from baseline according to task. SBP increases were higher after completion of the Stroop task and Bechara's gambling task (set 2) than after completion of the visual search and psychomotor vigilance tasks (set 1). The differences in BP reactivity may be attributed to task difficulty, where larger increases in BP were associated with increased task demand. In other words, the cognitive effects of GB extract were associated with amelioration of BP increases resulting from increased task demand. This suggests a single mechanism of action underlies both the cognitive and cardiovascular benefits of GB supplementation. Indeed, the vasodilatory effects of flavonoids has been proposed as one such mechanism and is discussed in detail in Section 1.6. The observation of GB extract benefits to cognitive performance in the more difficult tasks by Ong Lai Teik et al. (2016) provides further support for the hypothesis that the effects of flavonoids are apparent in more cognitively demanding situations, including those imposed by tasks with high cognitive demand, as evidenced by Whyte at al. (2016; 2017) and Elsabagh, Hartley, Ali, et al. (2005). However, it is worth noting that a relatively short washout period of 48 hours was used to separate the interventions,

compared to a 7 day washout used by Kennedy et al. (2000; 2002; 2007) and Elsabagh, Hartley, Ali, et al. (2005). The rationale for this washout period in relation to GB metabolism and excretion is unclear from the paper. The influence of visit order was not analysed and therefore the possibility of a carry-over effect cannot be ruled out. In such a case, the effects of GB extract would likely have been understated.

Interestingly, in addition to gender-specific cognitive responses, gender differences in cardiovascular reactivity to testing were also revealed. Composite cardiovascular scores of blood pressure and heart rate showed increases in cardiovascular scores were higher in females than males. It could be argued that in males the task battery was insufficient to bring about a cardiovascular response and the associated cognitive effects. The authors suggest that the blunted cardiovascular reactivity found in males may have been due to reduced levels of engagement in the tasks. However, this was not reflected in differences in performance between genders. The exact reason for the gender differences in cardiovascular response here is yet to be elucidated and is a potential consideration in future studies.

In summary, mixed effects of GB extract have been demonstrated in young adults. Whilst initial research suggests that the effects of GB extract in young adults are primarily evident in EM and attention in a dose-dependent manner, more recent findings indicate that even the typically assessed lower doses can elicit an effect on EF. Further the effects of GB extract on mood have been inconsistent and firm conclusions cannot be reached from the existing literature. One strength of the 0GB research reviewed here is the use of standardised extracts to enable direct comparisons of doses across studies. Further, in each of the studies, bias due to blinding has been minimised through the formulation of the interventions as capsules or tablets. Although GB flavonoids may contribute to the acute cognitive effects found in young adults, crucially cognitive benefits may also be attributed to the terpenoid content of the extracts.

Acute cocoa flavanol (CF) supplementation has also shown positive cognitive effects in young adult populations. Improved attention was demonstrated in a study by A. B. Scholey et al. (2010) in which thirty healthy young adults (mean 21.9 years) completed a three-arm crossover-design study, with a 3 day washout period. Subjects consumed chocolate drinks containing 994mg, 520mg and matched control containing 46mg of CF respectively. The Cognitive Demand Battery (CDB) was employed to place high cognitive load on participants and exclude ceiling effects for CF benefits to manifest. This was comprised of six repeats of a 10 minute cycle consisting of two serial subtraction tasks (Serial 3s and Serial 7s), a Rapid Visual Information Processing (RVIP) task and a 'mental fatigue' scale, commenced at 90 minutes post-intervention. Performance in the Serial 3s was significantly higher following 520mg CF than control for all 6 repetitions. In comparison, performance was only better than control for the first four repetitions following 994mg CF. Although following 994mg CF fewer errors for Serial 3s were found during the second repetition, more errors for Serial 7s were shown during the same repetition, as well during the third repetition. The absence of positive effects for Serial 7s following CF treatment could be explained by the higher EF component of the task compared to Serial 3s, which arguably has a greater psychomotor element and therefore assesses slightly different cognitive domains. Faster responses for RVIP were shown during the third and fourth repetition following 994mg CF. Better mental fatigue ratings were found for all test timepoints, except during the third repetition, following 520mg CF. Overall 520mg CF was shown to be more beneficial to attention and mood than the higher dose of 994mg. This suggests that a dose-relationship between CF and attention is likely to be non-linear and the exact shape of the dose-response curve is yet to be clarified. Benefits to cognition and mood found by Scholey et al. (2010) were most apparent during the fourth repetition (130minutes post-intervention) which coincided with the 2 hour timepoint at which peaks in cerebral blood flow (CBF) were found by Francis, Head, Morris, and Macdonald (2006) following CF treatment. This finding suggests support for increased CBF as a mechanism through which flavonoids elicit improvements to cognitive

performance, however direct measures of CBF were not taken, yet would be required to make causal links between the two outcomes.

Similarly, Field, Williams, and Butler (2011) also found improvements in attention with CF in young adults, demonstrated via a task battery which included Choice Reaction Time (CRT) and Visual Spatial Working Memory (SWM) task. Thirty participants (18 to 25 years) took part in the crossover study with one week washout period which compared the consumption of 35g of dark chocolate (773mg of CF) and 35g of white chocolate (trace flavanols). Participants were tested two hours after chocolate bar consumption to correspond with peak increases in CBF following CF treatment (Francis et al., 2006). Significantly faster reaction times were found in CRT following dark chocolate consumption compared to white chocolate. In addition to the benefits of CF to attention found by A. B. Scholey et al. (2010), Field et al. (2011) also showed positive effects on working memory. A small (3.6%) improvement in accuracy following the high flavanol dark chocolate over the low flavanol white chocolate was demonstrated in the SWM task. Scholey et al. (2010) interpreted the positive effects of CF to Serial 3s as benefits to psychomotor function in relation to attention. However, Serial 3s also draws on working memory, such that the findings of Field et al. (2011) and Scholey et al. (2010) may be more similar than first appears. Whilst Scholey et al. (2010) found some of the benefits to cognition of a lower dose CF 520mg were lost at the higher dose of 994mg, the positive effects of 773mg CF demonstrated by Field et al. (2011) suggest that these benefits are maintained at an intermediate dose and further support a non-linear dose-response curve.

These two studies (Field et al., 2011; A. B. Scholey et al., 2010) demonstrate cocoa benefits to attention and reduce self-reported mental fatigue. However, these cannot be attributed to cocoa flavonols exclusively. Other bioactives found in cocoa have also been shown to have positive effects on cognition, namely caffeine (Boyle, Lawton, & Dye, 2018; Lorenzo Calvo, Fei, Domínguez, & Pareja-Galeano, 2021) and theobromine (Cova, Leta, Mariani, Pantoni, & Pomati, 2019). Indeed, Karabay,

Saija, Field, and Akyürek (2018) addressed this limitation by utilising a caffeine and theobromine-matched placebo and further controlled for their effects through an inert 'baseline' intervention. Forty-eight young adults aged between 18 and 29 years (mean 22.1 years) were supplemented with a high dose flavanol cocoa drink (747mg CF), low dose flavanol cocoa drink (373.5mg CF), placebo (alkalised cocoa powder with no CF content) and a sugar-matched baseline (sugar and water only). The study adopted a crossover design where interventions were separated by a 7 day washout period. Rapid serial visual presentation task (RSVP) and Visual Search (VS) task were used to assess cognitive performance at 2 hours post-intervention. Visual search speed was improved by 373.5mg CF and 747mg CF compared to baseline intervention and placebo in orientation non-target trials in target present trials and following 373.5mg CF in the colour and orientation non-target trials of the target absent condition of the VS task, without detriment to VS accuracy. This suggests visual search efficiency was improved by CF supplementation and was achieved using a lower CF dose than previously tested (520mg tested by Scholey et al. 2010). However, no evidence for CF effects on temporal attention were found by RSVP. In keeping with the CF benefits to reaction times without effects on accuracy found by Scholey et al. (2010) and Field et al. (2011), the results of Karabay et al. (2018**)** indicate that improvements to spatial attention efficiency may have contributed to these prior findings. In terms of the methodology adopted by Karabay et al. (2018), timing of the study test day varied between subjects, in that four possible start times were used; 10.00h, 11.00h. 14.00h or 15.00h. In turn, the subjects who received the interventions in the afternoon are likely to have undergone cognitive testing during daily performance peaks (May, 1999). In contrast, subjects who commenced the test day in the late morning may have been tested during the post-lunch dip when cognitive performance is known to decline (Valdez, 2019). In light of the suggestion that flavonoid effects may be more apparent during cognitively demanding and compromised situations, it would be interesting to understand if time of testing modulated the CF effects shown here and whether CF cognitive effects were more pronounced during the post-lunch dip.

Acute changes to cognitive performance have been attributed to increased CBF following flavonoid ingestion, including cocoa flavanols. Other mechanisms of action through which flavonoids exert their benefits to cognition, include interactions with cell signalling pathways, such as through increased levels of brain-derived neurotrophic factor (BDNF). (For a more detailed review of the proposed mechanisms of action see section 1.6). Decroix et al. (2016) therefore aimed to investigate changes in BDNF and CBF following CF supplementation and cognitive testing. In a crossover design with a 7 day washout period, twelve men regularly undertook high impact physical training aged between 20 and 35 years (mean 30 years) were supplemented with 903mg CF chocolate milk and a control (low dose 15mg CF). The control was matched for caffeine, theobromine, sugar and taste. Cognitive performance was assessed by Stroop task (duration 5 minutes) at baseline prior to consumption of lunch and the intervention and at 95 minutes post-intervention. Blood samples were taken at the start of each test day and after cognitive testing. Interestingly, serum BDNF was measured rather than plasma BDNF levels. Platelet-bound BDNF found in serum is unable to cross the blood-brain barrier (BBB), therefore it could be argued that free BDNF found in plasma is a more accurate reflection of the concentrations of BDNF reaching the brain (Gejl et al., 2019). Serum levels of BDNF are generally higher than those found in plasma, which should be considered when comparing absolute BDNF measurements across studies. Decroix et al. (2016) recorded acute changes in cerebral blood volume as a measure of CBF and oxygenation by near infrared spectroscopy (NIRS) at rest and during cognitive assessment. This showed increased cerebral oxygenation following 903mg CF compared to control. However, this did not translate to positive effects on EF. Changes to serum BDNF concentrations were not detected. Decroix et al. (2016) suggest that these results are in contrast to previous findings of CF benefits to cognitive function (Field et al., 2011; A. B. Scholey et al., 2010). However, the 903mg CF dose used by Decroix et al. (2016) is in fact similar to the high dose (994mg CF) tested by Scholey et al. (2010) which also revealed limited benefits to cognition. Alternatively, the benefits of CF may not extend to EF as measured by the Stroop task or that the 5 minute Stroop task did not place sufficient demand on

subjects to elicit an CF effect (Decroix et al., 2016). Further, it should be noted that the interventions were consumed with lunch in the form of a chocolate milk drink. The potential for milk proteins to reduce flavonoid absorption has already been discussed and may have contributed to the null cognitive findings here.

Together these acute studies in young adults indicate that cognitive benefits of CF supplementation (namely attention and working memory) are observed at doses between 373.5mg and 773mg CF. Doses above 900mg CF have not revealed consistent positive effects to cognitive performance. The cognitive effects of CF have exhibited a non-linear dose-response with the shape of the curve still to be determined.

Cerebral blood flow has also been measured following treatment with citrus flavonoids in young adults. In a crossover design study with a one week washout, Lamport, Pal, et al. (2016) supplemented 24 adults aged between 18 and 30 years with 500ml high flavanone (HF) orange and grapefruit juice drink (70.5mg flavonoids; 42·15mg hesperidin, 17·25mg naringin, 6·75mg narirutin) and an energy and vitamin C matched placebo. Cognitive performance was assessed by Freiburg Vision Test, Immediate Word Recall, Logical Memory, Sequence Learning Task, Digit Symbol Substitution Test (DSST), Stroop Test, Letter Memory Test, Go-NoGo Task, Spatial Delayed Recall, delayed Word Recall and delayed Logical Memory at baseline and 2 hours post-intervention. DSST performance at 2h improved from baseline and compared to placebo following the HF citrus drink. The benefits to psychomotor speed demonstrated here are in keeping with the positive effects on attention following GB extract (Kennedy et al., 2000; 2002) and CF (Field et al., 2011; Karabay et al., 2018; A. B. Scholey et al., 2010) which appear to be underpinned by improvements to psychomotor function.

The lack of positive effects for all of the other measured cognitive outcomes (measures of EF and memory) may be due to the insufficient sensitivity of the tasks employed. However, ceiling effects were not apparent. More likely the dose tested was too low to have an effect given this was a relatively low dose of flavanones compared to the experimental doses of CF employed, for example 373.5mg to 994mg CF (Karabay et al., 2018; A. B. Scholey et al., 2010).

BP measured prior to cognitive testing at baseline and post-intervention showed diastolic blood pressure (DBP) reductions at 2h post-intervention from baseline and compared to placebo. This echoes the concomitant cardiovascular and cognitive benefits of flavonoids demonstrated by Ong et al. (2016) following GB supplementation. In a separate arm of the study, Lamport, Pal, et al. (2016) measured CBF in a further 16 subjects by functional magnetic resonance imaging arterial spin labelling (fMRI ASL) at baseline, 2h and 5h post-intervention. This revealed increased CBF at 2 hours following HF citrus drink consumption compared with placebo. This suggests that the vasodilatory effects of flavonoids on the peripheral vasculature may extend to blood vessels in the brain and the positive effects of HF citrus drink on psychomotor function, blood pressure and increased CBF may be attributed to this mechanism of action. (See section 1.6 for details of proposed mechanisms of action). Despite temporal correlation between these three outcomes, direct associations cannot be made from the findings of Lamport, Pal, et al. (2016) in the absence of concurrent recordings of CBF, cognitive performance and BP. Indeed, cognitive performance was not assessed at 5h, when no changes to CBF were shown in the ASL arm of the study.

Further approaches have been taken to understand the mechanisms through which flavonoids exert their effects on cognition. Several studies have investigated biomarkers of such proposed mechanisms of action alongside measures of cognitive performance following acute berry flavonoid treatment in young adults. Watson et al. (2015) employed a crossover design with a 1 week washout period to test two blackcurrant interventions. Thirty-six 18 to 24 year olds (mean 24.8y) were

treated with cold compress juice, blackcurrant extract (both providing a polyphenol dose of 525mg/60kg body weight) and sugar-matched placebo. A test battery which was designed to induce mental fatigue was comprised of 7 repetitions of DVT, Stroop task and RVIP, followed by a logical reasoning task and visual analogue scales to assess mood, mental fatigue and perceived task difficulty, were administered at baseline and 65 minutes post-intervention (corresponding with detectable levels of blackcurrant anthocyanins in plasma). Peripheral monoamine oxidase inhibition was measured by platelet monoamine oxidase levels, and blood glucose levels were also monitored. A decline in RVIP accuracy scores was shown to be improved across all repetitions following the blackcurrant extract compared to placebo. The slowing of Digit Vigilance reaction times was attenuated by blackcurrant juice at repetitions 1, 4 and 7 only. In keeping with findings of benefits to psychomotor function and vigilance following GB, CF and citrus flavonoids (Field et al., 2011; Karabay et al., 2018; Kennedy et al, 2000, 2002; Lamport, Pal, et al., 2016; Scholey et al., 2010), these results show berry flavonoids also benefit attention. However, positive effects were not evident for Stroop task, logical reasoning, mental fatigue and perceived task difficulty following either blackcurrant intervention. Despite results showing inhibition of MAO-B by the blackcurrant juice, corresponding improvements to mood were not detected. In comparison to placebo, the blackcurrant juice showed higher plasma glucose levels at 60 minutes and 150 minutes post-intervention, in keeping with flavonoid modification of the immediate postprandial glucose response (Proença, Ribeiro, Freitas, & Fernandes, 2022). Notably, the effects on monoamine oxidase and blood glucose were only evident following blackcurrant juice but not the extract. Although both blackcurrant interventions contained the same quantity of total flavonoids, plasma anthocyanin levels were higher following the juice than the extract at 2.5h, indicating possible greater bioavailability of anthocyanins from juice. This may be due to the different food matrix in which the anthocyanins were consumed. In particular, fibre content has been found to influence flavonoid absorption and can aid flavonoid bioavailability (Kamiloglu, Tomas, Ozdal, & Capanoglu, 2021). More likely, the differences in the measured physiological effects may be explained by the different flavonoid profiles, for example the juice

contained greater concentrations of epigallocatechin and myricetin, but lower levels of quercetin than the extract. Despite MAO inhibition and glucose absorption modulation by the blackcurrant juice intervention, cognitive benefits to attention were detected across more repetitions following blackcurrant extract. This indicates that additional mechanisms beyond MAO inhibition and blood glucose modulation are likely responsible for berry flavonoid effects.

Watson et al. (2018) continued to investigate the cognitive effects of blackcurrant juice in young adults. In a crossover design study, subjects (mean age 23 years) were supplemented with cold pressed blackcurrant juice (500mg polyphenols) and a sugar and vitamin C matched placebo, with 1 week separating interventions. Electroencephalography (EEG) recordings were taken during cognitive testing at baseline and 45 minutes post-intervention, to determine associations between cognitive performance and changes in neuronal activity. Post-intervention testing consisted of three repetitions of a 7 minute test battery comprised of Simple Reaction Time, Digit Vigilance and CRT. Visual analogue scales after each test battery repetition were used for self-reporting of mood, mental fatigue and physical fatigue. EEG readings during performance of SRT and DVT revealed suppression of α waves and increased β wave strength localised in the prefrontal cortex, demonstrating increased attention and lower fatigue across repetitions following blackcurrant juice. However, the observed modulations of brain waves did not translate into cognitive or mood effects. Rather a slowing of CRT was found, which could be explained by increased δ wave strength showing increased relaxation and may be attributed to anxiolytic effects due to MAO inhibition (Watson et al., 2015). The null cognitive findings by Watson et al. (2018) do not reflect the positive effects on attention demonstrated in their earlier study (Watson et al., 2015). Cognitive testing commenced at 45 minutes post-intervention and was completed at 66 minutes. This means that the majority of cognitive performance was assessed prior to the known peak in plasma blackcurrant anthocyanins (Costello et al., 2022) when cognitive effects are most likely to occur. The cognitive demand battery induced progressive mental fatigue across 7 repetitions (Watson et al., 2015) in contrast mental

fatigue scores did not increase across 3 repetitions of the test battery used by Watson et al. (2018). This suggests that insufficient cognitive load was placed on subjects for a cognitive effect of blackcurrant juice to become apparent and is in keeping with findings of Whyte et al. (2016; 2017), Elsabagh, Hartley, Ali, et al. (2005) and Ong et al. (2016) where flavonoid benefits were revealed in more challenging trials of cognitive tasks. These two studies by Watson et al. (2015; 2018) demonstrate physiological changes which may underpin cognitive changes can be evident in the absence of behavioural effects.

Conversely, behavioural effects have been shown in the absence of measured physiological outcomes. For example, in a crossover design study with a 1 week washout period, Philip et al. (2019) supplemented 30 adults aged 18 to 24 years (mean 22 y) with 600mg of polyphenols-rich extract of grape and wild blueberry (PEGB) providing 260mg flavonoids and placebo in the form of 2 capsules. Cognitive performance during six repetitions of the Cognitive Demand Battery (CDB) consisting of Serial 3s, Serial 7s, RVIP and VAS, was assessed commencing at 90 minutes post-intervention. Blood samples and cardiovascular measures of BP and HR, plus FMD (26 subjects) were taken at the start of the test day and 15 minutes after completion of the CDB (3.5h post-intervention). Changes in performance from the first CDB repetition showed improved performance in Serial 3s; number of total answers, correct answers, number of errors, percentage of correct answers and net score but not Serial 7s. This mirrors the findings of Scholey et al. (2010) in relation to Serial 3s and Serial 7s performance following CF treatment, and further supports findings of psychomotor function benefits of flavonoid treatment that extends from GB, CF, citrus to PEGB. A decline in RVIP accuracy across repetitions showed a trend towards attenuation following PEGB, which reflects the findings of Watson et al. (2015) following blackcurrant treatment.

Philip et al. (2019) showed an increase from baseline of plasma flavan-3-ols and gut microbiota metabolites at 3.5h following PEGB. However, this single measurement informs little on the timing

of peak plasma levels following the combined PEGB intervention. Indeed, plasma metabolite concentrations of individual grape and blueberry polyphenols have shown to peak at different times. Grape polyphenol metabolites were found to peak around 4 hours after ingestion (Castello et al., 2018) and blueberry polyphenol metabolites have been found to peak between 1 and 2 hours and again at 6 hours (Rodriguez-Mateos et al., 2013). Philip et al. (2019) measured FMD to assess pre and post intervention changes. FMD readings were not taken concurrently with cognitive testing such that causal links between FMD (endothelial function and vasodilation) cannot be made. Philip et al. (2019) in fact found no effect of PEGB on FMD and explained this may have been due to mental fatigue induced by the task battery which can reduce vasodilation (Ghiadoni et al., 2000). Further, FMD was measured outside of known peaks in polyphenol metabolites found in plasma such that increases to blood flow may have been missed. The contribution of the individual grape and blueberry components to the cognitive effects of the combination PEGB were not assessed by Philip et al. (2019). Whilst a small number of grape interventions have been investigated, studies into the cognitive effects of blueberry treatment in young adults is sparse.

Haskell-Ramsay, Stuart, Okello, and Watson (2017) supplemented twenty young adults (18-30 years) with a drink made up of 200mL of purple grape juice and 30mL of blackcurrant cordial, compared with a sugar-matched control (200mL of white grape juice mixed with 10mL of blackcurrant cordial and 20mL of water) in a crossover design separated by 1 week washout. The purple grape drink contained 138.3mg/L of anthocyanins and the control contained 1.04mg/L of anthocyanins. Composite scores of memory accuracy, memory reaction time, attention accuracy and attention reaction times were derived from immediate and delayed word recall, numeric working memory, word recognition, picture recognition, simple reaction time, CRT and digit vigilance at 20 minutes post-intervention. Mood was assessed by Bond-Lader VAS to give scores of alertness, calmness and contentment. Reaction times were found to be significantly faster in attention tasks following purple grape juice drink in further support of flavonoid benefits to attention in young adults. Higher calm

ratings were reported following grape juice. Further cognitive changes may have been missed since testing was conducted only 20 minutes post-intervention and may have been insufficient time for peak bioavailable levels to be reached.

In contrast, a study of thirty-five young adult smokers (18 to 50 years, mean 26 years) found no improvement to performance in implicit memory tasks (word fragment completion task) and mood (Profile of Mood States questionnaire- POMS) following treatment with a grape juice intervention (Hendrickson & Mattes, 2008). The volume of grape juice consumed was calculated as 10mL/kg of body weight. Each litre of grape juice provided 2.1g of total phenolics. Cognitive testing took place at four timepoints during the day, starting with baseline before lunch and subsequent testing after lunch to assess the effects of grape juice on the post-lunch dip. The intervention was taken with lunch such that the meal itself may have influenced the cognitive performance of the study participants through elevated blood glucose levels, in turn masking the effects of the grape juice. Together these studies indicate that grape juice elicit benefits to attention in young adults, but not other cognitive domains.

Further to studies of whole grape juice, an extract of grape seed has been tested in young adults (18 – 30 years) in an acute-on-chronic parallel groups design (Bell et al., 2022). Sixty subjects were supplemented with 400mg grape seed polyphenol extract (GSPE), containing catechin, epicatechin, proanthocyanidins, and derivatives of catechin and epicatechin (epicatechin gallate) or 400mg maltodextrin in a single capsule. Cognitive performance was assessed by a battery consisting of Auditory Verbal Learning Test (AVLT), Serial Subtraction 3s and 7s, the Modified Attention Network Test (MANT), Simple and Complex Finger Tapping and the Switching Task. Self-reported mood was measured by PANAS-NOW. Limited cognitive benefits were demonstrated following GSPE treatment. Testing during the acute phase of the study completed at baseline, 2h, 4h and 6h post-intervention revealed faster Switching task reaction times in subjects receiving GSPE than those who received

placebo at 2h and 4h. The acute findings are in keeping with previous studies which have shown flavonoid benefits to psychomotor function speed. However, for several measures performance in the placebo group was better than subjects in the GSPE group.

Taken together these studies show inconsistent cognitive and mood findings between grape products which may be attributed to varying flavonoid profiles and therefore metabolism and bioavailability, as shown with blackcurrant juice and extract (Watson et al., 2015; 2018). However, the polyphenol content of the interventions in each grape study have been characterised to varying degrees of detail; anthocyanins (Haskell-Ramsay et al., 2017), total polyphenols (Hendrickson & Mattes, 2008; Bell et al., 2022), thus making direct comparisons of profiles and dose difficult. Furthermore, the food matrix varies between whole fruit and extract, to influence flavonoid absorption and subsequent bioavailability and in turn may explain differences in apparent cognitive changes.

Acute cognitive and mood effects have also been investigated following a blended berry drink. In a parallel design, Whyte, Cheng, Lamport and Williams (2019) supplemented young adults (20-30 years; mean 22.8y) with 400ml of a blended berry drink made of 75g each of whole strawberries, blueberries, blackberries and raspberries mixed with 100ml water (n = 20) or a sugar and vitamin C matched placebo (n = 20). The berry drink provided 14.3g of polyphenols (569.7mg total flavonoids; 254.6mg anthocyanins and 256.4mg proanthocyanins). Subjects completed MANT, TST and PANAS-NOW at baseline, 2h, 4h and 6h post-intervention. Benefits of the berry drink were evident for both the Switching task and MANT. Switching task reaction times were shown to improve across the day following the berry drink but not placebo. Faster MANT reaction times were observed following the berry drink than placebo. Results also showed that a decline in MANT accuracy across the day was attenuated by the berry drink. In particular for incongruent trials, accuracy was greater at 6h for subjects who received the berry drink than those who had received placebo. This result supports the

hypothesis that flavonoid effects are most apparent during situations of high cognitive load, such as more challenging trials of a cognitive task. However, no effects of the berry drink on mood were detected.

Existing research provides evidence for the positive effects of a range of flavonoid-rich foods on cognition in young adults. However, clear benefits of soya isoflavones on cognition has yet to be demonstrated in young adults. In a parallel design study, male and female young adults (mean 20.1y) were supplemented with 50g soy protein (54mg total isoflavones) or 50g whey protein control (no isoflavones) (Vanata & Metzger, 2007). After testing visual-spatial memory, word recall and word recognition tests at baseline, post-intervention assessment was completed at 105 minutes. The results showed no effects of soy treatment on measured cognitive outcomes. This indicates that soy isoflavones do not induce acute effects on memory in young adult populations. Given that benefits of flavonoids in young adults have most consistently been observed in relation to measures of psychomotor function, it is plausible that soy isoflavones may also have positive effects in this domain not assessed here, and therefore worth investigation.

Overall, acute studies in young adults have shown mixed cognitive results following flavonoid supplementation. Where positive effects have been found, these were most commonly reported for measures of attention and psychomotor function. However, attempts to demonstrate improved cognitive performance concurrently with changes in biomarkers of potential mechanisms of action have generally been unsuccessful.

1.4.2.3.3 Acute Intervention in Middle Aged Adults (40-59 years)

The current research into flavonoid effects in middle-aged adults is somewhat limited and have provided less compelling evidence than that found for children and young adults. Further to the investigations of citrus treatment in young adults by Lamport, Pal, et al. (2016), Alharbi et al. (2016) supplemented twenty-four middle-aged males (30-65y; mean 51 SD 6.6y) with 240mL of orange juice (272mg of flavonoids) and a sugar and energy matched control in a crossover study with a 2 week washout period. A 45 minute test battery comprised of Immediate Word Recall, Simple and Complex Finger Tapping, DSST, Continuous Performance Task (CPT), Serial Sevens, Contrast Sensitivity, Delayed Word Recall and Positive and Negative Affect Scale, was used to assess cognitive performance and mood at baseline plus 2h and 6h post-intervention. Change from baseline scores revealed improvements in the Continuous Performance Task at 6 hours post-consumption and fewer errors were recorded in the Finger-tapping Task at both 2 hours and 6 hours. There was a larger reduction in self-reported alertness scores following the control than orange juice, indicating maintenance of alertness by citrus flavonoids. The positive effects on psychomotor function shown here support findings by Lamport, Pal, et al. (2016) following a lower dose of total flavonoids (70.5mg) despite not replicating benefits to DSST specifically. The larger dose of citrus flavonoids tested by Alharbi et al. (2016) elicited further benefits to EF, which suggests that larger doses are required to produce effects on higher cognitive domains. Interestingly, while effects were observed during the known peak in plasma hesperidin and narirutin metabolites between 4 and 7 hours post-ingestion (Manach, Scalbert, Morand, Rémésy, & Jiménez, 2004; Mullen, Archeveque, Edwards, Matsumoto, & Crozier, 2008) benefits were also seen at 2 hours, indicating effects were apparent even at sub-peak plasma levels when coinciding with CBF increases Lamport, Pal, et al. (2016).

Whilst treatment with citrus flavonoids in middle aged adults has demonstrated acute benefits to measures of psychomotor function, EF and alertness, similar effects have not been evident following

treatment with other flavonoid-rich foods. In an acute-on-chronic parallel design study, middle-aged adults (40 to 65 years) were treated with a dark chocolate drink containing 500mg or 250mg of polyphenols or a taste, energy, caffeine and theobromine matched placebo (Pase et al., 2013. In the acute phase of the study (n = 79), cognitive performance and mood were measured at baseline, 1h, 2.5h and 4h post-intervention, according to the cognitive drug research (CDR) assessment system (comprised of Immediate Word Recall, Simple Reaction Time, Digit Vigilance, CRT, Tracking, Spatial Working Memory, Numeric Working Memory, Delayed Word Recall, Delayed Word Recognition and Delayed Picture Recognition) and Bond-Lader VAS. Composite scores were derived from results of the CDR for Quality of Working Memory, Quality of Episodic Secondary Memory, Continuity of Attention, Speed of Memory and Power of Attention. There were no significant differences in performance in the cognitive test battery and mood scores across the interventions and across acute time points.

The low dose chocolate drink (250g polyphenols) tested by Pase et al. (2013) provided a lower dose of CF than those which have shown positive effects on cognitive performance in young adults (between 373.5mg and 773mg) (Karabay et al., 2018; Field et al,. 2011; Scholey et al., 2010). This could indicate 250mg polyphenols was insufficient to elicit acute changes to cognition and mood. However, the high dose chocolate drink (500mg polyphenols) lies within the range in which effects are seen and therefore might be expected. Although ceiling effects were not specifically reported, Pase et al. (2013) acknowledge that the study subjects were highly educated such that the test battery may have been insufficiently challenging to detect acute treatment effects to explain the absence of positive results, as evidenced by Whyte at al. (2016; 2017) and Elsabagh, Hartley, Ali, et al. (2005).

Null findings in relation to cognitive and mood outcomes in middle aged adults have also been reported by Bondonno et al. (2014). In a crossover design with a washout of 1 week, thirty

participants (mean age 47.3 years) were treated with apple and control, to investigate the effects of flavonoids on cognitive performance and nitric oxide status. The apple treatment consisted of homogenised apple skin and flesh containing 184mg quercetin and 180mg epicatechin compared to a control of blended apple flesh only (less than 5mg of quercetin and epicatechin). Two doses of these were consumed 4 hours apart: with breakfast and with lunch. At 150 minutes post-lunch, a 20 minute CDR battery comprised of word presentation, simple reaction time, digit vigilance, CRT, spatial working memory, numeric working memory and delayed word recognition, and Bond-Lader VAS were administered. Nitrates and nitrites were tested in saliva at 120 minutes and in plasma at 140 minutes after lunch. Urinary samples were also collected throughout the test day (8 hours). Raised plasma nitrates compared to control were detected following apple treatment, indicating vasodilatory effects which corroborate earlier FMD and blood pressure results published by Bondonno et al. (2012) . However, these changes in physiological biomarkers did not translate into behavioural effects. The results showed no effect of treatment on cognitive and mood measures. This was possibly due to a relatively short test battery that was not challenging enough for a treatment effect to become apparent.

Modulation of vascular function by flavonoids has been further demonstrated in middle aged adults in the absence of cognitive effects following tart cherry treatment. Twenty-seven middle-aged adults (45 to 60 years) were treated with 60ml of Montmorency tart cherry concentrate (MC) (68mg cyanidin/l) in water and a matched placebo (fruit-flavoured cordial) separated by 14 days in a crossover design study (Keane, Haskell-Ramsay, Veasey, & Howatson, 2016). Cognitive performance was assessed by Computerised Mental Performance Assessment System (COMPASS) comprised of two repetitions of a 9 minute battery (DV, Stroop and RVIP) and VAS of task difficulty and fatigue were administered at baseline, 1h, 2h, 3h and 5h post-intervention. Cerebrovascular response (NIRS and transcranial Doppler) and BP were also monitored. Acute supplementation with MC did not show significant effects on digit vigilance, Stroop, RVIP or mood despite significant reductions in SBP

at 1h, 2h and 3h following MC and improved cerebral oxygenation. NIRS revealed higher concentrations of oxygenated haemoglobin (Hb) at rest between 30 and 40 minutes post-intervention, as well as during task performance at 1h post-intervention following MC compared to placebo. Higher concentrations of total Hb were also found during task performance at 1h post-intervention. Transcranial doppler results showed no effect of MC treatment on cerebral blood flow velocity. Positive cognitive effects have been shown following acute berry treatment and are attributed to their anthocyanin content, for example 127mg WBB anthocyanins (Whyte et al., 2016) and 254mg anthocyanins from mixed berries (Whyte et al., 2019). The anthocyanin dose tested by Keane et al. (2016) (73.5mg of cyanidin) is therefore low in comparison and may have been too low to elicit effects on measured cognitive outcomes. Further, taking into account findings of cognitive effects apparent in demanding situations (Whyte et al., 2016; 2017; Elsabagh, Hartley, Ali, et al., 2005; Ong et al., 2016) it is likely the short test battery (18 minutes duration) employed by Keane et al. (2016) did not challenge participants at a level for cognitive effects to manifest. Together, the studies by Bondonno et al. (2014) and Keane et al. (2016) demonstrate that acute doses of flavonoid-rich interventions can modulate components of vascular function in middle-aged adults. Although this may underpin cognitive performance, it is detectable in the absence of measurable cognitive improvements and echoes findings in young adults by Decroix et al. (2016).

In this limited number of studies the effects of a small range of flavonoid-rich foods on cognition in middle-aged adults have been examined. Overall there is insufficient evidence for positive effects of acute flavonoid treatment in this age group. Careful consideration of task difficulty and flavonoid dose should be integral to future studies to maximise opportunities to detect cognitive effects.

1.4.2.3.4 Acute Intervention in Older Adults (60 years and over)

Much like the acute flavonoid-cognition studies in children, the acute studies in older adults have focused on the effects of berry flavonoids. Bell and Williams (2019) compared three doses of haskap berry extract (freeze-dried powder) containing 100mg, 200mg and 400mg of anthocyanins, and a sugar-matched placebo. In a pilot study, twenty older adults (62 to 81 years; mean 70.5 years) received the interventions according to a crossover design with 1 week washout. Cognitive performance was measured by Serial 3s and 7s, ANT and AVLT, and mood by PANAS-NOW at baseline and 1.5h post-intervention. The effects of haskap berry on working memory and EF were mixed, with both beneficial and detrimental effects on Serial 3s and Serial 7s performance. Surprisingly, lower alertness scores were reported with both the higher doses which is contrary to previous findings of alerting effects with flavonoid treatment (Kennedy et al., 2002; Alharbi et al., 2016). Positive EM effects were revealed as better word recall performance following both 200mg and 400mg of haskap berry anthocyanins, and improvements to word recognition accuracy following the 400mg dose compared to the control. No effects on EM were found with 100mg haskap berry anthocyanins, which indicates that these benefits are dose dependent. Indeed, 100mg anthocyanins falls below typical experimental doses used in berry cognitive research. Further, reductions in DBP recorded alongside cognitive effects following 400mg anthocyanins are in keeping with observations in young adults following ginkgo biloba (Ong et al., 2016) and citrus flavonoids (Lamport, Pal, et al., 2016).

Dodd, Williams, Butler, and Spencer (2019) further demonstrated EM effects following acute berry anthocyanins in older adults. In another small study, eighteen older adults aged between 60 and 75 years (mean 68.7y) were supplemented with a high bush blueberry (BB) drink (508mg anthocyanins and 71mg pro-cyanidins) and a sugar and vitamin C matched placebo in a crossover design. A comprehensive cognitive test battery consisting of Go-NoGo, Stroop, Digit Switch, Continuous

Performance Task, Digit Symbol Substitution Test, Random Word Generation, Three-Word Sets Task, N-back, Letter memory, Location Task, immediate and delayed recall and recognition was used to assess EF, memory and to derive a combined score of global cognitive function. The test battery was administered at baseline, 2h and 5h post-intervention. Measures of plasma BDNF, BP and arterial stiffness by digital volume pulse were recorded at baseline and 1h after intervention consumption. Global cognitive function scores revealed improvements from baseline following BB at 2h and 5h, compared with a drop in performance at 2h following placebo. This result suggests that BB consumption can facilitate maintenance of cognitive performance throughout the day and attenuate the cognitive decline observed following placebo. Individual test performance indicate that this was driven by improved word recognition. The EM effects support the findings in children by Whyte et al. (2015; 2016; 2020) and Barfoot et al. (2019). Similar to the methodology employed by Whyte et al. (2015), Dodd et al. (2019) also used semi-skimmed milk as the vehicle for the interventions. As already discussed, this may have reduced anthocyanin absorption and in turn reduced the bioavailable levels of active metabolites such that improvements were only apparent in 1 out of 14 individual tests. Attenuation of systolic blood pressure (SBP) increases from baseline and reductions in plasma BDNF concentrations by BB did not reach statistical significance. Whilst this trend supports previous findings which have shown that berry flavonoids can modulate cardiovascular function and interact with cell signalling pathways, in the absence of direct measurements of CBF here, it is not possible to make conclusions around the mechanistic links between these observations and the cognitive effects shown.

In older adults, investigations of the acute effects of flavonoids on cognition have been limited to those derived from berries which have shown promise for benefits to EM in particular. This mirrors the EM effects of berry flavonoids demonstrated in child populations. As observed in a review by Bell, Lamport, Butler, and Williams (2015), children and older adults may be more susceptible to EM

changes by way of lower performance during EM development in children in contrast with EM decline in older adults.

1.4.2.4 Chronic Studies

Investigations into the medium- and longer-term effects of flavonoids on cognition have been conducted in both healthy populations as well as those experiencing cognitive deficits. Key research studies on flavonoid supplementation beyond 24 hours duration are presented in the following section.

1.4.2.4.1 Chronic Intervention in Children

To date, only one study has examined the chronic effects of flavonoid treatment on cognitive performance in children. In a pilot study, Barfoot et al. (2021) used a parallel design in which fifteen subjects aged between 7 and 10 years consumed 200mL daily of WBB drink (253mg anthocyanins) or a sugar and vitamin C-matched placebo for 4 weeks. Cognitive performance and mood were assessed at baseline prior to intervention, at 2 weeks and 4 weeks by MANT, RAVLT and the Positive and Negative Affect Schedule for Children (PANAS-C). Significantly greater accuracy scores were found on medium load trials of MANT for the WBB group compared to the placebo group. Similar results were shown for high load trials which trended towards significance. WBB-treated subjects also performed more accurately on incongruent trials of MANT relative to the placebo group. Improved accuracy was maintained across trials of increased difficulty following WBB, in that poorer accuracy was observed for incongruent trials compared to congruent trials and lower accuracy scores were recorded for faster 120ms trials than slower 500ms trials in the placebo group, whereas no differences were found in WBB-treated subjects and were seemingly untroubled by the increased difficulty these trials. Furthermore, subjects who received placebo performed more slowly in high

load incongruent trials than in medium load incongruent trials of MANT. In comparison, reaction times were not affected by load in the WBB group. These results further demonstrate WBB benefits in MANT trials of greater cognitive demand (Whyte et al., 2017; Barfoot et al., 2019). This study extends the research into the effects of blueberry treatment in 7- to 10-year-olds by Whyte et al. (2015; 2016; 2017; 2020) and Barfoot et al. (2019) which to date have all been acute trials investigating effects in the immediate post-dosing period. In particular consistent findings of EF effects following acute WBB treatment (Whyte et al., 2016; 2017; 2020) and Barfoot et al. (2019) as well as chronic (Barfoot et al., 2021) with the same anthocyanin dose (253mg) indicate that these cognitive outcomes are robust. Despite evidence of benefits to EM following WBB supplementation in larger acute studies, the same effects were not shown following chronic treatment in children (Barfoot et al., 2021).

As a measure of the effects of WBB treatment on circulating polyphenol metabolites coinciding with measures of cognitive effects, 24-hour urine samples were collected prior to each test visit. At 2 weeks significantly higher concentrations of hippuric acid were detected in the WBB group compared to placebo. Together with previously observed increases in plasma hippuric acid following between 4 and 12 weeks berry treatment (Rodriguez-Mateos et al., 2019), Barfoot et al. (2021) suggest that hippuric acid is a candidate metabolite of interest in relation to the cognitive benefits of flavonoids. However, significant differences in urinary hippuric acid between treatment groups were not detected at 4 weeks which was attributed to inter-individual variation in polyphenol metabolism and polyphenol consumption external to the study intervention permitted under a low-flavonoid diet.

These results are promising but are however limited to the examination of blueberry effects in child populations. No other flavonoid-rich foods have been investigated in chronic repeat dose studies in children.

1.4.2.4.2 Chronic Interventions in Young Adults (18 – 39 years)

In contrast to the acute flavonoid-cognition RCTs in young adults which overall have shown positive associations between flavonoid consumption and short-term improvements to cognitive performance, the outcomes of chronic studies have been less compelling. In a short study, Moulton, Boyko, Fitzpatrick, and Petros (2001) supplemented sixty young males (mean age 20 years) with either two 60 mg tablets of Ginkgo biloba extract (GBE) containing 27% flavonoid glycosides and 7% terpenoids daily or placebo (tablet excipients) for 5 days. Blood pressure and heart rate readings taken on all five days of the study showed no differences between the GBE-treated group and placebo group. Benefits of GBE to memory were shown to be limited to fewer errors for probe present word set size 2 trials of the Sternberg memory scanning test when subjects are required to memorise a set of 2 digits. In fact, the placebo group performed better in the more difficult probe present word set size 4 trials when required to memorise a set of 4 digits. No further treatment effects were found for a reaction time control test, the vocabulary and digit span subtests of the Wechsler Adult Intelligence scale revised (WAIS-R), reading span test and prose recall test. The overall absence of positive effects on memory following GBE by Moulton et al. (2001) could be attributed to the short study duration, in that changes to memory performance may require longer treatment periods to be detectable. Interestingly, Ong et al. (2016) found GBE cognitive effects to be gender-specific to female subjects. A sample of male subjects only may have contributed to the lack of positive outcomes found by Moulton et al. (2001). It is worth noting that pre-intervention measures of cognitive performance were not recorded, this means baseline differences between the intervention groups may have existed and have not been accounted for nor considered in the authors' interpretation of the results. Further, cognitive testing took place on the final day of treatment at 2 hours post-intervention, such that the observed outcomes are likely to have been acute effects of GBE rather than chronic.

Similarly, Elsabagh, Hartley, Ali, et al. (2005) performed cognitive testing at between 3 to 5 hours after a final dose of GBE following 6 weeks of treatment. Thus, any effects were likely to have been due to acute treatment rather than chronic cognitive changes. This was experiment 2 of the study, in which twenty 18- to 26-year-olds were supplemented with 120mg GBE (25% flavonoids and 6% terpenoids) daily with a single tablet and twenty subjects with placebo. Cognitive performance was assessed at baseline and after 6 weeks by the Paced Auditory Serial Addition Task (PASAT), the pattern recognition memory (PRM), the spatial recognition memory (SRM) test, delayed word and picture recall, the spatial working memory (SWM) test and the Intra Dimensional/Extra Dimensional set shifting task (IDED). Self-ratings of mood were recorded using Bond-Lader VAS. No effects of treatment were demonstrated.

In a longer study by Burns, Bryan, and Nettelbeck (2006), one hundred and four adults aged between 18 and 43 years (mean 30.4 years) consumed one 40mg tablet of GBE (10.7mg Ginkgo biloba flavonoid glycosides and 2.7mg terpenoids) three times a day or a placebo matched for appearance and taste for 12 weeks. A comprehensive cognitive battery comprised of Concept-Formation, Visual Matching, Memory for Names and Visual-Auditory Learning (from Woodcock-Johnson Psycho-Educational Battery revised), Raven's Progressive Matrices, Information, Digit Span, Picture Recognition, Digit Symbol, PASAT, the Stroop Test, Odd-man-out and Inspection time. Mood was assessed by the Profile of Mood States prior to joining the study and before the final test visit. The only outcome to show a benefit of GBE treatment was Digit Symbol, a measure of cognitive processing speed, which is in keeping with the effects shown for acute GBE treatment.

Taken together these three studies do not provide clear evidence of positive effects of regular supplementation with 120mg GBE daily on cognition in young adults, except for some evidence of benefits to psychomotor function. This is unsurprising given that acute treatment with 120mg did not elicit clear benefits to cognition and effects on attention were demonstrated at doses of 240mg

and 360mg, with further effects on EM shown with 360mg GBE. It is possible that similar doses are required for chronic effects to be evident. These studies examined the effects of GBE treatment for durations ranging from 5 days to 12 weeks, however the schedule of Moulton et al. (2001) and Elsabagh, Hartley, Ali, et al. (2005) may have reflected acute testing, and reliable conclusions around duration of longer term treatment cannot be made. In keeping with the findings of the acute studies reviewed in section 1.4.2.3.2, these three chronic studies do not provide evidence of benefits to mood following GBE supplementation in young adults, which indicates that longer treatment duration does not increase likelihood of mood effects.

A variety of other flavonoid-rich interventions have been investigated for their cognitive effects in healthy young adults for periods of up to 12 weeks. These have also failed to demonstrate positive effects on measures of cognitive performance. In a within subject crossover design study by Francis et al. (2006), female subjects aged between 18 and 30 years were supplemented with a high flavanol cocoa drink (172mg cocoa flavanols) and a low flavanol cocoa drink (13mg cocoa flavanols). Brain activation during a letter-digits pair task (a task-switching paradigm) was measured by fMRI following 5 days of treatment, with 14 days separating scans. No significant differences were found between the two CF drinks with respect to switch cost, error rate or reaction times. However, following 5 days' treatment with the high flavanol cocoa drink, increased BOLD signalling was measured during the switch condition. The increased activation occurred in task-related brain loci; the dorsolateral prefrontal cortex, parietal cortex, anterior cingulate cortex and cerebellum. Since subjects consumed the fifth dose of intervention at 1.5 hours prior to the fMRI scan, the neurological effects reported are likely to have been due to acute CF effects rather than chronic. Subsequently, the authors continued to investigate the acute effects of CF on CBF in a pilot study of four subjects. Arterial spin labelling (ASL) fMRI was measured at baseline, 2 hours, 4 hours and 6 hours following a single dose of a high flavanol cocoa drink (516mg flavanols) and a low flavanol cocoa drink (39mg flavanols) on separate test days. No significant changes in CBF were detected following the LF drink.

In contrast following the HF drink, increases to CBF reached a peak at 2 hours, returning to baseline levels by the 6 hour timepoint. This suggests that that the increase in cortical activity following CF may be due to improved oxygen delivery resulting from increased CBF. However, these physiological changes did not coincide with improved cognitive performance in the first part of the study. As detailed in Section 1.4.2.3.2, Lamport, Pal, et al. (2016) also used ASL fMRI to detect increased CBF following high flavanone supplementation. However, in both studies by Lamport, Pal, et al. (2016) and Francis et al. (2006) CBF and cognitive performance were not assessed concomitantly, thus limiting the conclusions which can be drawn regarding flavonoid mechanisms of action.

In a small study, eighteen young adults (20 to 31 years) consumed either 24g per day of dark chocolate (containing 540mg polyphenols including 34.8mg epicatechin) or white chocolate matched for energy, protein, fat and carbohydrate content for 30 days (Sumiyoshi et al., 2019). Cognitive testing at baseline, 30 days and at 3 week follow-up after the intervention period was by Stroop test and digital cancellation test. At 30 days, significant improvements in correct responses for the Stroop test were observed in the dark chocolate group relative to the white chocolate group. These improvements persisted at the 3 week follow-up despite not reaching statistical significance. For trial 3 of the digit cancellation test, increased performance by the dark chocolate group at 30 days remained significant at follow-up compared to the white chocolate group. However, prefrontal CBF measured by NIRS during cognitive testing and plasma BDNF levels showed no difference between interventions. These findings are unable to support the proposals that flavonoids act via improved CBF and modulate cell signalling via BDNF to improve attention and processing speed.

In the chronic arm of an acute-on-chronic parallel groups design study, Bell et al. (2022) supplemented sixty subjects (18 to 30 years) with 400mg grape seed polyphenol extract (GSPE) or 400mg maltodextrin in a single capsule daily for 12 weeks. Cognitive performance was assessed by Auditory Verbal Learning Test (AVLT), Serial Subtraction 3s and 7s, the Modified Attention Network

Test (MANT), Simple and Complex Finger Tapping and the Switching Task at baseline, 6 weeks and 12 weeks. Self-reported mood was measured by PANAS-NOW and mental fatigue by a nine-point Likert scale. GSPE was found to maintain performance from week 6 to week 12 in the Simple Finger Tapping task, compared to a significant decline in performance following placebo. This is in keeping with the findings of acute flavonoid-cognition studies in young adults which commonly show benefits to psychomotor function and attention. However, no other benefits to cognitive outcomes were shown following 12 weeks of GSPE treatment.

Interestingly, positive effects on cognitive performance in young adults were found by Mohamed, Lee Ming, and Jaffri (2013) who examined the effects of catechin-rich oil palm leaf extract (OPLE) on cognition in young adults using a parallel groups design. Thirty 22 to 24 year olds consumed either one 500mg capsule of OPLE (22.5mg of polyphenols) daily for 2 months or a placebo capsule. At baseline, 1 month and 2 months a picture recall task was used to assess short term memory, processing speed was measured by letter-matching task and paired-associative recall task, and the Card Rotations test assessed spatial visualisation learning. At 1 month, short term memory was found to be significantly improved following OPLE relative to baseline and placebo. This benefit was sustained at 2 months with further improvements to processing speed and spatial visualisation. More positive effects on cognitive performance were observed with longer duration of OPLE supplementation which may be due to accumulation of flavanols and their metabolites. This is speculative and would require measures of plasma or urinary concentrations to confirm.

Together these studies show that there is little evidence of positive effects, other than for psychomotor function, of longer term flavonoid treatment on cognitive performance in healthy young adults. Null findings in this population may be explained in that these subjects are at their cognitive peak. One interpretation is that there is therefore limited scope for improvement with flavonoid treatment or that subjects are performing at ceiling in the cognitive tasks, such that testing

is insufficiently sensitive to any cognitive changes. This limitation may be further exacerbated in that most studies have recruited university students who are likely to perform above average in cognitive assessments. Few studies have investigated the effects of flavonoids in young adults with cognitive deficits or delayed cognitive development.

One such study was conducted by de la Torre et al. (2016), in which 87 young adults (18 to 34 years) with Down's syndrome received three sessions of cognitive training plus either green tea extract or placebo (rice flour) in capsule form. The dose of green tea extract was calculated to provide epigallocatechin-3-gallate (EGCG) 9mg/kg body weight. Cognitive performance was assessed at baseline, 3 months, 6 months and 12 months, according to Motor Screening Test (MOT), Simple Reaction Time task (SRT) the Spatial Span forward recall (SSP), Digit Span forward recall, Paired Associates Learning (PAL), the Pattern Recognition Memory test (PRM), Cued Recall Test (CRT), the semantic fluency word generation task, the SSP backward recall and the Digit Span backward recall, Tower of London, Weigl Color-Form Sort Test, the Cats and Dogs Test, the Boston Naming Test and the Token Test. At baseline, 6 months and 12 months fMRI was used to assess resting state connectivity in a sub-group of 20 subjects. At 12 months, EGCG-treated subjects showed a preservation of accuracy in PRM immediate recall where a significantly greater decline in correct responses was recorded for the placebo group. EGCG also improved correct responses and reaction times for the Cats and Dogs Test compared to placebo. These outcomes provide evidence of the positive effects of EGCG on EM and EF in young adults with Down's syndrome. Subjects with Down's syndrome may represent a population which is more susceptible to EM and EF changes akin to EM development during childhood and EM decline in older adults in which benefits of flavonoids to EM have been shown. It is also worth noting that subjects underwent a much longer period of supplementation in this study relative to other studies. As proposed by Mohamed et al. (2013), cognitive effects may be more apparent following longer periods of intervention. The fMRI scans revealed EGCG was associated with increased connectivity in the frontal, somatosensory and

occipito-temporal cortices, with significant interactions in the posterior cingulate cortex, precuneus and cerebellum at 6 months. At 12 months only changes in the frontal cortex achieved statistical significance. Despite observed increases in brain activation, measurements were not taken concurrently with cognitive testing and therefore the relationship between increased connectivity and behavioural outcomes remains unclear.

Overall, chronic flavonoid supplementation in young adults has not provided clear evidence for positive effects on cognitive outcomes. Whilst it may appear that the acute effects in this age group are not sustained after medium to longer term treatment, attention should be given to the methodology employed by future studies. In particular, when assessing chronic outcomes there should be careful scheduling of testing in relation to consumption of the final dose of the intervention to eliminate potential acute effects, cognitive tests selected should be of an appropriate difficulty to avoid ceiling effects and sufficiently sensitive to detect potentially small changes in performance.

1.4.2.4.3 Chronic Interventions in Middle Aged Adults

Cognitive changes, including decline in executive functioning, has shown to commence in middle age even in the absence of pathological signs and symptoms. This population is therefore of interest in relation to the search for interventions for the prevention of neurocognitive disease with long prodromal periods in later life.

Cieza, Maier, and Pöppel (2003) recruited 50 to 65 year olds onto a 4 week intervention of either 120mg GBE twice daily (24% flavonoid glycosides and 6% terpene lactones) or placebo, both as tablets. Cognitive performance was assessed by Increment threshold for Visual Stimuli test (ITVS), Digit connection test–G (DCT-G), Word list test (WL), Finger tapping test, Auditory choice reaction

time (ART), Colour word test (CWT), Incidental learning test (ILT), Auditory order threshold test (AOT), Temporal reproduction test (TR) and Sensorimotor synchronisation test (SMS), at baseline and 4 weeks. Mood was also measured using POMS, subjective intensity scale-mood (SIS-M) and self-rating depression scale (SDS) at baseline then weekly. Benefits of GBE were revealed for reaction times in the Finger tapping test and ART relative to placebo. These findings demonstrate improvements to psychomotor function following chronic GBE treatment, in-keeping with GBE treatment of young adults in acute studies by Kennedy et al. (2000; 2002; 2007) and Elsabagh, Hartley, Ali, et al. (2005) and a chronic study by Burns et al. (2006). Mood was shown to improve as measured by SIS-M, in which changes at 2 weeks were particularly pronounced. However, GBE showed no effect on memory performance.

The effects of GBE have been investigated in postmenopausal women, a population in which complaints of memory and concentration difficulties are often reported (Hogervorst et al., 2022). Hartley, Heinze, Elsabagh and File (2003) supplemented thirty-four post-menopausal women aged between 53 and 65 years with either 120mg daily of GBE (25% Ginkgo flavonoids and 6% terpenoids) or a placebo tablet for one week. Cognitive assessment was completed at baseline and after 7 days of treatment. Subjects completed PASAT, Immediate and delayed paragraph recall test, Delayed Matching-to-sample (DMTS), Picture recall task, Stockings of Cambridge task and IDED. Self-rated mood and sleepiness scores were determined using Bond-lader VAS, Stanford sleepiness scale (before and after cognitive battery) and Epworth sleepiness scale (after cognitive battery). Improvements relative to baseline and placebo were revealed for performance in DMTS, in the slower 1.6s trials of PASAT and accuracy in Stage 2 of IDED. This suggests that sub-chronic GBE treatment can improve EM, sustained attention and aspects of mental flexibility. GBE supplementation was also associated with faster completion times for Stage 1 of IDED. However, these results were confounded by baseline scores such that the true effect of GBE treatment may be minimal. No effects of GBE were detected for mood and sleepiness.

As a follow-up to this study, Elsabagh, Hartley, and File (2005) treated post-menopausal women (51 to 67 years) with the same GBE as Hartley et al. (2003) for a period of 6 weeks. Cognitive decline increases with time post-menopause, in order to investigate any differential effects based on post-menopausal status subjects were grouped into Stage 1 menopause (within 5 years of last menstrual period) and Stage 2 menopause (more than 5 years since last menstrual period). Cognitive performance was assessed at baseline and after 6 weeks' intervention using a test battery comprised of PASAT, immediate and delayed paragraph recall test, DMTS, picture recall task, category generation, Stockings of Cambridge task and IDED. Mood and sleepiness were scored as per Hartley et al. (2003). GBE treatment was shown to have no effect on postmenopausal women overall. However, for Stage 2 menopausal women better performance was demonstrated in that fewer trials were required to complete the task and fewer errors were recorded in the GBE group compared to the placebo group. This effect was not seen in Stage 1 menopausal women who demonstrated better performance in IDED than Stage 2 menopausal women at baseline. This indicates that GBE benefits are more likely to be evident in subjects with greater cognitive decline. However, in this study it was not possible to distinguish poorer baseline performance due to menopausal status from cognitive decline due to ageing.

Taken together the results of the studies by Hartley et al. (2003) and Elsabagh, Hartley, and File (2005) show that the positive effects of GBE for mental flexibility were sustained from 7 days to 6 weeks of treatment in a subgroup of Stage 2 menopausal women. However, the improvements to EM and sustained attention were not sustained. Elsabagh, Hartley, and File (2005) suggest that this may be due to tolerance to GBE intervention in that prolonged repeated administration is no longer effective. This would mean cognitive domains are differentially influenced by tolerance, the plausibility of this notion therefore requires further exploration. In keeping with Hartley et al. (2003), no effects of GBE on mood or sleepiness were revealed. This is in contrast to the positive findings of Cieza et al. (2003) which may be explained by the different sensitivity of the instruments used. It

should be noted that both Hartley et al. (2003) and Elsabagh, Hartley, and File (2005) conducted cognitive testing between 4 and 5 hours post intervention consumption. Therefore the cognitive outcomes found are likely to be due to acute GBE effects rather than chronic. This limitation to GBE research has been previously noted in this thesis and highlights the need to interpret these results with caution.

In a large parallel design study, R. Kaschel (2011) supplemented one-hundred and eighty-eight male and female adults (45 to 56 years) with either 240mg GBE (24% flavonoid glycosides and 6% terpene lactones) or placebo tablets for 6 weeks. Measurements at baseline and after 6 weeks' intervention with GBE showed benefits to performance in the more complex Appointments test, with no GBE effect on the simple Driving route film test. This supports the findings of Whyte et al. (2016; 2017), Elsabagh, Hartley, Ali, et al. (2005) and Ong et al. (2016) following acute flavonoid supplements, where flavonoid effects were most apparent in more cognitively challenging tasks. At 6 weeks, GBE-treated subjects demonstrated superior performance in immediate and delayed recall and better ratio of incorrect to correct responses for delayed recall in the Appointments test. Subjective memory ratings, mood and well-being showed no effect of GBE treatment.

Further to investigations of the cognitive effects of GBE in postmenopausal women, the benefits of isoflavones have also received much attention. For example, Duffy, Wiseman, and File (2003) supplemented thirty-three post-menopausal women (50 to 65 years) with 60mg daily of soy IF (mean age 58.8 years) or colour-matched placebo capsules containing lactose (mean age 56.8 years) for 12 weeks. A cognitive test battery comprised of Story recall, Delayed matching to sample (DMTS), Picture recall, Category generation, IDED, SoC and PASAT was administered at baseline and 12 weeks, alongside measures of mood by Bond-Lader VAS. Change from baseline performance showed significantly improved scores in delayed picture recall and immediate story recall following soy IF compared to placebo. Reaction times were also improved by soy IF treatment for slower 1.6ms trials

of PASAT and Stage 2 of IDED in which time taken to learn rule reversal was measured. This indicates regular consumption of 60mg soy IF can improve EM, EF and attention. Further evidence supporting this was provided by Casini et al. (2006). In a crossover design study, seventy-eight post-menopausal women (mean age 50 years) were supplemented with 60mg isoflavones as aglycones daily and placebo tablets matched for appearance for 6 months, separated by a one month washout period. Subjects completed cognitive assessment at the end of each intervention arm which showed soy IF supplementation was associated with better recall of pairs in the Digit Symbol test and a greater number of digits recalled in reverse order in the Digit Span test compared to placebo, with no effects of IF on Visual scanning test. Mood and depression were scored on the Beck Depression Index (BDI), Hamilton Rating Scale for Depression, the Spielberger State-Trait Anxiety Inventory, POMS and Bond-Lader VAS. Improved mood was shown following soy IF as lower depression scores on the BDI and better scores with the VAS relative to placebo. These findings suggest that positive effects on EM, EF and attention found following 3 months of 60mg IF consumption are sustained at 6 months with further mood benefits becoming apparent.

Despite these positive outcomes following 60mg soy IF, the cognitive benefits of higher doses of IF are less clear. Santos-Galduróz, Galduróz, Facco, Hachul, and Tufik (2010) supplemented thirty-eight post-menopausal women (50 to 65 years) with either one 80mg isoflavone (IF) tablet daily (60.8mg genistein, 16mg daidzein, 3.2mg glycitein) (mean 54.5 years) or a placebo tablet (mean 56.6 years) for 4 months. Performance on the Visual-Spatial test, Digit span test Digit symbol test, Similarity test and Verbal Paired Associates test were used to assess cognitive ability and level of depression was measured by the Geriatric Depression Scale (GDS). Significantly better scores for delayed recall of semantically non-related pairs in the Verbal Paired Associates test were revealed following IF relative to placebo, demonstrating benefits of IF to EM only. In particular the IF effects on Digit Span test and Digit Symbol test found by Casini et al. (2005) were not detected by Santos-Galduróz et al. (2010). These disparate results could be explained by differing methodologies, for example, Casini et

al. (2005) tested aglycone forms of IF which are more readily absorbed and therefore more bioavailable than glycoside equivalents, such that even at a lower dose this may have been more effective at producing detectable behavioural changes.

Further to consideration of the chemical form in which soy IF are presented, Fournier, Ryan-Borchers, Robison, and Wiediger (2007) aimed to investigate the effects of consuming soy IF in the matrix of a whole food compared to isolated IFs. Seventy-nine post-menopausal women (mean age 56.1 years) were randomised to receive one of three interventions comprised of a supplement tablet and milk drink. The soy milk group consumed 353mL soy milk twice a day (72mg IF; 31mg daidzein, 37mg genistein, 4mg glycitein) plus placebo tablet (maltodextrin), the purified IF group consumed one soy IF tablet (70mg IF; 30mg daidzein, 33mg genistein, 7mg glycitein) plus 353mL of cow's milk (matched for colour and flavour to the soy milk) twice a day and the placebo group consumed 353mL cow's milk and placebo tablet twice a day for 16 weeks. Cognitive scores on the Stroop test, Digit Ordering, Pattern Recognition, Benton Visual Retention Test, Colour Match task, Forward Digit Span and Corsi Blocks task were recorded at baseline and following 16 weeks of intervention, whilst mood was assessed by BDI. Neither of the soy IF interventions were associated with positive effects on cognitive outcomes. Indeed following soy milk a greater decline in Digit Ordering was observed relative to purified IF and placebo. Although the authors suggest that these null findings may have been due to a relatively short intervention period, positive effects after 3 and 4 months of treatment have been shown (Duffy et al., 2003; Santos-Galduróz et al., 2010) which indicates that duration of IF treatment cannot fully explain the lack of effects. Further, the absence of IF benefits were found despite increased urinary concentrations of total IF following consumption of soy milk and following purified IF, relative to baseline and placebo reported in a subset of forty-five women.

Whilst habitual IF consumption was not estimated in these studies, existing research has shown typically Western diets to be lower in soy and soy products, and therefore soy IF, compared to Asian

diets (Jaceldo-Siegl, Burke, & Fraser, 2006; Messina, Nagata, & Wu, 2006), such that the subjects recruited by Duffy et al. (2003), Casini et al. (2005), Fournier et al. (2007) and Santos-Galduróz et al. (2010) are likely to have had low levels of IF intake in their background diets. In order to examine the effects of soy IF treatment in a population with high soy intake (Ho et al., 2007) recruited one-hundred and ninety-one Chinese post-menopausal women (55 to 75 years) to receive either 80mg soy IF daily in capsule form or a placebo capsule (matched for appearance) for 6 months. A cognitive test battery consisting of Hong Kong List Learning test, Rey-Osterrieth Complex Figure test, Wechsler memory scale – revised (WMS-R), TMT, Verbal fluency test, Digit span test, Digit vigilance test, Finger tapping test and Visual perception test, was administered at baseline and following 6 months' intervention. A composite score was derived from outputs from the component tests of the cognitive battery and global cognitive performance was measured by MMSE. No effects of soy IF were shown for cognitive outcomes for the complete sample, nor for subgroups according to age (65 years and younger, and over 65 years). This suggests that long term background consumption of soy IF may be more influential than short to medium term supplementation, such that cognitive protection was maintained even in the placebo group.

In middle-aged adults, the investigations into the chronic cognitive effects of soy IF other than in post-menopausal women are limited. One such study was conducted by Thorp, Sinn, Buckley, Coates, and Howe (2009) in which healthy men aged between 30 and 80 years (mean 49 years) were recruited to a two-arm crossover study. Subjects consumed four capsules per day (two in the morning and two at night) containing 116mg of soy IF (68 mg daidzein, 12 mg genistein, 36 mg glycitein) followed by placebo capsules (30mg raftilose and fibre) each for 6 weeks, or in the reverse order. Cognitive performance was recorded at baseline and following 6 weeks of treatment. This was assessed by a test battery designed to target memory, executive function and visuospatial processing composed of RAVLT, PAL, Backwards Digit Span, Letter-Number Sequencing, Novel Spatial Working Memory, Letter Fluency, TMT and mental rotation task. Improved performance

following soy IF was revealed in the spatial working memory task for overall score and each of its elements; pairs viewed, memory error and time to complete. In addition to the benefits of soy IF to EM, EF and attention found in post-menopausal women, these results provide evidence that soy IF effects extend to WM in middle-aged adults. Interestingly, Thorp et al. (2009) did not utilise a washout period between treatment phases. The pseudo half-lives of genistein and daidzein have found to be 9.2 hours and 8.2 hours respectively following a single dose of soy IF in healthy men (Busby et al., 2002) and are known to be extended with repeated dosing. This suggests that soy IF and their metabolites were likely to have remained in circulation following the termination of 6 weeks of IF supplementation, such that carryover effects into the placebo phase were possible.

In summary, these soy IF studies demonstrate benefits to EF, EM, attention and WM in middle-aged adults, which appear to be influenced by interindividual characteristics, such as gender (WM apparent in men but not women populations) and habitual intake and treatment characteristics, including chemical form, duration, food matrix and dose. Indeed, most cognitive benefits were demonstrated following a dose of 60mg soy IF, above which positive effects were less apparent. This could indicate a non-linear dose response where increased doses are less favourable which would require specific dose-response examination.

Direct comparisons of chronic flavonoid doses have been investigated with cocoa interventions by M. P. Pase et al. (2013) and D. Camfield et al. (2012). In an acute-on-chronic parallel design study, middle-aged adults (40 to 65 years) were treated with a dark chocolate drink containing 500mg or 250mg of polyphenols or a taste, energy, caffeine and theobromine matched placebo (Pase et al. 2013). In the chronic phase of the study (n = 72), cognitive performance and mood were measured at baseline and following 30 days' treatment according to the cognitive drug research (CDR) assessment system (comprised of Immediate Word Recall, Simple Reaction Time, Digit Vigilance, CRT, Tracking, Spatial Working Memory, Numeric Working Memory, Delayed Word Recall, Delayed

Word Recognition and Delayed Picture Recognition) and Bond-Lader VAS. Composite scores were derived from results of the CDR for Quality of Working Memory, Quality of Episodic Secondary Memory, Continuity of Attention, Speed of Memory and Power of Attention. No effects on cognitive outcomes were found following 30 days of either CF treatment compared to placebo. Self-rated scores of calmness and contentedness showed significant increases from baseline following the high dose chocolate drink. No effect on mood following low dose chocolate drink and placebo, could suggest that higher doses are needed for mood effects to be measurable.

Camfield et al. (2012) adopted a parallel groups design to investigate the effects of 30 days of CF supplementation on neural activity during cognitive testing. Sixty-three subjects aged between 40 and 65 years (mean age 52.3 years) were randomised to receive one of three dark chocolate drinks containing 500mg CF, 250mg CF or placebo (trace CF content). Steady state probe topography (SST) was used to record changes in phase and amplitude of steady state visually-evoked potential (SSVEP) as a measure of neural activity during a spatial working memory task at baseline and following 30 days of CF. There were no effects of CF on spatial working memory (accuracy and reaction times) relative to baseline and between doses. SSVEP amplitude in the posterior parietal region showed a significant increase following placebo compared to reductions following 250mg and 500mg CF. This indicates reduced neural activity following CF treatment. Previously, increased posterior parietal SSVEP amplitude was found to be associated with increased task difficulty, leading the authors to conclude that subjects in the 250mg and 500mg CF groups did not find the task as challenging as the placebo group. Less activity was also detected in fronto-central regions, where less SSVEP phase advance was measured following 250mg and 500mg compared to placebo. In the absence of behavioural differences between treatment groups, the lower brain activity was interpreted as the CF groups showing improved neural efficiency.

In a parallel groups design study, Brickman et al. (2014) examined the effects of two different doses of CF on cognition following a longer period of 3 months treatment. Subjects aged between 50 and 69 years (mean age 57 years) were randomised to receive either 900mg CF or 45mg CF. A modified version of the Benton Visual Retention test (ModBent) was developed to avoid ceiling effects in this healthy sample. Testing at baseline and 3 months showed significantly improved reaction times relative to placebo, indicating benefits to spatial recognition. However, no effects were shown for delayed retention. Further, measures of cerebral blood volume (CBV) in the dentate gyrus (DG) by CBV-fMRI revealed increased CBV following 900mg CF. The DG is of particular interest because of known DG-dependent cognitive ageing compared with more general hippocampal dysfunction found in AD. Cerebral blood volume is modified by blood flow and capillary density. The increased CBV shown here suggests that CF consumption may act by improvements to CBF or capillary density or both.

Collectively these three studies indicate that CF doses of 250mg and 500mg do not elicit improvements to cognitive performance when consumed for 30 days. CF treatment at higher doses including 900mg, for longer periods may be required for benefits to spatial recognition speed to become apparent. Interestingly, this is contrast to the doses at which acute effects of CF have been observed (373.5mg and 773mg CF) and specifically doses of approximately 900mg were found to have no acute benefits to cognition.

Further evidence for flavonoid benefits to spatial memory in middle-aged adults was demonstrated by Lamport, Lawton, et al. (2016). Mothers of pre-teen children were identified as a group of adults with a stressful lifestyle which negatively impacts cognition and blood pressure. The cognitive effects of daily Concord grape juice (CGJ) consumption was investigated in twenty-five healthy working mothers aged between 40 and 50 years (mean 43.2 years). Subjects consumed 355mL of concord grape juice (777mg total polyphenols including 167 mg anthocyanins and 334 mg proanthocyanidins)

and an energy, taste, appearance and sugar matched placebo for 12 weeks followed by the alternate intervention after a 4 week washout. Cognitive ability was assessed using a 45-minute test battery comprised of visual verbal learning test (VVLT), visual spatial learning test (VSLT), rapid visual information processing (RVIP), Grooved Pegboard and Tower of Hanoi. Mood (measured by VAS) and blood pressure were also recorded. In a subgroup of 17 subjects, performance in a longitudinal and a lateral driving task was completed during a simulated driving sequence. Better immediate recall on all trials of VSLT were found following CGJ relative to placebo, indicating benefits of CGJ to spatial memory. Immediate recall of trial 1 to 3 of VVLT was shown to be higher when the placebo was consumed in arm 2 of the study compared to arm 1. Further for the Tower of Hanoi, faster completion times were recorded for CGJ compared to placebo in arm 1, whereas no significant differences were found in arm 2 of the study. Together, these findings suggest that the effects of CGJ were sustained to influence performance during placebo treatment. Significantly better coherence during the longitudinal tracking (car following) task was found following CGJ than placebo, demonstrating real-life benefits of regular flavonoid consumption. However, acute effects cannot be excluded from these observations since intervention drinks were consumed on the day of testing prior to commencement of the cognitive battery. No effects on mood or BP were found. The authors attributed the lack of BP effects to study subjects being healthy such that there may have been insufficient scope for a BP effect. However, as might be expected of a group of adults with stressful lifestyles, the mean baseline BP readings fall above the normal range into the elevated BP category (SBP: 122mm/Hg, DBP: 77 mm/Hg) and stage 1 hypertension category (SBP: 120 mm/Hg, DBP 81mm/Hg) for the placebo and CGJ arms respectively (Flack & Adekola, 2020). Chai, Davis, Wright, Kuczmarski, and Zhang (2018) found reductions in SBP following 12 weeks of tart cherry juice consumption in subjects with higher mean BP readings in the Stage 1 and Stage 2 hypertension categories. This suggests that in the study by Lamport, Lawton et al. (2016) BP effects may have been apparent in a subgroup of participants whose BP fell into the stage 1 hypertension category. However this analysis was not completed by the authors.

As can be seen by the studies reviewed here, investigations into the effects of chronic flavonoid treatment have been conducted in healthy middle-aged subjects and this may have influenced the predominantly null findings. One study to examine the effects of berry flavonoid consumption in an at-risk group for dementia was completed by Krikorian, Skelton, Summer, Shidler, and Sullivan (2022). In a parallel groups design study, thirty-three overweight middle-aged adults (50 to 65 years) with insulin resistance and subjective cognitive decline (SCD) were randomised to receive whole fruit freeze-fried blueberry powder (BB) equivalent to half a cup of blueberries, or a placebo powder matched for sugars, glycaemic load, appearance and taste. Subjects were recommended to take one sachet of powder in water once daily with either a morning or evening meal for 12 weeks. Cognitive performance was assessed by Controlled oral word production, CVLT and VPAL. Mood was measured by BDI and subjects reported memory problems using the Everyday Memory Questionnaire (EMQ). Metabolic measures of serum glucose, insulin and lipid profiles were recorded at baseline and following 12 weeks of intervention. Mitochondrial dysfunction is found to be widespread in AD, therefore changes in mitochondrial function in peripheral platelets were measured as an indicator of this risk factor. Blueberry treatment was associated with improved phonemic access for controlled oral word production. Fewer intrusion errors in CVLT following BB compared to placebo demonstrates the chronic benefits of flavonoid treatment to EF in this group of subjects with increased risk of developing dementia. Lower self-reported scores of forgetfulness and memory encoding difficulties following BB provide further support for the cognitive results of the objective test scores. At 12 weeks, a significant reduction in fasting insulin was detected in the BB group compared to placebo. Importantly, this was found in the absence of significant increase in blood glucose. In light of the links between insulin resistance and elevated insulin levels with neurocognitive disease development, insulin reduction could be a plausible mechanism through which flavonoids exert their cognitive effects. Interestingly the flavonoid composition of the blueberry intervention was not described in the paper.

1.4.2.4.4. Chronic Interventions in Older Adults (60 years and over)

As already described, effective pharmacological treatments for dementia are not easily accessible to patients. Recent development of monoclonal antibody therapies have shown to reduce brain amyloid burden in early AD (Van Dyck et al., 2023). However, despite showing statistically significant slowing of depreciation in CDR-SB score compared to placebo, the clinical relevance of this difference has come under question (Walsh, Merrick, Richard, Nurock, & Brayne, 2022). Furthermore, the anticipated substantial cost of this therapy, side effect profile and administration by iv infusion may be prohibitive for both patients and health services. This means the search for non-pharmacological therapies remains pertinent. Although existing epidemiological literature is suggestive of the benefits of flavonoid treatment in this patient group, evidence from intervention trials is required to confirm these effects.

In a parallel design study, Gleason et al. (2015) supplemented sixty-five Alzheimer's disease patients (mean age 76.3 years) with either 100mg soy IF (taken as one 50mg tablet twice a day) or placebo (maltodextrin and caramel food colouring) for 6 months. Despite significant increases in total plasma IF following soy IF treatment relative to baseline and placebo at 6 months, results of cognitive testing using a battery consisting of List Learning, Paragraph Recall, Benton Visual Retention test, Complex Figure Recall, phonemic fluency, animal fluency, Digit Symbol, Digit Span, Stroop Colour Word test, Mazes, Trail Making Test A and B, Complex Figure Copy and Grooved Peg Board, showed no effect of soy IF treatment after 3 and 6 months. Global cognitive function measured by MMSE and mood (scored by POMS and GDS) were also unaltered following soy IF consumption.

More positive results have been found with berries and GB extract treatment. K. Kent et al. (2017) examined the effects consuming 200mL of cherry juice (138mg anthocyanins) daily for 12 weeks compared to control apple juice (0.04mg anthocyanins) in older adults (mean age 79.8 years) with

mild-to-moderate Alzheimer's type dementia. Cognitive performance was measured at baseline, 6 weeks and 12 weeks using RAVLT, the self-ordered pointing task (SOPT), Boston naming test, TMT, digit span backwards, category and letter verbal fluency. At 6 and 12 weeks, significant improvements were found for category verbal fluency, RAVLT total words recalled, delayed recall and 20 minutes delayed recall following cherry juice treatment compared to control. No effects were found on mood measured by GDS. With respect to physiological measures, significant effects were found for BP but not inflammatory markers; CRP and IL-6. At 6 and 12 weeks significant reductions were shown in SBP in the cherry juice group compared to control. The benefits of regular cherry juice consumption to verbal fluency, short and long term memory in conjunction with improved SBP in AD patients are suggestive of vasodilatory effects of anthocyanins as a common mechanism underpinning these observations. However, this study employed a relatively small sample size and more importantly only treated patients for a short duration. It would be prudent to examine the effects of extended intervention periods to understand if the benefits to cognitive performance following cherry juice are sustained.

In a large parallel groups design study by Ihl et al. (2011), four-hundred and ten patients (mean age 65 years) with mild or moderate dementia (all types) with neuropsychiatric features were recruited and randomised to receive either 240mg GBE (EGb 761) in a once daily formulation or placebo for 24 weeks. Cognitive performance was measured by The Syndrom-Kurz test (SKT) and Verbal Fluency test at baseline, 12 weeks and 24 weeks. However, only the results following 24 weeks treatment are reported in the paper. Statistically significant improvements in SKT and Verbal Fluency were found following 240mg GBE. Importantly, the size of this improvement in SKT is comparable to that achieved by drug treatment with cholinesterase inhibitors, meaning these findings are of clinical significance. Herrschaft et al. (2012) successfully replicated these results in a follow-up which adopted the same methodology in a multi-centre trial. Four-hundred and ten patients (mean age 65 years) with mild or moderate dementia were recruited to the study. Following 24 weeks of

intervention, 240mg was again associated with greater improvements in SKT and Verbal Fluency, indicating robustness of these findings. Interestingly, Ihl et al. (2011) and Herrschaft et al. (2012) included dementia patients with neuropsychiatric symptoms, in contrast Gleason et al. (2015) and Kent et al. (2017) excluded patients with neuropsychiatric symptoms. However, these are often co-presented in dementia, which could limit the ecological validity of findings by Gleason et al. (2015) and Kent et al. (2017).

Further to the studies in dementia patients by Ihl et al. (2011) and Herrschaft et al. (2012), Gavrilova et al. (2014) extended the investigations of GBE to MCI patients. One-hundred and sixty MCI patients (mean age 64 years) were randomised to receive either 240mg GBE for 24 weeks or placebo. Measurements at baseline and 24 weeks, showed significant improvements in TMT-A and TMT-B reaction times as well as improved scores in the State-Trait Anxiety Inventory following GBE compared to placebo. No effects of treatment were observed in relation to GDS scores. Improved TMT-A and B reaction times were also evident following CF in MCI patients. Desideri et al. (2012) conducted a parallel-arm study, in which ninety subjects with MCI consumed either a high, intermediate or low-flavonoid-containing cocoa drink (990mg, 520mg and 45mg of CF respectively) for eight weeks. Cognitive assessment by MMSE, TMT-A and TMT-B and verbal fluency task, was completed at baseline and 8 weeks. A combined composite score was derived from individual test results. Improved reaction times for TMT-A and TMT-B were found following the 990mg and 520mg CF drinks compared to the 45mg CF drink. The 990mg CF drink was also associated with better Verbal fluency than the 45mg CF drink. Measures of BP showed significant reductions in SBP and DBP following 990mg CF and 520mg CF drinks, similar BP effects were not found following 45mg CF. Similarly, significant reductions in plasma glucose concentrations and HOMA-IR were revealed for subjects who received 990mg CF and 520mg and not for those in the 45mg CF group. These findings demonstrate significant improvements in measures of working memory, executive function and

processing speed in MCI patients concurrently with benefits to BP and glucose concentrations and therefore in keeping with proposed mechanisms of vasodilatory and glucose metabolism effects.

Indeed, the role of glucose metabolism and insulin resistance in flavonoid effects in MCI patients was investigated by Krikorian, Nash, Shidler, Shukitt-Hale, and Joseph (2010). In a small study, twelve subjects with MCI (mean age 78.2 years) were supplemented either with concord grape juice (CGJ) (6-9 mL juice per kg body weight) daily for twelve weeks or placebo (matched for appearance, taste, carbohydrates and energy). The juice was consumed in divided doses with morning, midday and evening meals. Cognitive performance at baseline and 12 weeks was measured by California Verbal learning test (CVLT) and Spatial Paired Associate Learning Test. Improved item acquisition on the CVLT demonstrates CGJ can benefit memory function in MCI patients (with memory decline). No effects on mood were detected by GDS. Fasting blood samples were taken to determine serum glucose and insulin concentrations. Surprisingly, at 12 weeks elevated insulin levels found in the CGJ group are contrary to the beneficial effects of flavonoid treatment on glucose metabolism found elsewhere (Bell, Lamport, Butler, & Williams, 2017; Giovambattista Desideri et al., 2012; Krikorian et al., 2022). This was however a small sample and the effect of CGJ on blood glucose needs further investigation.

Krikorian et al. (2012) further demonstrated CGJ enhancement of cognitive performance in MCI patients. Twenty-one older adults with MCI (mean age 76.9 years) were randomised to receive either CGJ (6.3−7.8 mL juice per kg body weight) daily for 16 weeks or placebo beverage (matched for colour, taste, sugars and energy) in three divided doses. The Concord grape juice contained 2091mg/L total polyphenols, 425mg/L anthocyanins and 888mg/L proanthocyanidins. Performance in CVLT measured at baseline and 16 weeks showed reduced interference from non-target foils, suggesting that CGJ can enhance inhibitory control. No effects on mood were detected by GDS. A subgroup of 8 subjects, underwent fMRI scanning whilst completing the n back task which revealed

significantly increased activation of the right middle frontal cortex and marginal increase in the right superior parietal cortex. These areas are associated with WM, therefore greater activity here may explain the improved inhibitory control exhibited in CVLT following CGJ. However, behavioural changes were not detected for n-back which was completed concurrently with fMRI scanning. Interestingly, 16 weeks of CGJ treatment did not confer any preferential effects over previous findings following 12 weeks of CGJ consumption (Krikorian, Nash, et al., 2010). Indeed different CVLT results were found across the two studies (item acquisition compared with interference) which implies that these outcomes were not replicated and therefore further evidence of robustness is required.

Further neuroimaging techniques have shown increased metabolic activity in brain regions following grape supplementation in MCI patients. In a pilot study Lee, Torosyan, and Silverman (2017) treated ten MCI patients (mean age 72.2 years) with 36g of freeze-dried grape powder (178.2mg total polyphenols) in water twice a day for 6 months or placebo that was matched for appearance, flavour, smell and sugars. At 6 months FDG-PET scanning revealed treatment with grape formulation protected against significant metabolic decline from baseline following placebo in the right posterior cingulate cortex and left superior posterolateral temporal cortex, areas known to be affected in the early stages of AD. Cognitive testing at baseline and 6 months was carried out according to a battery comprised of Verbal Learning Test, BVRT, Rey-Osterreith Complex Figure Test delayed and copy, Boston Naming Test, Letter Fluency, Category Fluency, Stroop test, TMT-A, TMT-B, Wisconsin Card Sorting Test, Digital Symbol, Symbol speed, Block Design, Symbol search, Letter-Number Sequencing, Digital Span, Wechsler Test of Adult Reading, Alzheimer's Disease Assessment scale - Cognitive subscale (ADAS-Cog), MMSE, Memory Functioning Questionnaire, Hamilton Depression Rating Scale and Hamilton Anxiety Rating Scale. No effects of grape powder treatment were found for any of the cognitive measures despite the increased brain activity, which is in keeping with the findings of Francis et al. (2006). Overall, these three studies provide some evidence of memory effects and

brain activation following grape intervention in MCI patients. However, all three studies were small and larger investigations would be required to confirm the preliminary outcomes revealed here. The same limitation of small sample size applies to investigations of blueberry supplementation in MCI patients (Krikorian, Shidler, et al., 2010).

Krikorian, Shidler, et al. (2010) supplemented nine MCI patients (mean age 76.2 years) with wild blueberry juice (6-9 mL juice per kg body weight) daily for twelve weeks. The juice was consumed in divided doses with morning, midday and evening meals. VPAL, CVLT and GDS were administered at baseline and after 12 weeks. Wild blueberry juice was associated with significant improvements in VPAL cumulative learning and CVLT word list recall at 12 weeks compared to baseline. In the absence of a blueberry-matched placebo, the blueberry intervention was compared with the CGJ -matched placebo adopted in an earlier study by the same research group (Krikorian, Nash, et al., 2010). This indicated superior performance in VPAL following blueberry juice. The effects of blueberry treatment on fasting insulin and glucose did not achieve statistical significance. Krikorian et al. (2020) continued to investigate the effects of blueberry supplementation in older adults. Thirty-seven subjects with MCI (mean age 77 years) were randomised to receive 12g of freeze-dried blueberry powder twice daily (total polyphenols 401mg, 258mg anthocyanins) or an energy-matched placebo (containing purple and red colouring, blueberry flavouring, maltodextrin, fructose, glucose, and citric acid) for 16 weeks. Cognitive ability was objectively assessed by performance in TMT A and B, controlled oral word association, Hopkins verbal learning test and Spatial paired associate learning test and subjectively reported by Dysexecutive Questionnaire, at baseline and 16 weeks. Insulin resistance was estimated from measures of serum blood glucose and insulin. Urine samples after an overnight fast were taken at baseline and 8 weeks to determine levels of the anthocyanidins (cyanidin, delphinidin, malvidin, pelargonidin, peonidin, petunidin). Blueberry treatment was associated with improved performance compared to placebo in the Controlled oral word association test, specifically for semantic access where losses are characteristic of MCI and early stages of AD.

Better SPAL performance indicated benefits of BB to non-verbal memory. However, no effects of treatment were found for measures of verbal memory, which is in contrast to the positive effects found following CGJ consumption (Krikorian, Nash, et al., 2010). Further, self-perceived cognitive efficiency in daily activities was not affected by BB treatment. No intervention effects on insulin resistance were detected which suggests that, together with the negative effects of CGJ on blood glucose (Krikorian, Nash, et al., 2010), the cognitive effects observed in MCI patients following berry treatment may not be due to modulation of glucose metabolism. Although there were no significant effects of blueberry treatment on levels of total anthocyanins or individual anthocyanidins, cognitive effects were found to correlate with concentrations of parent and glycoside forms of anthocyanins (Krikorian et al., 2020). This suggests that the cognitive benefits of BB supplementation are related to regular consumption of parent compounds rather than phase 2 metabolites. However, urine samples and cognitive measures were taken at separate times, 8 and 16 weeks respectively, such that the metabolites detected may not have accurately reflected those in circulation at the time cognitive effects were observed.

The same research group went on to examine the effects of BB treatment on brain activation during cognitive testing in older adults with MCI. In a parallel groups study, Boespflug et al. (2018) treated twenty-one MCI patients (mean age 78 years) with 12.5g of freeze-dried whole fruit blueberry powder twice a day (20.37mg/g total polyphenols; 14.53mg/g anthocyanins) or 12g of a colour, taste and sugar matched placebo twice daily, for 16 weeks. Working memory was assessed by n-back task during BOLD fMRI scanning. Following 16 weeks of BB supplementation increased BOLD signal was revealed in the left inferior parietal and left pre-central gyri in the 2-back condition relative to placebo. No treatment effects were detected for the less challenging 0- and 1-back conditions. Despite evidence of increased neural activation following BB treatment, this did not coincide with significantly improved WM performance measured by n-back. Other studies that have found increased neuronal activation without associated behavioural changes following flavonoid treatment

include in young adults (Francis et al., 2006) and older adults with MCI (Krikorian et al., 2012 and Lee et al., 2017).

In summary, despite some promising outcomes for verbal fluency, short and long-term memory and attention following flavonoid-rich supplementation in dementia patients, there are too few RCTs to draw conclusions over their efficacy in improving symptoms in this group of patients. In MCI patients, the larger studies have shown consistent benefits to WM, EF and processing speed following GBE and CF supplementation, as measured by TMT-A and TMT-B which are most likely to be affected by cognitive decline. Although there is some emerging evidence of positive effects of berry treatments on cognitive ability in MCI patients, these findings have been mixed and likely due to the small sample sizes recruited. Further, measures of increased neuronal activation following berry treatment support the proposed mechanism of improved vascular function to enhance CBF and neuro-vascular supply to task-related brain regions, even in the absence of measurable behavioural changes.

There is some evidence to suggest that flavonoid benefits to cognition are not limited to older adult subjects with cognitive impairment or deficit, and that these extend to healthy ageing. As with middle-aged adults isoflavones in older adults predominantly investigated cognitive effects in post-menopausal women. For example, The SOy and Postmenopausal Health in Aging (SOPHIA) Study treated fifty-six postmenopausal women with one 55mg soy IF tablet twice daily (mean age 59.9 years) or placebo of inert ingredients which was matched for appearance (mean age 61.5 years) for 6 months (Kritz-Silverstein, Von Mühlen, Barrett-Connor, & Bressel, 2003). Performance in TMT A and B, category fluency, logical memory and recall were used to assess cognitive ability at baseline and 6 months. Daily consumption of 110mg soy IF was associated with a significantly greater improvement in category fluency relative to baseline and placebo. This demonstrates the benefits of soy IF to verbal memory.

However, these effects were not replicated in a longer study, Kreijkamp-Kaspers et al. (2004) treated two-hundred and two post-menopausal women aged between 60 and 75 years (mean 66 years) with 25.6g soy protein in powder (99 mg of isoflavones; 52 mg genistein, 41 mg daidzein, and 6 mg glycetein as aglycones) or 26.6g milk protein in powder which was matched for appearance, taste and non-IF nutrients for 12 months. Changes in cognitive performance from baseline to 12 months were measured by MMSE, RAVLT, the Digit Span forward and reversed, the Doors test, Digit Symbol Substitution and TMT A and B, Verbal Fluency and the Boston Naming Task. No effects of treatment were found for any of the cognitive outcomes. Indeed, ceiling effects were shown for RAVLT recognition and digit span. Effects of oestrogen treatment on cognition have been most noticeable in younger perimenopausal women (LeBlanc, Janowsky, Chan, & Nelson, 2001). This could explain why cognitive effects were not found in this older group of post-menopausal women by Kreijkamp-Kaspers et al. (2004) compared to those studied by Kritz-Silverstein et al. (2003). Indeed the role of age on soy IF response was investigated by sub-group analysis in the Women's Isoflavone Soy Health trial (Henderson et al., 2012). Three hundred and fifty healthy postmenopausal women aged between 45 and 92 years (mean 61 years) were randomised to receive 25 g of soy protein daily (91mg soy IF; 52 mg genistein, 36 mg of daidzein and 3 mg glycitein) or a matched placebo (milk protein) for 2.5 years. A cognitive test battery comprised of symbol digit modalities test, TMT-B, Shipley abstraction, Letter-number sequencing, block design, judgement of orientation, category fluency, Boston naming test, CVLT, East Boston memory test, Faces I (immediate recall) and Faces II (delayed recall), was used to derive scores for four factors (verbal episodic memory (list learning) factor, verbal EM (logical memory) factor, visual EM factor and Executive/expressive/visuospatial factor) and composite cognitive score. Mood was assessed with the Center for Epidemiologic Studies-Depression scale. Adherence to treatment protocol was measured by plasma and urinary genistein, daidzein, glycitein, and equol at baseline and every 6 months. The only treatment effect that was found was for the visual memory factor, where significantly greater improvements in the soy IF group was detected compared to placebo. Sub-group analysis revealed no significant effect of

age, time since menopause, type of menopause (natural or surgical) and severity of baseline vasomotor symptoms on composite cognitive score. The composite cognitive scores of regular equol producers in the soy IF group (subjects with plasma equol levels greater than 20 nmol/L at all post-intervention visits) were better than placebo but not statistically significant. Interestingly, Kreijkamp-Kaspers et al. (2004) and Henderson et al (2012) treated subjects with similar doses of soy IF protein. Effects on visual memory only became apparent after 2.5 years consumption, which suggests that 12 months intervention was insufficient duration to exert detectable effects.

Outside of post-menopausal women, Gleason et al. (2009) investigated the effects of purified soy IF on the cognitive performance of thirty-four older men and women (mean age 73.7 years). In a parallel groups design, subjects were assigned to receive 100mg soy IF daily (85% daidzein and genistein) or placebo capsules containing maltodextrin and caramel food colour for 6 months. Neurocognitive assessment was completed at baseline, 1 month, 3 months and 6 months using a task battery consisting of Buschke Selective Reminding test, Paragraph Recall, Rey Complex Figure test, Visual Spatial Learning test, Boston Naming test, category fluency, Rey Complex Figure test, Grooved Pegboard, Stroop test, Mazes and Trail Making Test B. Improved performance in Complex Figure test (immediate and delayed recall) and Grooved Pegboard were found following soy IF relative to baseline and placebo. At 3 and 6 months better Category fluency and accuracy in rejecting incorrect designs in the Visual Spatial Learning test were shown following soy IF compared to placebo. These findings demonstrate the benefits of soy IF to visuo-spatial memory, motor function and category fluency. However, placebo treatment was associated with superior performance in TMT-B, Stroop test and more accurate in recalling correct stimuli in Visual Spatial Learning test. Non-fasting blood samples were taken at baseline, 1 month and 6 months to determine plasma concentrations of genistein, daidzein and equol. These revealed large variations in the levels of circulating soy IF metabolites, indicating inter-individual differences in IF metabolism. In this particular sample, no significant equol producers were identified which the authors attributed to

age-related reduction in equol production. However this this cannot completely explain the lack of equol producers in the sample, since Gleason et al. (2015) found over 20% of subjects were equol producers in a sample of similar average age (76 years). Further, habitual IF intake was shown by FFQ to be little or none. Gleason et al. (2009) comment that this may influence metabolism of soy IF, the impact of which requires clarity from further investigation. It is also worth noting that interventions were consumed 4 hours before test visits. Whilst this standardised time elapsed between treatment and blood sampling, this does also mean that some cognitive effects may have been driven by acute mechanisms rather than chronic.

Further to studies into acute citrus interventions in young adults (Lamport, Pal, et al., 2016) and middle-aged adults (Alharbi et al., 2016, Kean et al., 2015) tested the effects of a flavanone-rich orange juice in older adults. In a crossover design study, thirty-seven men and women (mean age 67 years) consumed 500mL of high flavanone (305mg) orange juice daily for 8 weeks and a low flavanone (37mg) control, an orange-flavoured cordial which was matched for energy and sugar content, separated by a 4 week washout. Study days consisted of BP measurements before breakfast, followed by self-rated measures of mood by PANAS and cognitive testing. The cognitive battery was comprised of Go-NoGo, Consortium to Establish a Registry for Alzheimer's Disease (CERAD) (immediate and delayed), Letter Memory, Verbal Paired Associate (VPA) (immediate and delayed), Serial Sevens, Spatial Working Memory (SWM), DSST, Letter Fluency and simultaneous testing by both DSST and Letter Fluency. In addition to performance in each test, scores of EF, EM and global cognitive function were derived from the individual test outcomes. High flavanone orange juice was found to attenuate a significant decline in global cognitive function following control at 8 weeks. No effects on mood or BP were detected. Further, potential carryover effects of consuming the high flavanone orange juice were found for Go-NoGo reaction time and VPA immediate, whereby subjects performed significantly better when the high flavanone juice was consumed in the first arm of the study compared with performance when the control was consumed in the first arm.

In a large parallel groups design study, Solomon, Adams, Silver, Zimmer, and DeVeaux (2002) recruited two-hundred and thirty adults over the age of 60 years to consume either one 40mg GBE capsule three times daily (mean age 68.7 years) or a placebo capsule containing lactose (mean age 69.9 years) for 6 weeks. Cognitive assessment at baseline and 6 weeks showed no difference in outcome measures according to CVLT, Logical memory subscale and visual reproduction subscale, Digit span and Mental control of the Wechsler Memory scale (revised), Digit vigilance subscale of the Wechsler adult intelligence scale (revised), Stroop test, Controlled category fluency and Boston naming test. Interestingly, the flavonoid content or profile of the GB extract was not detailed in the paper.

A higher GBE dose was examined by Mix and Crews (2002). Two-hundred and sixty-two adults were supplemented with either one 60mg GBE three times a day (mean age 66.9 years) or placebo tablets (mean age 68.6 years) for 6 weeks. Change from baseline performance at 6 weeks showed GBE was associated with improved delayed recall and recognition in Selective Reminding Test (SRT) and delayed recognition in Faces II (WMS-III FII) subtests relative to placebo. No effects of treatment were found for Wechsler Adult Intelligence Scale-III Block Design (WAIS-III BD) and Digit Symbol-Coding (WAIS-III DS) subtests and Wechsler Memory Scale-III Faces I (WMS-III FI). These findings suggest that doses higher than 120mg GBE are required for EM effects to become apparent.

Burns et al. (2006) examined the same daily dose of GBE as Solomon et al. (2002) and extended the intervention period from 6 weeks to 12 weeks. Ninety-three healthy older adults aged between 55 and 79 years (mean 61.7 years) were randomised to receive one 40mg GBE tablet (containing 10.7mg GB flavonoid glycosides and 2.7mg ginkgolides) three times a day with meals, or a taste and appearance matched placebo. Cognitive performance in a range of sub-tests of the Woodcock-Johnson Psycho-Educational Battery (WJR) were grouped into broad categories and scores averaged;

fluid ability – assessed by Analysis-Synthesis and Concept-Formation, crystallised ability – assessed by Picture-Vocabulary and Oral Vocabulary, short-term memory – assessed by Memory for Sentences and Memory for Words, processing speed – assessed by Visual Matching and Cross Out, long term storage and retrieval – assessed by Memory for Names and Visual-Auditory Learning and delayed recall was assessed by delayed recall versions of Memory for Names and Visual-Auditory Learning. Additionally, Spot-the-Word was used to assess vocabulary, Self-Ordered Pointing for EF, speed of information processing was assessed by 'odd-man-out' for which measures of decision time and movement time were derived. Subjects were also required to rate their mood during the past week by POMS. Following 12 weeks of intervention significantly better scores were found for measures of long-term storage and retrieval in the GBE supplemented group compared to placebo. Taken together with the findings of Solomon et al. (2002), who found 120mg GBE for 6 weeks showed no cognitive effects, this suggests that longer intervention durations are required in that effects were found after 12 weeks supplementation (Burns et al., 2006). The positive effects on EM are in-keeping with Mix and Crews (2002) where a higher dose of 180mg GBE for a shorter period. The optimal dose and duration of treatment have therefore yet to be elucidated.

In a parallel design study, Santos et al. (2003) recruited forty-eight men aged between 60 and 70 years to consume one 80 mg GBE capsule daily at night or a placebo capsule for 8 months. A comprehensive task battery was comprised of tests from WAIS-R – information, digit span (forward and backward), vocabulary, arithmetic, comprehension, similarities, picture arrangement, picture completion, block design, object assembly and digit symbol, tests from WMS-R – information, orientation, mental control- time and errors, logical memory and verbal paired associates, Corsi blocks test, Rey-Osterrieth complex figure test, Wisconsin card sorting test, Toulouse-Piéron Concentrated Attention and Verbal Free Recall. At 8 months, change from baseline scores showed significant improvements to performance in the Wisconsin card sorting test, as well as measures of attention (Toulouse-Piéron Concentrated Attention - cancellation in relation to number of errors and

digit symbol) and information processing speed (mental control reaction time) following GBE compared to placebo, which coincided with increased cerebral perfusion in pre-frontal areas of the brain in GBE treated subjects relative to the placebo group measured by Single Photon Emission Computed Topography (SPECT). Further positive effects of GBE treatment were shown for change from baseline scores in measures of visuospatial ability (block design, object assembly, Corsi blocks test and Rey-Osterrieth complex figure test) relative to placebo, which were found alongside improved CBF in parietal lobe and right frontoparietal areas. These findings are supportive of the proposal that flavonoids act by improvements to CBF to exert positive effects on cognition by improved delivery of oxygen and glucose to task-related brain regions. Increased cerebral perfusion was also detected in left temporal regions associated with verbal memory following GBE treatment. However, behavioural outcomes were mixed, in that accuracy in simple verbal free recall was not affected (benefits only shown for specific types of errors - intrusions, perseverations, repetitions). In contrast, in the Verbal Paired Associates test improvements following GBE were only seen in the more challenging not semantically related pairs. These findings are in keeping with previous research that have shown cognitive benefits of flavonoid treatment to be most likely apparent in more cognitively demanding situations, including more challenging tasks. In conjunction with increases in CBF, blood viscosity measured using a rotational viscosimeter, revealed significant decreases in blood viscosity following GBE treatment compared with placebo. The authors hypothesised that increases to blood flow resulting from GBE supplementation may in turn reduce blood viscosity. However, this notion remains speculative.

In a parallel groups design study by Crews Jr, Harrison, and Wright (2008), one-hundred and one older adults (mean age 68.7 years) consumed either a 37 g dark chocolate bar (397mg cocoa proanthocyanins) and 273-ml cocoa beverage (357 mg proanthocyanins) daily for 6 weeks or a placebo bar (0.2mg/g proanthocyanins) and drink (40.87mg/g proanthocyanins) matched for appearance, scent, flavour and energy. Cognitive testing by a battery comprised of Selective

reminding test, Wechsler Memory scale III Faces I and Faces II subtests, TMT, Stroop test, Wechsler Adult Intelligence Scale III Digit Symbol-Coding subtest, Activation-Deactivation Adjective Checklist and General Activation subscale (A-DACL) were administered at baseline and 6 weeks. Serum lipid profiles, C-reactive protein (CRP) and BMI were measured at baseline and 6 weeks. At 3 weeks, an interim assessment by A-DACL and BP readings were also completed. No treatment effects were found for cognitive outcomes, lipid profile, inflammatory markers or BMI. Although subjects were required to exclude chocolate, cocoa products and flavonoid-rich foods prior to commencement of the study, cognitive effects may have been confounded by habitual flavonoid consumption. Crews et al. (2008) suggest that the null findings may have been due to the short duration of treatment. Indeed this would be in keeping with results from cocoa cognitive studies in middle-aged adults, where treatment duration of 30 days found no effects, but were apparent following 3 months of CF consumption and in healthy older adults.

In addition to the examination of inflammatory markers as indicators of flavonoid mechanisms of action, levels of BDNF and glucose metabolism have also been investigated in older adults. In a cross-over design study, Neshatdoust et al. (2016) supplemented forty older adults (62 to 75 years) with a high-flavanol (494mg) and a low-flavanol (23mg) cocoa beverage for 4 weeks with a 4 week washout period. The interventions were matched for nutrients, caffeine, theobromine and calories. Cognitive function was assessed at baseline and following each intervention arm by Go-NoGo task, Stroop, plus-minus task, trail making tasks, letter memory task, free and delayed recall, word recognition and face recognition, serial sevens task, spatial delayed recall task (SDRT), a virtual 3D radial arm maze task, word stem completion task, digital symbol substitution task (DSST) and rapid visual information processing (RVIP) task. A combined measure of global cognitive function increased significantly following 12 weeks of high-flavanol cocoa drink consumption compared to the low-flavanol drink. Importantly, the improved cognitive performance was paralleled by increase

serum concentrations of mature BDNF, which support proposals that flavonoids modulate cognitive function via BDNF-related synaptic plasticity and signalling.

Further to this, Mastroiacovo et al. (2015) successfully demonstrated positive effects of CF on cognition following an intervention period of 8 weeks. In a dose-response study, Mastroiacova et al. (2015) recruited ninety older adults (mean age 66.9 years) to consume one of three cocoa powders for reconstitution with water for 8 weeks. The three interventions differed in CF content; high flavanol (HF) (993mg), intermediate flavanol (IF) (520mg) and low flavanol (LF) (48mg) and were fully nutrient matched including similar caffeine and theobromine content. Individual test scores and a composite z score were derived from MMSE, TMT-A, TMT-B and verbal fluency test at baseline and 8 weeks. Significantly faster completion times for TMT-A and TMT-B were revealed following HF and IF relative to LF. This extends the results of CF treatment in MCI patients (Desideri et al., 2012). Greater improvements were shown in verbal fluency in the HF group compared to the IF and LF groups. HF and IF were also associated with greater improvements in composite z score relative to LF. Blood pressure readings and plasma 8-iso-prostaglandin F2α as an indicator of lipid peroxidation, fasting glucose, insulin and lipids were measured from blood samples taken at baseline and following 8 weeks of daily intervention. Significant SBP reductions were revealed in HF and IF groups compared to LF. At 8 weeks significantly reduced total 8-iso-prostaglandin F2α and lipid profiles were found following HF and IF compared to baseline and LF. Insulin resistance estimated from insulin and glucose readings showed significantly greater improvements following HF and IF relative to baseline and LF. Regression analysis found approximately 17% of the variation in composite z score was attributable to a change in insulin resistance which support the hypothesis that flavonoids influence cognitive function through changes to glucose metabolism (Bell et al., 2017, Krikorian et al., 2022 and Desideri et al., 2012).

These findings indicate that regular CF consumption may protect against age-related cognitive decline. However, non-consumers of chocolate and cocoa were selected to participate in the study and therefore naïve to CF. Given the widespread consumption of chocolate and cocoa products this potentially limits the ecological validity of the findings. Regulation of glucose metabolism as a pathway for flavonoid effects on cognition has also been investigated following berry interventions. In a crossover design study Nilsson, Salo, Plaza, and Björck (2017) forty healthy older adults consumed 600mL daily (200mL three times a day) of a mixed berry drink and placebo for 5 weeks, separated by a 5-week washout. The mixed berry intervention was a mixture of 150g blueberries, 50g blackcurrants, 50g elderberries, 50g lingonberries, 50g strawberries and 6g tomato powder, diluted with water to 600mL daily (414mg/litre anthocyanins and 155mg/litre flavonols). The placebo was matched for pH and carbohydrate content. On test days at the end of each intervention arm, Verbal WM test was administered at 30 minutes, 90 minutes and 150 minutes after breakfast, alternating with Sustained attention test at 60 minutes and 120 minutes. Better WM test performance at 30 minutes was found for the berry condition, particularly when the control drink was consumed in the first arm of the study. No effects of treatment were found for sustained attention test, however significant practice effects were observed. BP, fasting glucose, insulin, lipids, inflammatory markers and oxidative stress were measured at baseline and following 5 weeks of treatment. Total cholesterol and LDL levels were reduced in the berry condition compared to baseline and control. Significant increases from baseline fasting glucose concentrations were found following 5 weeks of the control beverage. Further, fasting insulin and subsequent insulin resistance (HOMA-IR) were significantly increased following control relative to berry intervention. This suggests that the berry intervention was able to overcome the detrimental effects of the control drink on glucose metabolism. Thus, providing support for improved glucose metabolism as a potential pathway through which flavonoids benefit cognitive function, more specifically Nilsson et al. (2017) suggest that this may be mediated via improved insulin sensitivity or insulin receptor signalling in brain. However, it should be noted that although Nilsson et al. (2017) acknowledge the potential

benefits of carotenoids to cognitive performance (Davinelli, Ali, Solfrizzi, Scapagnini, & Corbi, 2021), the carotenoid content of the interventions was not established. In particular, tomatoes are a rich source of carotenoids, in which lycopene concentration of up to 18mg per 100g of ripe fruit have been found (Martí, Roselló, & Cebolla-Cornejo, 2016). It is therefore not possible to exclude carotenoid effects from the observed WM improvements. Further, subjects were not asked to identify the order in which they received the two interventions. This would have been useful for interpretation of the findings in light of the likely obvious differences in taste and texture between the whole fruit berry drink and water-based control. Indeed, this limitation is generally applicable to studies which test whole fruit interventions.

In contrast blueberry intervention had no effect on glucose metabolism in a small study in which McNamara et al. (2018) recruited older adults aged between 67 years and 80 years (mean age 67.5 years) with subjective cognitive impairment. Nineteen subjects consumed 12.5g of whole freeze-dried blueberry powder twice daily with meals (269mg anthocyanins daily) for 24 weeks and twenty subjects consumed 12g of colour, taste and sugar-matched placebo powder twice per day. At baseline and 24 weeks, the Dysexecutive Questionnaire (DEX) was used for self-assessment of cognitive symptoms and objective cognitive ability was measured by TMT A and B, Controlled Oral Word Production and Hopkins Verbal Learning Test (HVLT). Fasting blood insulin and glucose were also measured during these test days. Urine samples following an overnight fast were collected at baseline and 12 weeks for analysis of anthocyanin content. Additionally, cognitive assessments were carried out 24 weeks following treatment cessation (48 weeks) in order to examine whether any cognitive effects were sustained. At 24 weeks, improved discrimination in recognition memory was found on the HVLT and subjects reported fewer cognitive symptoms in relation to everyday activities in the BB group compared to the placebo group. These findings demonstrate the benefits of BB to WM. At 48 weeks cognitive symptoms remained improved, however the changes to HVLT were not sustained. No effect of BB on glucose, insulin and HOMA2-IR were found. Overall, the effects of

berry interventions on glucose metabolism in cognitive studies have been mixed. Improvements were demonstrated in middle-aged adults with insulin resistance following BB treatment (Krikorian et al., 2022) and in healthy older adults following a mixed berry intervention (Nilsson et al., 2017). However, in MCI patients neither grape (Krikorian, Nash, et al., 2010) or blueberry interventions (Krikorian et al., 2020; Krikorian, Shidler, et al., 2010) have shown beneficial effects to blood glucose or insulin levels. This could reflect the extent to which insulin resistance Is associated with the pathophysiology underlying MCI. Further, the studies in MCI patients may have been insufficiently powered to detect changes in glucose metabolism due to the small sample sizes employed.

Total anthocyanin excretion was not significantly different at 12 weeks compared to baseline in either intervention group (McNamara et al., 2018). The absence of a significant decrease in the placebo group indicates anthocyanin metabolites are maintained for extended periods of time and persist in the circulation. However, change from baseline concentrations showed a 2-fold increase in native anthocyanin forms (parent forms found in food) in the BB treated group compared to a significant reduction in the placebo group. This suggests that the benefits of BB to WM are resultant from consumption of anthocyanins in their native (food form) rather than metabolites which were also detected in the placebo group. Indeed, concentrations of native forms of anthocyanins were shown to be correlated with improved semantic access and non-verbal memory (Krikorian et al., 2020). However, in the study by McNamara et al. (2018), cognitive performance and self-assessment was completed 12 weeks after collection of urine sample. It would be more useful to analyse anthocyanin excretion concurrently with cognitive testing.

The correlation between urinary excretion of phenolic metabolites and cognitive effects has been further explored in a combined berry intervention. In a study by Bensalem et al. (2019), two-hundred and fifteen older adults aged between 60 and 70 years (mean 64.6 years) were selected with limits on flavonoid in habitual diet and allocated to receive a daily dose of 600mg polyphenols-

rich extract of grape and wild blueberry (PEGB) (258mg flavonoids) as one 300mg PEGB capsule twice daily for 6 months or a placebo containing 300mg of maltodextrin. Interestingly, intervention capsules were taken at least one hour after meals. This may have avoided interactions with food matrices affecting many other flavonoid studies. Assessment of memory performance was completed by cognitive tests selected from the Cambridge Neuropsychological Test Automated Battery (CANTAB); paired associate learning total errors adjusted (PALTEA), Verbal Recall Memory free recall (VRMFR) test, Verbal Recognition Memory recognition (VRMR) test, Spatial Span (SSP) and the Reverse SSP tests at baseline and after 6 months of daily intervention. Benefits of PEGB to EM were found to be dependent on cognitive performance at baseline, in that analysis of data from all subjects only showed superior scores for VRM free recall following PEGB. However, in subjects with the lowest baseline PALTEA performance (4^{th} quartile), PEGB was associated with improved PALTEA, VRMFR and delayed version of the VRMR scores. This indicates that the positive cognitive effects of PEGB are most apparent in subjects with the greatest age-related cognitive decline in EM. Profiling of urinary PEGB phenolic metabolites revealed concentrations of native (−)-epicatechin and its conjugates were strongly correlated with VRMFR improvement. Further, subjects with the greatest cognitive decline (4^{th} quartile baseline PALTEA) exhibited higher excretion of PEGB metabolites, which was found to correlate with poorer cognitive performance. The authors therefore suggest that excessive flavonoid excretion may play a role in the progression of age-related cognitive deficits. Importantly, PEGB treatment was able to overcome this elevated excretion to produce a cognitive effect.

The cognitive effects of grape and blueberry treatments have been investigated individually in separate studies. In a parallel design study, Calapai et al. (2017) recruited one-hundred and eleven adults aged between 55 and 75 years to receive one 250mg grape extract capsule once a day (mean age 66.8 years) or a placebo capsule containing maltodextrin (mean age 66.9 years) for 12 weeks. In order to assess cognitive performance and mood, the Repeatable Battery for the Assessment of

Neuropsychological Status (RBANS), MMSE, BDI and Hamilton Anxiety Rating Scale (HARS) were used. Total score for RBANS (measure of attention, language, short and long term memory) was significantly improved following the grape intervention relative to baseline and placebo. Grape treatment was also associated with significant reductions in BDI score compared to baseline. Further, significant reductions in HARS were found in the grape-treated group relative to both baseline and placebo. These findings suggest that the cognitive improvements found with grape treatment may have contributed to the mood effects (reduced anxiety) since anxiety often co-presents with memory decline (Mantella et al., 2007).

In a small study by Bowtell, Aboo-Bakkar, Conway, Adlam, and Fulford (2017), twenty-six adults aged over 65 years were pair-matched according to Addenbrooke's Cognitive Examination III Questionnaire (ACE-III) and randomised to receive 30mL of blueberry concentrate (387mg anthocyanidins) every morning diluted with tap water (mean age 67.5 years) or an energy-matched control of synthetic blackcurrant and apple cordial (mean age 69.0 years) for 12 weeks. Subjects completed a cognitive test battery comprised of a detection task, Groton maze timed chase test and learning test, Identification task, International shopping list task and n-back tasks. The Numerical Stroop test was completed during ASL-fMRI scanning to examine the effects of BB on cerebral perfusion. Significant increases in brain activation were detected in several task-associated regions (Brodman areas 4, 6, 10, 21, 40,44, 45, precuneus, anterior cingulate, insula and thalamus) following BB treatment relative to baseline and control. This could reflect increased effort to focus on completing the Stroop task or larger increases in blood flow to task-related regions. However, there were no significant effects of treatment on any of the measured cognitive outcomes, including Stroop test during ASL-fMRI scanning. No significant effects of BB were found on serum levels of brain-derived neurotrophic factor (BDNF) and the inflammatory marker CRP relative to baseline or control. These findings do not support associated mechanistic pathways of neural synaptic plasticity (BDNF) and reduced inflammation. However, non-fasting samples were used which may have

affected BDNF status. The absence of cognitive effects is likely due to the small sample size. The authors highlight that the study was not powered to detect cognitive changes.

In contrast, benefits of BB to some cognitive outcomes has been demonstrated by Miller, Hamilton, Joseph, and Shukitt-Hale (2018) and Whyte, Cheng, Fromentin, and Williams (2018) using different forms of BB interventions. In another small study, Miller et al. (2018) supplemented twenty-seven older adults with 12g of whole freeze-dried BB powder twice daily or placebo powder for 90 days. Both interventions were taken in water with meals. The BB intervention provided approximately 804mg total polyphenols including 460.8mg of anthocyanins. The placebo powder contained maltodextrin, fructose, blueberry flavouring, artificial colours and citric acid, designed to match the active treatment for colour, energy and taste. Cognitive assessment was conducted at baseline, 45 days and 90 days using a Task-switching test (TST), Digit span task (DS), virtual Morris Water Maze, Attention Network task. Mood was measured by GDS and POMS. TMT and CVLT were administered at baseline and 90 days only. The results showed repetition errors for CVLT increased from baseline to 90 days in the placebo group, in contrast to reduced errors in the BB group. This suggests regular treatment with BB was able to improve long term memory performance. Importantly, Miller et al. (2018) demonstrated the positive effects of BB treatment on aspects of EF in older adults, in contrast to the majority of previous findings have shown benefits to memory. BB treatment was associated with a greater reduction in switch trial errors across visits compared to placebo. The findings by Miller et al. (2018) suggest that blueberry consumption may protect against the early sign of cognitive ageing in executive functioning decline. However, the interventions were consumed with breakfast on test days, such that the observed behavioural changes may in part be attributed to acute effects rather than chronic. No other cognitive effects were evident.

Bowtell et al. (2017) and Miller et al. (2018) used similar sample sizes and intervention periods. The limited treatment effects across these two studies may have been due to insufficient power and

duration of treatment to detect further BB effects on cognitive outcomes. In a larger and longer parallel design study, Whyte et al. (2018) recruited one-hundred and twenty-two older adults aged between 65 and 80 years (mean 70.8 years) with subjective memory complaints to receive one of four supplements formulated as capsules for consumption with dosage instructions to take two capsules with water every morning at breakfast for 6 months. The four treatment arms consisted of purified wild blueberry extract containing 7mg anthocyanins (WBE111) and two doses of whole wild blueberry powder, 500mg containing 1.35mg anthocyanins (WBP500) and 1000mg containing 2.7mg anthocyanins (WBP1000), and placebo (1g of maltodextrin, red and blue colouring). The antioxidants L-cysteine and L-glutathione were included in the formulation of the active blueberry treatments to enhance the stability of the blueberry anthocyanins and therefore increase anthocyanin bioavailability. Cognitive, mood and cardiovascular outcomes (blood pressure and pulse rate) were measured at baseline, 3 months and 6 months. A cognitive task battery was employed to detect cognitive changes. This was comprised of Rey's Auditory Verbal Learning task (RAVLT), an object recognition task and the Corsi Blocks task, to measure effects on episodic memory. Outcome measures recorded for serial subtractions and Sternberg memory scanning were used to assess working memory. Executive function was evaluated using the Modified Attention Network Task (MANT) and Stroop task. Finally, subjective mood was assessed by the Positive and Negative Affect Schedule—NOW (PANAS-NOW). The results showed significant improvements to delayed word recognition in RAVLT and a trend towards significance for total number of sequences correctly recalled in the Corsi Blocks task at 3 months following WBE111. No other significant treatment-related effects were observed with respect to cognitive measures at 3 months or 6 months for WBE111. These findings are in keeping with Dodd et al. (2019) who also found improved word recognition in RAVLT, observed following acute BB treatment in older adults. Significant reductions to systolic blood pressure (SBP) at 3 months, which were sustained at 6 months, were also recorded following WBE111 treatment. The concurrent benefits to SBP and EM at 3 months in the WBE 111 group are in keeping with proposals that flavonoid may act through vasodilation in the vasculature

to increase cerebral perfusion in task relevant areas. The cognitive improvements following WBE 111 recorded at 3 months were not sustained at 6 months. This may have been due to practice effects or tolerance to WBE. Elsabagh, Hartley, Ali, et al. (2005) also suggested the lack of cognitive effects following chronic cocoa supplementation, in which acute effects were evident could be explained by tolerance to treatment. Together, this suggests that timescales of chronic treatment require further exploration as existing findings could indicate insufficient as well as excessive durations. On test days, intervention capsules were consumed after testing to exclude measurement of acute cognitive changes and more confidently attribute behavioural outcomes to chronic effects. Indeed, these were found with a very much lower dose compared to those following which cognitive effects have previously been demonstrated. This suggests that the inclusion of L-cysteine and L-glutathione in the WBE formulation successfully stabilised the anthocyanin components to increase bioavailability and therefore produce measurable changes in cognitive performance.

In summary, whilst there is some evidence from human intervention studies of the beneficial effects of dietary flavonoids on cognitive function, research findings remain equivocal. Some of these null findings may be explained by the demographic of the subjects selected for study participation. In general, volunteers for flavonoid-cognition research may be more interested in health, nutrition and cognition. The impact of this is that subjects may follow healthier diets that are already high in flavonoids with little scope for improvement following additional supplementation. They are likely to be well-educated and therefore perform better than average on the cognitive tasks. In the case of young adults, subjects were often recruited from university student populations at their cognitive peak and potentially performing at ceiling. The magnitude of flavonoid effects on cognition may therefore be understated. Further, the majority of research has taken place in Western populations, such that findings cannot be generalised to all ethnicities and patterns of habitual flavonoid intake due to cultural differences.

Overall, cognitive outcomes appear to be determined by subject characteristics and intervention protocols. The extent to which these impact on flavonoid effects warrants further quantitative examination. Where positive effects have been demonstrated these have been in relation to psychomotor function, episodic and working memory and executive function in particular.

1.5 Metabolism and Bioavailability

Only a small proportion of ingested flavonoids are absorbed via the stomach and small intestine. The majority of a flavonoid dose undergoes extensive transformation including degradation by enzymes and gut microbiota, and phase II metabolism (Tena, Martín, & Asuero, 2020). The efficient metabolism of parent flavonoids means that plasma levels are often absent and their biological activity is likely mediated by their metabolites (Luca et al., 2020). Knowledge of the metabolites in circulation is therefore key to understanding the mechanisms for physiological changes which are responsible for health and the cognitive benefits outlined earlier in this chapter in section 1.4.

1.5.1 Absorption

Dietary flavonoids are most commonly present in glycoside forms which have low bioavailability. The presence of glycoside groups increases hydrophilicity of the flavonoid molecule to reduce membrane permeability, as demonstrated by intestinal cellular models (Yi, Akoh, Fischer, & Krewer, 2006). With only 1-2 % of anthocyanins remaining in their parent form following ingestion (Tena et al., 2020) anthocyanins were found to have the lowest bioavailability, followed by galloylated catechins, flavonols, flavanones, flavanols and isoflavones (Manach, Williamson, Morand, Scalbert, & Rémésy, 2005). Cell models of the gastric border show anthocyanins are absorbed from the human stomach by passive diffusion (Fernandes, de Freitas, Reis, & Mateus, 2012). In the acid environment (pH 1.5-3) anthocyanins are found as flavylium ions, which carry a positive charge at the oxygen atom of C

ring. Absorption rates are low due to the acid conditions, limited absorptive surface area and rapid transition through the stomach. The main site of absorption of flavonoids is the small intestine, where hydrolysis by lactase phlorizin hydrolase (LPH) releases bioactive aglycone forms which are more bioavailable than their parent molecules by way of increased lipophilicity. These may directly enter epithelial cells by passive diffusion. Alternatively, flavonoid glycosides are transported by the Na+ dependent glucose transporter SGLT1 into epithelial cells. Rodent studies have shown that the type, position of and degree of glycosylation affects flavonoid absorption. For example, acylated anthocyanins were found to be more easily absorbed than non-acylated forms (Harada, Kano, Takayanagi, Yamakawa, & Ishikawa, 2004) and quercetin- 3-β-galactoside, 3-β-rhamnoside and 3-α-arabinoside were found to poorly absorbed in comparison to quercetin-4-0-β-glucoside and quercetin-3-β-glucoside when taken at the same dose (Morand, Manach, Crespy, & Remesy, 2000). Further, the stereochemistry of anthocyanin diglucosides meant that they were better absorbed than their monoglucoside counterparts (He et al., 2006).

Further to the physicochemical properties of the flavonoid molecule, macronutrients and micronutrients when co-ingested with flavonoids in the food matrix have also shown to affect their bioavailability. Although findings from individual studies varied, a review by Kamiloglu et al. (2021) found that overall the presence of food proteins, dietary fibre and minerals negatively affected the bioavailability of flavonoids. For example, flavonoid binding to milk proteins through non-covalent links may decrease the absorption of flavonoids from tea and cocoa (Xiao et al., 2011). Conversely, fats, carbohydrates and vitamins were shown to improve flavonoid bioavailability. High carbohydrate foods may promote gut motility and blood flow to the gut mucosa to enhance flavonoid deglycosylation and absorption.

1.5.2 Metabolism

Once absorbed, flavonoids undergo phase II metabolism which commences in the wall of the small intestine, followed by transportation via the portal vein to the liver where further phase II conjugation continues or efflux back into the lumen of the small intestine. Unabsorbed flavonoids delivered to the colon may undergo bacterial deglycosylation, hydrolysis, demethylation, and ring fission of the flavonoid backbone to produce low molecular weight metabolites, such as phenolic acids, and c-valerolactones which are then absorbed or excreted (Wan, Co, & El-Nezami, 2021). The type and bioavailability of the metabolites produced depends on the diversity of the colonic microbiota, largely determined by an individual's habitual diet (van Duynhoven et al., 2011).

In the liver phase II enzymic metabolism by glucuronidation, sulfation and methylation to polar groups, such as hydroxyl groups, in the flavonoid molecule (parent or metabolites) produce hydrophilic conjugates which are readily excreted in urine or enter the systemic circulation. Therefore, the greater the number of hydroxyl groups in the flavonoid molecule, the greater the potential for rapid conjugation (Chen, Cao, Huang, Xiao, & Teng, 2021). The position of conjugation can influence subsequent phase II metabolism. For example, glucuronide conjugation on the B or C rings of EGCG was shown to inhibit further methylation of the benzene ring. This was not found for glucuronide conjugation on the A ring (Luo et al., 2017).

1.5.3 Blood Plasma

Once in the systemic circulation peak plasma levels of flavonoids have shown to coincide with cognitive effects. Peak plasma concentrations of cocoa flavanols have been found to occur between 2 and 3 hours after intervention consumption (Actis-Goretta et al., 2012; Ottaviani, Kwik-Uribe, Keen, & Schroeter, 2012; Richelle, Tavazzi, Enslen, & Offord, 1999) and improved cognitive

performance have been demonstrated at the same timepoint (Scholey et al. 2010, Field et al. 2011 and Karabay et al. 2018). Berry flavonoid metabolites have also exhibited similar patterns between blood plasma peaks and positive cognitive effects. Blueberry polyphenol metabolites were found to peak between 1 and 2 hours, and again at 6 hours (Rodriguez-Mateos et al., 2013) which are temporally aligned to observed cognitive improvements following blueberry treatment (Whyte et al. 2015, 2016, 2020; Barfoot et al. 2019 and Dodd et al., 2019). Watson et al. (2015) demonstrated acute cognitive benefits following blackcurrant treatment corresponding to an initial peak in plasma metabolites detected at 1.5 hours (Costello et al. 2022). However, Watson et al. (2015) did not conduct cognitive testing during a second observed peak in plasma concentration at 4 hours (Costello et al. 2022). Despite limited cognitive effects demonstrated following acute grape seed extract consumption (Bell et al. 2022), some were found to coincide with peak plasma concentrations of grape flavonoid metabolites at 4 hours (Castello et al. 2018). The citrus flavanone hesperidin has shown to reach peak concentrations in the plasma between 4 and 8 hours post-intervention (Ávila-Gálvez, Giménez-Bastida, González-Sarrías, & Espín, 2021; Manach & Donovan, 2004). More pronounced cognitive effects found following citrus juice drinks at 6 hours than at 2 hours (Lamport, Pal, et al. 2016; Alharbhi et al. 2016) are in-keeping with this pharmacokinetic data. The timing of post-intervention peaks of Ginkgo biloba flavonoid metabolites have not been directly measured, rather GB terpenoids have received focus in this regard (Briskey & Rao, 2022). However, metabolites of quercetin, a key GB flavonoid, have shown to peak in the plasma at 5 hours post-consumption (Jaganath, Jaganath, Mullen, Edwards, & Crozier, 2006), which may explain the commonly observed cognitive effects of GB treatment around this timepoint (Kennedy et al. 2000, 2002, 2007 and Elsabagh et al. 2005). Interestingly, a general absence of evidence for positive effects following acute soy IF supplementation, may in part be due to the mismatching of testing schedules and peak plasma concentrations in the blood. For example, Vanata and Metzger (2007) examined the effects of soy IF at 105 minutes post-intervention, whereas concentrations of soy IF metabolites have shown to peak in blood plasma much later at between 12 and 16 hours (Jang et al., 2020).

1.5.4 Blood Brain Barrier

In vitro and in vivo studies have shown flavonoids are able to cross the blood brain barrier (BBB) (Faria et al., 2014; Youdim, Qaiser, Begley, Rice-Evans, & Abbott, 2004). Faria et al. (2014) demonstrated methylated forms of flavanols and anthocyanins were more efficiently transported across the BBB than their parent forms. Their findings also suggest that the mechanisms through which different flavonoid subclasses cross the BBB vary. Flavanol metabolites were found to move through the BBB via both passive diffusion and membrane transporters. Anthocyanin crossing of the BBB was heavily dependent on their lipophilicity, indicating the role of passive diffusion. Further, flavonol transport was shown to be modulated by treatment with phosphatase activators, whereas anthocyanin and flavanol transport were unaffected. This suggests flavonol crossing of the BBB may be regulated by a phosphorylation/dephosphorylation mechanism. (Yang et al., 2014) further demonstrated mechanisms of passive diffusion and specific transporters.

Rodent studies have consistently demonstrated the crossing of flavonoid subclasses of the BBB. Margalef, Pons, Bravo, Muguerza, and Arola-Arnal (2015) administered an oral gavage of grape seed polyphenol extract (GSPE) to rats found methyl-sulfated forms can more efficiently cross the BBB than other conjugates in a dose dependent manner. The stereo specific penetration of the BBB suggests that the process is transporter-mediated rather than via passive diffusion. Further, Ferruzzi et al. (2009) used an intragastric gavage of (GSPE) showed low molecular weight flavonoids such as catechin monomers, bioaccumulate in the brain. In particular, Lin, Wang, Tseng, Sung, and Tsai (2007) found EGCG metabolites in the cortex, brain stem, hippocampus, cerebellum and striatum in the brain of rats orally fed EGCG. Importantly, polarity and molecular structure of anthocyanins were revealed to affect entry across the BBB. It was discovered that in rats orally supplemented with tart cherry powder, peonidin-3-rutoside but not cyanidin-3-rutinoside, was able to cross the BBB. The

two compounds differ structurally by a single methoxyl group in the B ring only (Kirakosyan et al., 2015).

There is emerging evidence from human subjects of flavonoids traversing the BBB. Grabska-Kobylecka et al. (2020) found caffeic acid of exogenous source (i.e. food origin) in the cerebrospinal fluid sampled by lumbar punction in 28 patients. Plasma concentrations of caffeic acid in peripheral blood samples were similar to that found in CSF but were not significantly correlated. Discrepancies between plasma and CSF concentrations of flavonoids were found by Zini et al. (2006). Despite detection of green tea flavonoids in plasma following an acute dose, no flavanols or their metabolites were detected in CSF samples from the same subjects. These findings support proposals that flavonoids benefit cognition through both direct and indirect mechanisms of action on the CNS which are discussed in the following section of this chapter.

1.6 Mechanisms of Action

The limited bioavailability and diverse metabolic pathways of the flavonoid subclasses and individual compounds within them, suggest multiple mechanisms exist through which flavonoids may modulate cognitive function. Early animal studies to investigate the effects of flavonoid-rich foods on cognition found protection from and reversal of age-related cognitive decline in young and aged rats respectively (Joseph et al., 1998; Joseph et al., 1999). The effects of chronic supplementation with either strawberry extract, spinach extract and vitamin E were compared to control diet for 8 months on spatial memory was measured by Morris water maze performance in 6-month old rats (Joseph et al., 1998). The strawberry and spinach extracts comprised between 1 and 2% w/w of the rats' diets and were matched for anti-oxidant activity with the vitamin E supplemented feed. Decline in spatial memory found in control-fed rats was shown to be attenuated by spinach and vitamin E supplementation. In particular, prevention of this age-related cognitive deficit was most prominent

in spinach-fed rats despite the vitamin E diet showing the most protection from oxidative stress (OS), indicating the cognitive effects of the flavonoid-rich spinach extract were not explained by anti-oxidant effects alone.

Joseph et al. (1999) continued investigating the effects of flavonoid-rich diets in aged rats. In this study, 19 month old rats were supplemented with strawberry, spinach or blueberry extracts, compared to a control diet for 8 weeks prior to psychomotor (rod walking, wire suspension, plank walking, inclined screen and accelerating rotarod) and cognitive testing (MWM). All flavonoid-fed animals showed significant improvements in MWM performance compared to control. Further, improved rod walking and accelerating rod performance in BB-fed rats compared to other diets. These findings indicate that chronic intake of flavonoid-rich diets may reverse age-related cognitive decline. Importantly, as shown in young rodents (Joseph et al., 1998), these effects were found irrespective of OS protection, in that reduced OS was found following supplementation with strawberry and blueberry extract only here (Joseph et al., 1999). Taken together, these two studies suggest that flavonoids do not modulate cognitive function by way of their anti-oxidative properties, rather involvement in cell signalling pathways, neuroinflammation, cerebrovascular function, glucose metabolism and interplay with the gut microbiota have been posited as potential mechanisms and are discussed herein.

1.6.1 Modulation of Signalling Cascades

Flavonoids have been shown to interact with cell signalling cascades to promote cell survival via activation of cAMP response element binding protein (CREB) and increases in brain-derived neurotrophic factor (BDNF) in rodent models (Rendeiro et al., 2012; Rendeiro et al., 2013; Williams et al., 2008). CREB is a transcription factor activated by phosphorylation to regulate downstream gene expression and protein synthesis involved in long-term memory formation including BDNF

(Bourtchuladze et al., 1994; Lonze & Ginty, 2002), a neurotrophin which regulates synaptic plasticity, structure and transmission (Kowiański et al., 2018). In the hippocampus BDNF has shown to stimulate axon and dendrite growth (Cohen-Cory & Fraser, 1995; Pang & Lu, 2004) by promoting dendritic protein synthesis (Schratt, Nigh, Chen, Hu, & Greenberg, 2004).

A 12-week blueberry supplemented diet (2% w/w of diet) in aged rats (18 months old) was compared to a control diet fed to aged rats and young rats (6 months old) (Williams et al., 2008). At baseline, significantly poorer cross-maze performance in both groups of aged rats compared to young rats indicated age-related decline in spatial working memory. While the cross-maze accuracy of control-fed aged rats remained depleted, BB-fed aged rats showed improved accuracy to a level matching the control-fed young rats, which became apparent at 3 weeks and maintained test sessions at 6 weeks, 9 weeks and 12 weeks. This finding suggests that blueberry supplementation is able to reverse age-related cognitive decline. Levels of CREB phosphorylation and both pro and mature types of BDNF (pro BDNF and mBDNF) were significantly lower in the hippocampus of aged rats compared to young rats at baseline. Following blueberry supplementation concentrations of phosphorylated CREB and BDNF were significantly increased in the hippocampus of aged rats. This indicates increased neuronal survival in brain regions associated with memory formation. Importantly, SWM performance was found to correlate with both phosphorylation of CREB and concentrations of proBDNF and mBDNF. Further investigation found increased phosphorylation of extracellular signal-related kinase 1 and 2 (ERK1/2) associated with BDNF levels. ERK 1/2 are MAP kinases critical to the transduction of extracellular signals into cellular responses and are involved in the activation of CREB (Karin & Chang, 2001). These findings suggest that the positive BB effects on ERK-CREB-BDNF signalling pathway may be linked to the improvements to spatial memory observed in aged rats. Mammalian target of rapamycin (mTOR) is an important protein kinase involved in synaptic plasticity (Hoeffer & Klann, 2010). Therefore increased levels of phosphorylated Akt and phosphorylated mTOR in the hippocampus of BB-fed aged rats found by Williams et al. (2008),

suggest particular involvement of the PI3 kinase/Akt pathway in benefits of BB supplementation to spatial memory in aged rats. In keeping with the increased activation of BDNF and ERK 1/2, levels of activity-regulated cytoskeletal-associated protein (Arc/Arg3.1), a protein involved in the regulation of long-term synaptic plasticity and long-term potentiation (H. Zhang & Bramham, 2021), were significantly elevated in the hippocampus of BB-fed aged rats compared to control-fed aged rats. Importantly, the observations of increased protein synthesis downstream from BDNF activation were found in the hippocampus where spatial memory is processed.

Rendeiro et al. (2013) successfully replicated the findings of improved spatial working memory and increased hippocampal BDNF in BB-fed aged rats. Further, diets containing pure anthocyanins or pure flavonols at similar levels to that present in blueberries, elicited similar benefits to spatial memory and BDNF levels, suggesting flavonoids are the components of blueberries responsible for their molecular and cognitive effects. Eighteen month old rats were fed one of four diets: whole freeze-dried BB powder, pure anthocyanins, pure flavonols or control feed (matched to the BB diet for sugars and vitamin C) for 6 weeks. All interventions were supplemented at 2% w/w of diet. Assessment of SWM by cross-maze performance at baseline and weekly showed improved accuracy following in BB-fed, anthocyanin-fed and flavonol-fed rats compared to the control-fed rats and baseline, which attained statistical significance by week 4 and was sustained at weeks 5 and 6. Increased hippocampal BDNF concentrations were found following all intervention diets compared to control. As found by Williams et al. (2008), SWM performance and hippocampal BDNF levels were positively correlated, further supporting flavonoid actions on cell signalling cascades as a mechanism of modulating cognitive function. Measures of BDNF mRNA, an indicator of de novo synthesis, only showed significant increases in hippocampal regions of anthocyanin-fed rats (in dentate gyrus, polymorphic cell layer, Cornu Ammonis 1 and 3). This suggests that the increased levels of BDNF in the hippocampus could not be explained by synthesis alone, and that stabilisation of existing BDNF may be possible. The benefits of blueberry supplementation are not limited to aged animals.

Rendeiro et al. (2012) fed the same BB-supplemented diet to young rats (8 weeks old) compared to a control feed for 7 weeks. Spatial memory was assessed by delay non-match task (DNMT) in an 8 arm radial maze twice a week. By the final week of testing, BB-fed rats were found to be more accurate and demonstrated a faster rate of learning than control-fed rats. This increased accuracy was seen alongside increased time taken to complete the task, indicating a trade-off between speed for accuracy. Increased hippocampal levels of phosphorylated ERK 1 and ERK 2, CREB and BDNF were found in BB-fed rats compared to control-fed rats, providing further support that flavonoids act on ERK-CREB-BDNF pathway to improve spatial memory, not only in aged rats but young animals too.

Extending the effects shown for blueberry flavonoids on spatial memory and CREB-BDNF in rats, green tea catechins (GTC) have also been investigated. Li et al. (Li, Zhao, Zhang, Liu, Pei, Wang, Cai, et al., 2009) supplemented the drinking water of aged mice (14 months) with 0.025%, 0.05% or 0.1% w/v of GTC for 6 months, compared with control-fed aged mice and control-fed young mice (1 month old). Aged mice fed 0.05% and 0.1% GTC showed improved MWM performance compared to control-fed aged mice, in that time on target quadrant and number of platform crossings were increased and path lengths to target were reduced, showing similar performance to young mice. As found following blueberry supplementation in rats, this reversal of age-related cognitive decline was associated with increased hippocampal CREB phosphorylation and expression of BDNF (Williams et al. 2008; Rendeiro et al. 2013), further these effects were shown to be dose-dependent. In a subsequent study, Li, Zhao, Zhang, Liu, Pei, Wang, and Li (2009) examined the effects of 0.05% and 0.1% w/v GTC for 6 months compared to control in a murine model of AD (4 months old). A group of naturally-aged mice and a group of young mice (4 months old) acted as further controls. GTC supplementation was associated with improved spatial learning and memory demonstrated in MWM performance. Specifically, reductions in escape latencies and increased time in target quadrant and number of platform crossings in GTC-fed AD mice was found compared to control-fed AD mice, such that performance matched naturally-aged mice. In fact, the number of platform

crossings of BB-fed AD mice showed no difference from young mice. Deficits in hippocampal BDNF and phosphorylated CREB were found in control-fed AD mice compared to normal aged and young mice. The decline was prevented in the GTC-fed AD mice compared to control-fed AD mice. Further, Aβ oligomers were decreased in the hippocampus of GTC-fed mice compared to control-fed AD mice. These findings suggest that chronic flavonoid consumption may reverse dementia-related decline in spatial memory via modulation of CREB-BDNF signalling. Support for this notion was provided by Hou et al. (2010). In a murine model of AD, 8 month old mice were supplemented with Gingko biloba flavonols; quercetin and kaempferol, at a dose of 50mg/kg flavonols by gavage once per day for 4 months or an equivalent volume of carboxymethylcellulose sodium (vehicle). Non-demented wild type mice were employed as further control. AD mice showed substantially poorer performance in MWM latency compared to non-demented mice which was significantly improved by GB flavonols. Increased hippocampal BDNF was also associated with flavonol supplementation which was correlated with improved spatial memory. Interestingly, CREB activation was not affected by flavonol treatment which may have been due to an equilibrium of CREB phosphorylation in the absence of external stimuli.

Together these animal studies provide strong evidence of the relationship between flavonoids, improvements to spatial memory and modulation of the ERK-CREB-BDNF cascade as a potential mechanism of action. Translation of this to human research has been less convincing. Human interventions studies have generally been unable to demonstrate effects of flavonoids on BDNF levels. Chronic flavonoid intake in adults has not shown significant modulation of peripheral BDNF measurements. Sadowska-Krępa et al. (2019) supplemented sixteen healthy young males completing a 6 week programme of aerobic exercise training (mean age 22.6 years) with 2 capsules of 250mg green tea extract containing a daily dose of 490mg polyphenols, including 400mg catechins or placebo capsules containing microcrystalline cellulose, magnesium stearate and maltodextrin. Serum BDNF measured at baseline and following 6 weeks of green tea extract showed no significant effect

of green tea supplementation relative to placebo. Similar outcomes were found following 6 weeks of GB extract intake in eighteen young males (mean age 22.4 years) (Sadowska-Krępa et al., 2017). In this study, subjects consumed 2 capsules of 80mg of GB extract containing 38.4mg flavonoids or placebo capsules containing microcrystalline cellulose, magnesium stearate and maltodextrin. At 6 weeks no effects of GB extract were observed on basal serum BDNF levels

As reviewed in Sections 1.4.2.1 and 1.4.2.2, the modulation of cognitive function by flavonoid intake has been examined alongside their effects on BDNF levels.

Neshatdoust et al. (2016) showed significantly increased serum BDNF levels following consumption of high-flavanol (494mg) cocoa drink for 28 days compared to low-flavanol cocoa drink were correlated with improvements to global cognitive performance in older adults. However, in a similar population a daily dose of 30mL blueberry concentrate (387mg anthocyanidins) for 30 days did not reveal differences in plasma BDNF levels nor cognitive performance relative to control (Bowtell et al., 2017). This could be due to difference in bioavailability of flavonoids; cocoa flavanols more bioavailable than berry anthocyanins. The two types of flavonoids were investigated by García-Cordero et al. (2022) who compared the effects of 12 weeks' consumption of cocoa powder, mixed berry powder and a combination of cocoa powder and berry powder on cognition and serum BDNF in sixty older adults (50 to 75 years). The mixed berry powder was comprised of a combination of dried berry powders; redcurrants (33.3%), blackcurrants (33.3%), raspberries (16.7%) and blueberries (16.7%). The prescribed daily dose of one tablespoon provided 100mg of anthocyanins. One tablespoon of cocoa powder contained 200mg of flavanols. Cognitive function was assessed using TAVEC (Verbal Learning Test Spain-Computense), a Spanish equivalent to RAVLT, Spatial recall test, Wechsler Adult Intelligence Scale (WAIS) IV letters and numbers, Stroop Task, Tower of London (TOL), WAIS-III number key, number cancellation, WAIS-III Digits. Composite scores for memory, processing speed and attention, and working memory derived from the individual tests showed no

differences between interventions. Individual test results showed improved performance relative to baseline across all interventions for time to start TOL, duration of test TOL and TAVEC word recognition. These improvements were most pronounced following consumption of the combination of cocoa and mixed berry powders. Although this could indicate synergistic effects of flavanol and anthocyanin consumption on cognitive ability in older adults, a flavonoid-free control was not employed. Further, the caffeine and theobromine content of the cocoa powder was not matched across interventions, such that the cognitive improvements may not be attributed to flavonoids entirely. Despite the observed cognitive effects, overall serum BDNF levels were not altered by cocoa, mixed berries or a combination of both powders, such that the cognitive improvements detected could not be explained by BDNF upregulation. Indeed, this indicates that multiple mechanisms may be responsible for the positive modulation of cognition by flavonoid consumption. In comparison to the elevated BDNF levels following cocoa flavanol supplementation found by Neshatdoust et al. (2016), the absence of similar changes may be due to the lower dose of CF tested by Garcia-Cordera et al. (2022), (494mg versus 100mg respectively) which suggests flavonoid effects on BDNF levels are dose-dependent, as revealed in animal studies (Li, Zhao, Zhang, Liu, Pei, Wang, Cai, et al., 2009). In women only, a negative correlation was found between serum BDNF and the number of moves required to complete TOL, in that higher BDNF was associated with better TOL performance a measure of executive function. Further to the effects on global cognition observed by Neshatdoust et al. (2016), these findings suggest that BDNF effects may be associated with executive functioning in particular, although this remains to be explored further. The potential gender differences in the effects of BDNF which these results infer are also currently unexplained.

In young adults, despite significant improvements to attention and processing speed following 30 days of dark chocolate (34.8mg epicatechin) consumption compared to a white chocolate control, changes to plasma BDNF were not observed (Sumiyoshi et al., 2019). This could be explained by the low flavonoid dose in the dark chocolate. Decroix et al. (2016) also supplemented young adults with

CF. They found a single dose of 903mg CF chocolate milk did not alter serum BDNF levels and did not improve executive function performance, while cerebral blood volume was acutely increased. Another acute study also failed to demonstrate statistically significant effects of flavonoid intake on BDNF levels. Improvements to global cognitive function were observed in older adults who consumed a blueberry drink containing 508mg anthocyanins and 71mg pro-cyanidins (Dodd et al., 2019). However, although blueberry intake showed an attenuation of the BDNF depletion observed following placebo, this did not reach statistical significance.

Taken together these human studies suggest that flavonoid effects on BDNF may depend on an interplay of several factors, including duration of supplementation, the bioavailability and dose of flavonoid. Variations in the methods used to determine peripheral BDNF levels may also influence study outcomes. There is a lack of consensus regarding whether plasma or serum BDNF more accurately reflect BDNF levels in the CNS.

1.6.2 Oxidative stress/ Neuroinflammation

Oxidative stress (OS) and chronic inflammation have been implicated in the development of a number of disorders such as neurodegenerative diseases and cognitive decline (Reddy, 2006). The reduction of OS and neuroinflammation by flavonoids via neurosignalling pathways may therefore play an important role in preventing onset and progression of cognitive dysfunctions.

The effects of flavonoid-supplemented diets have been examined in young rats (2 months old) in which OS and inflammation were induced by exposure to high-energy and charge (HZE particles) (Shukitt-Hale et al., 2013; Shukitt-Hale, Carey, Jenkins, Rabin, & Joseph, 2007). This type of irradiation causes accelerated brain ageing and cognitive deficits through disturbance of the dopaminergic system. Shukitt-Hale et al. (2007) supplemented the diets of young rats with 2% w/w

strawberry extract, 2% blueberry extract or a control diet for 8 weeks prior to irradiation by 1.5 Gy of 1 GeV/n high-energy Fe particles, with a further control group of non-irradiated animals. At 1 month following irradiation, impaired MWM performance and reduced dopamine release were observed in irradiated control-fed rats. Both flavonoid-supplemented diets were found to protect against these depletions. In particular, spatial memory decline detected in irradiated controls was ameliorated by the strawberry diet, in that strawberry-fed rats demonstrated better retention of location information (associated with hippocampal activity). Blueberry-fed rats showed improved performance in reversal learning trials of MWM which is linked to striatal integrity. Alterations to gene expression in the hippocampus responsible for these protective effects have been further investigated. In the first of two studies, Shukitt-Hale et al. (2013) analysed the effects of irradiation with 1.5 and 2.5 Gy of 1 GeV/n high-energy Fe particles, compared to no irradiation on the expression of genes involved in neuronal growth and differentiation in the hippocampus of young rats. This showed irradiation was associated with up-regulation of genes linked to apoptosis and down-regulation of genes which promote cell proliferation. In the second study, the effects of diets supplemented with 2% w/w strawberry extract, 2% blueberry extract or a control diet for 8 weeks prior to irradiation by 2.5 Gy of 1 GeV/n high-energy Fe particles in young rats were examined (Shukitt-Hale et al. 2013). A non-irradiated control group was also employed. Both flavonoid-enriched diets were found to protect against radiation-induced down-regulation of genes which modulate neuronal signalling (Jun, Mapk14, Raf1, and Gja1). This indicates flavonoid effects are mediated via improved cell signalling, which in this instance is preferential over increased proliferation of radiation-damaged cells.

Oxidative stress and inflammation have been induced by a high fat diet, which has shown to reduce neurogenesis as observed in ageing and impair cognitive performance. Carey, Gomes, and Shukitt-Hale (2014) investigated the effects of blueberry supplementation (4% w/w diet of whole freeze-dried blueberry powder) in low-fat (10% calories from fat) and high-fat (60% calories from fat) diets

fed to middle-aged (9 month old) mice for 5 months. Performance in the novel object recognition test (testing at months 2, 3 and 4) revealed significant improvements in mice fed the blueberry-supplemented high fat diet relative to training trials and compared to mice fed the high-fat diet at month 4. Mice fed the low-fat diet and blueberry-supplemented low-fat diet showed unimpaired novel object recognition during all test sessions. At 5 months, reduced MWM performance was observed in mice fed the high-fat diet compared to mice fed the low-fat diet and mice fed the blueberry-supplemented low-fat diet. This decline in performance was ameliorated by blueberry supplementation, in that blueberry-fed mice on the high-fat diet performed at a level equivalent to low-fat diet fed mice.

Together, these studies suggest that dietary flavonoids are able to protect against OS and inflammatory-related cognitive and neurological deficits through improved neuronal communication. In contrast, evidence from human intervention studies on the effects of flavonoid consumption on inflammation has been limited. Macready et al. (2014) recruited adults (26 – 70 years) who habitually consumed less than 4.4 portions of fruit and vegetables daily. Subjects were administered high-flavonoid (> 15mg/100g) or low-flavonoid (< 5mg/100g) fruits and vegetables or maintained on their usual diet (control). Incremental increases above habitual intake of the respective fruits and vegetables in the high and low-flavonoid groups, were by 2, 4 and 6 portions per day at 6 weekly intervals over a period of 18 weeks. Measures of inflammatory markers in fasting blood samples taken at baseline, 6, 12 and 18 weeks. At weeks 12 and 18, men in the high-flavonoid and low-flavonoid groups had significantly lower CRP than men in the control group. At week 12, lower concentrations of VCAM were detected in both fruits and vegetables groups compared to control. In the high-flavonoid group, reductions in E-selectin achieved significance at week 18 in men. In the low-flavonoid group, reductions in E-selectin were significant at week 12 in men and at week 18 in women. There were non-significant changes in IL-6 and TNF-α. These results provide some evidence for the anti-inflammatory effects of flavonoids and suggest that these may

be more pronounced in men. An explanation for this potential gender-specific effect is yet to be elucidated. In cognitive studies no effects of flavonoid supplementation on inflammatory markers have been found (Nilsson et al., 2017, Kent et al., 2017, Crews et al,. 2008 and Bowtell et al., 2017).

1.6.3 Cerebral blood flow

As discussed in section 1.4, evidence from human intervention studies suggest that the effects of flavonoids on BDNF concentrations may be dependent on duration of treatment. It is therefore reasonable to suggest that synaptic structural changes and increased plasticity modulated by BDNF activation are unlikely to take effect acutely (within 6 hours) following flavonoid consumption (Alharbhi et al., 2016). One of the purported mechanisms through which dietary flavonoids may modulate cognitive function both acutely and chronically is through increased CBF mediated by improved endothelial function.

Reductions in blood pressure associated with flavonoid consumption are attributed to their vasoactive actions (reviewed by Rees et al. 2018). These effects are mediated through improved endothelial function (reviewed by Ciumărnean 2020) as demonstrated by improved FMD (Alañón et al., 2020; Monahan et al., 2011; Njike et al., 2011; Rodriguez-Mateos et al., 2013). Flavonoids may increase nitric oxide (NO) production by enhancing expression of endothelial nitric oxide synthase (eNOS) or increasing eNOS activity (Mladěnka, Zatloukalová, Filipský, & Hrdina, 2010) to elevate levels of NO and in turn promote NO-mediated vasodilatation (Nehlig, 2013; Steffen et al., 2007). It is plausible that benefits to endothelial function will subsequently improve cerebral blood flow (CBF) and thus cognitive function by increasing the delivery of metabolic substrates required for brain function. Akazawa et al. (2021) demonstrated significant correlation between endothelial function and cerebral blood flow (CBF) in a cohort of middle-aged men.

Blood pressure, FMD and CBF may therefore be mechanistically linked to cognitive function by way of endothelial-dependent vasodilation. Indeed, improved cognitive performance has been associated with both acute (Philip et al., 2019) and chronic (Kent et al. 2017, Desideri et al. 2012; Mastroiacovo et al., 2014; Whyte et al. 2018) blood pressure reductions. Further, increased cerebral blood volume (an indicator of CBF) was shown to parallel benefits to spatial recognition (Brickman et al. 2014) following chronic cocoa flavonoid supplementation.

1.6.4 Blood glucose regulation and improved insulin sensitivity

Insulin resistance is associated with the onset of cognitive decline and progression of neurodegenerative disease (Bourdel-Marchasson, Lapre, Laksir, & Puget, 2010). There is evidence to suggest that flavonoids are able to improve cognitive function via improvements to glucose regulation and insulin sensitivity (Russo, Picconi, Malandrucco, & Frontoni, 2019). Modification of the immediate postprandial glucose response has been found following flavonoid consumption. Specifically, reductions in the magnitude of plasma glucose peaks and slowing of the subsequent decline were observed following blackcurrant (Watson et al. 2015), cranberry (Törrönen et al., 2012; Wilson et al., 2008) and blueberry (Bell et al., 2018) treatment. Importantly, these effects were paralleled with improved cognitive performance. Furthermore, insulin modulates both cerebral and peripheral circulation by increasing endothelial NO synthesis. Yet at elevated concentrations, insulin acts as a vasoconstrictor to increase blood pressure and reduce CBF. Chronic hyperinsulinemia in the peripheral circulation has shown to downregulate insulin receptors in the BBB, causing low levels of insulin in the brain of AD patients (Sartorius et al., 2015). Purported mechanisms through which flavonoids may improve insulin sensitivity include by decreasing insulin secretion from the pancreas (Hopper, Koch, &

Koch, 2013) or increased GLUT4 receptor expression. Chronic cocoa flavanol consumption has been found to improve insulin sensitivity in parallel with positive effects on cognitive performance. Desideri et al. (2012) showed improvements to working memory, EF and processing speed in MCI patients and Mastroiacova et al. (2015) found benefits to verbal fluency and composite cognitive score in healthy older adults. These studies provide evidence to suggest that acute cognitive effects of flavonoids may be attributed to attenuation of the immediate post-prandial peak in plasma glucose, and chronic cognitive effects may result from longer term improvements to insulin sensitivity.

In summary, animal studies have provided strong evidence that dietary flavonoids act by modulation of cell signalling pathways and reducing neuroinflammation to improve cognitive performance. However, rodent models tend to be limited to measures of spatial memory and domains such as verbal memory applicable to human participants cannot be assessed. Further, evidence of these mechanisms in human studies have been less compelling. This is likely driven by the use of peripheral surrogates in place of central markers and low bioavailability of active flavonoid metabolites following human consumption. It appears that measures of proposed indirect mechanisms have been more readily detected in parallel with cognitive improvements. However, a number of studies did not measure cognitive function and mechanistic indicators simultaneously. The cognitive effects of flavonoids likely depend on not one single mechanism, but an interplay between the range of purported mechanisms. Future research should continue to elicit the physiological changes which underpin cognitive flavonoid effects.

1.7 Literature Summary

In summary, the majority of findings from epidemiological research suggest that dietary intake of flavonoids support healthy cognitive ageing and reduce the risk of dementia. Overall, the human intervention studies reviewed in this chapter provide evidence of the benefits of both acute and chronic flavonoid supplementation on cognitive function. However, a number of studies have not shown a positive relationship between flavonoid consumption and cognitive performance. Where flavonoid supplementation has been found to benefit cognitive outcomes, these effects are seemingly modulated by a number of experimental factors. Improvements to the cognitive domains of psychomotor function, episodic memory, working memory and executive function have been demonstrated following flavonoid interventions, particularly when subjects are assessed under conditions of high cognitive demand, such as increased task difficulty. However, these effects have varied depending on subject characteristics such as age and cognitive health status. Acute studies have predominantly been conducted in young adults. In this age group flavonoid benefits have been demonstrated in relation to EM, EF, WM, attention and mood. In children, flavonoid supplementation has shown positive effects on EM, EF and mood. Improvements to EF in middle-aged adults and EM in adults have also been demonstrated following acute flavonoid treatment. In contrast, chronic flavonoid-cognition research has focused on older adult populations, both healthy and cognitively compromised dementia or MCI patients. Here, evidence of improvements to EM, EF, WM and attention have been shown. In middle-aged adults flavonoid supplementation has been found to benefit EM, EF and mood. In children, positive flavonoid effects were found in relation to EF and for attention in young adults. Cognitive development and decline across the human lifespan may account for some of the differences in the observed effects across age groups. However, the extent of these variations remains unclear due to limited examination of certain populations, such as chronic studies of flavonoids in children and young adults, and acute studies in older adults.

Further to subject characteristics, the studies reviewed in this chapter suggest that intervention characteristics also influence flavonoid-related cognitive effects. There are indications that flavonoid effects may be cumulative, and therefore benefits to cognition may be more likely following longer durations of chronic treatment. This is in keeping with the proposed mechanisms of CREB activation to increase BDNF production and reduce neuroinflammation, which may also depend on duration of flavonoid treatment. However, whilst animal models have provided strong evidence of these mechanisms of action, human studies have generally been unsuccessful in demonstrating relationships between biomarkers of these proposed mechanisms and behavioural outcomes. The cognitive benefits of acute flavonoid supplementation have been attributed to their vasodilatory effects and blood pressure reductions have been reported concomitantly with improved cognitive performance (Philip et al., 2019). Importantly, acute changes in cognitive performance have corresponded with plasma peaks in flavonoid metabolites.

The existing literature suggests that flavonoid effects on cognition are dose-dependent and non-linear. However, the exact shape of the dose-response curve remains unclear. Finally, the cognitive effects of flavonoids appear to vary with their dietary source and therefore flavonoid profile. The evidence for cognitive benefits following cocoa and berry supplementation is particularly promising and is attributed to their flavanol and anthocyanin content respectively. However, the intake of cocoa for cognitive benefits may contradict existing dietary guidelines, in that cocoa is often consumed in the form of chocolate with high fat and sugar content. In contrast, berry consumption is coherent with current health messages of eating fruit and vegetables, such as the UK's '5- a day' campaign (NHS England, 2022). Of the berry types which have been investigated, there is a growing body of evidence for the cognitive benefits of blueberries in particular. However, as per the cognitive-flavonoid literature in general, there is little research of acute effects in older adults.

In conclusion, a quantitative investigation of whether flavonoids positively impact cognitive outcomes is lacking. A meta-analysis of the existing literature is therefore warranted and furthers the

narrative review provided in this chapter. This meta-analysis will also investigate subject and intervention characteristics which appear to modulate the cognitive effects of flavonoids.

Blueberry supplementation is of particular interest in that they are a rich source of anthocyanins and found to benefit cognitive performance following chronic and acute treatment. However, there is a paucity of data with regards to their acute effects in older adults and will therefore be examined by this thesis. Further, acute cognitive effects have been demonstrated concomitantly with BP reductions and temporal associations with peak plasma concentrations of flavonoid metabolites. The cardiovascular effects of blueberry treatment will be measured alongside cognitive outcomes encompassing timepoints of known plasma peaks in blueberry flavonoid metabolites. The detailed plans for these investigations are outlined in Chapter 2.

Chapter 2: Plan of Investigations

2.1 Introduction

As documented in Chapter 1, there is a body of evidence for the positive effects of flavonoid consumption on cognitive function, however, not all research findings have shown significant effects. Where flavonoid-related effects on cognitive measures have been detected, these appear to be dependent on several experimental factors including dose, duration of treatment and the population examined. The first aim of this thesis was therefore to complete meta-analyses to investigate whether dietary flavonoids have a positive effect on cognitive outcomes and to see how these effects may be modulated by treatment and population characteristics.

Following this, given there is emerging evidence for acute cognitive effects of blueberry supplementation and the potential role of improvements in endothelial function to mediate this effect (see Chapter 1), the aim of the experimental investigations in this thesis were to explore the effects of an acute anthocyanin-rich blueberry extract on cognitive performance and cardiovascular measures in healthy older adults. There is some evidence to suggest that the cognitive and cardiovascular effects of flavonoids are dose-dependent. It was therefore of particular interest to understand the dose and temporal profile at which blueberry flavonoids elicit improvements to cognition and cardiovascular measures throughout the day.

2.2 Research questions

The research questions addressed in this thesis are:

Meta-analyses (Chapter 3): Do dietary flavonoids have a positive effect on cognitive outcomes? If so, are they modulated by intervention characteristics (dose, duration and flavonoid source), population characteristics (age and cognitive health status) and the cognitive domain investigated?

Rationale: The evidence for cognitive benefits of flavonoid intake remains equivocal, as shown in a narrative review of the literature detailed in Chapter 1. A need for a quantitative examination by meta-analysis of whether flavonoids have a positive effect on cognitive performance is timely. Previous meta-analyses have been limited to specific populations or particular flavonoid-rich food sources, resulting in conclusions regarding cognitive effects that cannot be generalised to flavonoids overall (for example; Ammar, Trabelsi, Boukhris, et al., 2020, Ammar, Trabelsi, Müller, et al., 2020, Camfield et al., 2014, Cheng et al., 2015, Cui et al., 2020, Potì et al., 2019). Review of the existing research also suggests that the flavonoid source, duration of flavonoid supplementation, characteristics of the population tested (such as age and cognitive health status) may determine cognitive outcomes following flavonoid consumption. Quantitative assessment of the influence of these factors is warranted.

Hypothesis: It was hypothesised that in accordance with the literature review detailed in Chapter 1, dietary flavonoids would show significant positive effects on cognitive measures. These are predicted to be most prominent in subjects with cognitive deficits including MCI and dementia patients and older adults likely affected by age-related cognitive decline. Dose-dependent effects

were expected and it was anticipated that these would increase with duration of flavonoid supplementation.

Study 1 (Chapter 4): Following intake of an acute wild blueberry extract in a healthy older adult population, are cognitive, mood or cardiovascular changes evident and, if so, are they dose-dependent and/or time-dependent?

Rationale: A review of the existing literature in Chapter 1, suggests acute blueberry intervention may produce positive effects on cognition and mood. Additionally, outcomes from meta-analyses (Chapter 3) also indicated blueberry treatment may be particularly beneficial for cognitive function. The meta-analyses also revealed cognitive effects may be most pronounced in older adult populations. In young adults, time and dose-dependent peaks in anthocyanin metabolism and associated improvements to vascular function following blueberry treatment were found at 1-2 hours and 6 hours (Rodriguez-Mateos et al., 2013), no similar study in older adults has been completed. Therefore, Study 1 of this thesis explored whether cognitive, mood and cardiovascular outcomes in a healthy older adult population were similarly dose-dependent and followed a similar time-course. Although an older adult sample was used, the findings by Rodriguez-Mateos et al. (2013) give some indication of the metabolites which may be present in the circulatory system following blueberry treatment.

Hypothesis: Wild blueberry extract (WBE) supplementation was shown to improve episodic memory and reduce systolic blood pressure following chronic 12-weeks daily consumption in healthy older adults (Whyte et al., 2018). However, acute dose response relationships between administration of WBE and its cognitive and cardiovascular effects have not been previously examined in this population. Based on existing evidence of cognitive improvements across 2h, 4h and 6h following berry consumption in a young adult population (Whyte et al., 2019), it was predicted that cognitive

performance would be improved compared to placebo across the same timepoints. The outcomes from the meta-analyses (Chapter 3) suggest that when flavonoids are consumed at the lower end of the dose range tested in cognitive research their effects on cognition are dose-dependent. It was therefore predicted that cognitive effects would increase as per increase in dose and be most pronounced for the highest dose of wild blueberry extract.

Therefore, given the appearance of a post-lunch dip in cognitive performance, and the apparent ability of the wild blueberry extract to maintain performance across the day, a subsequent study was undertaken:

Study 2 (Chapter 5): Can acute wild blueberry extract intervention overcome the post-lunch dip in a healthy older adult population?

Rationale: Outcomes from Study 1 suggested that WBE treatment was able to alleviate a post-lunch dip in cognitive performance observed following placebo. A dose of 222mg WBE reduced both systolic and diastolic blood pressure, in-keeping with purported vasodilatory mechanisms of both cognitive and cardiovascular effects of anthocyanins and was therefore identified as the optimal dose tested and selected for further examination. The post-lunch dip in cognitive function has been documented as most commonly commencing at 14:00h (Monk, 2005; Valdez, 2019). The aim of Study 2 was to replicate alleviation of cognitive decline during a predicted post-lunch dip whilst also improving cardiovascular outcomes following WBE 222 supplementation.

Hypothesis: It was hypothesised that in accordance with findings from Study 1, cognitive performance would be maintained and blood pressure reduced during the post-lunch dip, after WBE 222 consumption relative to a placebo.

Final Discussion (Chapter 6):

The findings from Chapters 3, 4 and 5 will be discussed collectively in Chapter 6 to draw overall conclusions of this thesis. The implications of these findings and how future research might be impacted by them will be considered in conjunction with the limitations of the research within this thesis.

Chapter 3: Dietary Flavonoids and Human Cognition: Meta-analyses

Data within this chapter has previously been published as: Cheng, N*., Bell, L*., Lamport, D. J., & Williams, C. M. (2022). (N. Cheng, Bell, Lamport, & Williams, 2022)Dietary flavonoids and human cognition: a meta-analysis. Molecular Nutrition & Food Research, 2100976.

* joint first authors

The meta-analyses were pre-registered on PROSPERO [CRD42019139022] and conducted and reported based on the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA 2020) statement (Page et al., 2021).

3.1 Introduction

Flavonoids have been extensively studied for their ability to influence cognition and to delay cognitive ageing due to their known bioactivity and their high concentrations found in certain food-types. The biochemistry of flavonoids and proposed mechanisms through which they exhibit their effects on cognition, including activation of neuronal signalling pathways, reduction of neuroinflammation, improvements to vascular function and interactions with the gut microbiome and neurotransmitter systems, have already been described in detail (Cichon, Saluk-Bijak, Gorniak, Przyslo, & Bijak, 2020; Flanagan, Müller, Hornberger, & Vauzour, 2018) and reviewed in Chapter 1 section 6 of this thesis. Differences in the molecular structure between flavonoid subclasses means that evidence of mechanistic variations are emerging. For example, isoflavones are thought to exert their cognitive effects through their particular affinity for estrogen receptors (Resende, de Oliveira, de Camargo, Vilegas, & Varanda, 2013), the degree of hydroxylation around the aromatic rings impacts on the antibacterial properties of flavonoids (Xie, Yang, Tang, Chen, & Ren, 2015) and there are differential effects on glucose metabolism (Hanhineva et al., 2010).

A number of published studies have investigated the cognitive benefits of the flavonoid family following supplementation with whole foods or supplements, including berries, cocoa, citrus fruits, tea, soya products and ginkgo biloba. A review of the existing literature, outlined in Chapter 1, found reported effects of flavonoids on cognition have been mixed and suggest that cognitive outcomes may be dependent on several experimental factors which warrant examination. The flavonoid source may be a particularly influential factor, for example Whyte et al. (2019) found a mixed berries intervention improved measures of executive function whereas Decroix et al. (2016) found acute cocoa supplementation failed to elicit an effect when also testing executive function in young adults. Duration of flavonoid supplementation is also likely to impact on the detection of cognitive effects, as evidenced by cocoa interventions, whereby chronic treatment in healthy adults typically benefits cognitive performance (Brickman et al., 2014) but not following acute treatment in a similar population (Crews et al., 2008). Characteristics of the population tested, such as age, may modulate the response to flavonoid intervention, for example, Burns et al. (2006) found that ginkgo was effective at improving long term memory in healthy older adults but had no effect on cognitive measures in young adults. Further to this, cognitive health status is also likely to influence cognitive outcomes following flavonoid treatment. This has been indicated by chronic cocoa supplementation, where benefits to cognitive performance were observed in healthy older adults (Brickman et al., 2014) but not in older adults with mild cognitive impairment (MCI) (Desideri et al., 2012).

Several systematic reviews have provided narrative assessment of the current research showing promise for the positive cognitive effects of flavonoids (Bell & Williams, 2021; Lamport & Williams, 2020; Travica et al., 2020). However, the few meta-analyses that have been conducted have been limited to specific populations or particular flavonoid-rich food sources, resulting in conclusions regarding cognitive effects that cannot be generalised more widely to broader populations or applied to other flavonoid-containing foods (Ammar, Trabelsi, Boukhris, et al., 2020, Ammar,

Trabelsi, Müller, et al., 2020, Camfield et al., 2014, Cheng et al., 2015, Cui et al., 2020, Potì et al., 2019). Further, in light of recent reports showing only 33% of the adult population in the UK consumes five or more portions of fruits and vegetables a day (NHS England, 2020) such targeted meta-analyses do not address the wider health benefits of consuming a range of beneficial plant-based foods in line with government dietary recommendations such as the UK's '5- a day' campaign (NHS England, 2022).Here, meta-analysis of the current literature was performed in order to gain a more systematic understanding of the broad nature and magnitude of flavonoid effects on cognition. Specifically, the primary objective of these meta-analyses is to investigate whether dietary flavonoids have a positive effect on cognitive outcomes. It is also of interest to understand how flavonoid effects may be modulated. The secondary objectives are therefore to identify the influence of study design (acute or chronic), population characteristics (age and cognitive health status), cognitive domain/ability investigated, and intervention characteristics.

3.2. Methods

3.2.1 Search Strategy

The studies included in our meta-analyses were identified through a systematic search performed electronically in PubMed, Web of Science, PsycINFO and Google Scholar databases, with no limitations placed on year of publication (from inception to start of January 2020). The keywords: flavonoid, polyphenol, flavanol, flavanone, anthocyanin, isoflavone, berry, blueberry, blackcurrant, acai, goji, cherry, grape, tea, nut, citrus, orange, grapefruit, ginkgo biloba, fruit, cocoa, chocolate, soya in combination with memory, mood, attention, executive function and cognition (including plurals or truncated forms) were used to conduct the search. These search terms were chosen to encompass the subgroups and dietary sources of flavonoids. In addition to the general term of cognition, specific cognitive domains were also used to broaden the search. Filters to exclude non-

human studies and limit the search to randomized control trials (RCTs) were applied to titles and abstracts. Searches of bibliographies of review articles and searches for additional published articles by major contributors in the field were also performed. Retrieved references were exported from the different databases to EndNote X9 for the removal of duplicate studies. A preliminary pool of relevant RCT trials were identified for consideration against the predetermined inclusion criteria below.

3.2.2 Study Selection

Titles and abstracts of studies retrieved through the search strategy were screened against the following inclusion criteria:

(1) randomized trials, parallel or cross-over design
(2) subjected to peer and/or editorial review
(3) human subjects - any health state, age or other demographic characteristics
(4) flavonoid content of intervention specified
(5) acute or chronic trials
(6) appropriate placebo-controlled design (or inclusion of a suitable control condition - see modifications below)
(7) studies that have utilized one or more cognitive tasks
(8) sufficient data to calculate effect size(s)
(9) written in English

If the above details could not be established from the title or abstract, then the full text was used to assess a study against the inclusion criteria. Studies that did not meet these criteria were excluded. Two investigators (NC and LB) independently completed screening of studies for inclusion. In the event of any discrepancies between study selections, a resolution was achieved through discussion

to reach a consensus or referred to a third reviewer (DL or CW) if a consensus was not initially achieved.

3.2.2.1 Modifications to Inclusion Criteria

During the study selection process, it became apparent that most studies compared one or more flavonoid supplement(s) with a control, rather than an inert placebo. A consensus was reached between two investigators (NC and LB) that the term "control" better reflected the study designs of interest. The minor modification to criterion 6 from "appropriate placebo-controlled design" to "appropriate control condition employed" was made. Studies that investigated a combination of flavonoid and non-flavonoid active treatments which did not isolate the cognitive effects of the flavonoid component were highlighted for discussion. Investigators NC and LB reached the conclusion that these studies did not specifically answer the research question of whether flavonoids have a positive effect on cognition. "Investigation of flavonoid active treatments only" was therefore added to the inclusion criteria.

3.2.3 Data Extraction

Full-text copies of the selected studies were obtained, and data extracted by two independent investigators (NC and LB) using a standardized Excel form. Any discrepancies were resolved by discussion to reach consensus with a third reviewer (DL or CW) consulted if a consensus could not be achieved. In cases where there was insufficient data present in the paper for analysis, authors were contacted via email using the contact information supplied on the paper. If no reply was received, then the study was excluded. Fourteen studies were excluded for this reason. To inform the primary objective of whether dietary flavonoids have a positive effect on cognitive outcomes the following data were extracted from each study for a global analysis: first author's name, year of publication,

number of participants, cognitive scores (means and standard deviations/standard errors) and correlations between repeated data. For the purpose of our secondary objective, to identify the influence of study design, population characteristics, cognitive ability investigated and intervention characteristics, the following data were extracted from each study for moderator analysis: study design – acute/chronic, parallel/crossover; type of nutritional intervention – dietary source; dose of administered intervention (expressed as flavonoid or polyphenol content); treatment duration; age and health status of study participants; and cognitive ability/task type. It was acknowledged that meta-analyses commonly select either parallel or crossover design studies for review both for compatibility purposes and to limit heterogeneity. However, in order to successfully answer the main research question posed by this review, both study designs were incorporated such that the meta-analyses remained as inclusive as possible. Inclusion of both types of study designs permitted the comprehensive assessment of the current literature since both designs contribute to the research question posed here. Exclusion of either type would have reduced the number of studies significantly and limited the generalizability of our results. The heterogeneity of the effect sizes between parallel and crossover studies was computed as part of these meta-analyses to assess their impact on the overall estimated effect size.

3.2.4 Data Coding

Cognitive tasks employed in the selected studies were extracted and then coded according to the Cattell-Horn-Carroll (CHC) classification (Jewsbury, Bowden, & Duff, 2017; Matthew P Pase & Stough, 2014) in order to investigate whether any specific cognitive abilities are selectively responsive to the effects of flavonoids. Individual studies often reported data for multiple cognitive outcomes, across multiple cognitive abilities. In such cases, all data were included in the meta-analyses. Where multiple timepoints were reported in a single study, all timepoints were also included in the data extraction. Coding of study design, flavonoid source, flavonoid dose, duration of supplementation,

age of participant, participant cognitive health status, are described under Section 3.2.7 Moderator Analysis. All data was collated in a standardized form in Excel.

3.2.5 Risk of Bias (Quality) Assessment

Risk of bias/quality of the included studies was assessed by two investigators (LB and NC) using the Cochrane 'risk of bias tool' for randomized trials (Higgins et al., 2011) as part of the data extraction procedure recorded using Excel. Any discrepancies were resolved through discussion. If agreement could not be reached, a third investigator was consulted (see Results Section 3.3 for outcomes).

3.2.6 Data Synthesis

The software package 'Comprehensive Meta-analysis' (CMA for Windows, version 3, Biostat, Englewood, NJ 2013, USA) was used to conduct a meta-analysis of the aggregate data. Due to the varied methodology employed by the included studies, a random-effects model was used in the global analysis. Hedge's g corrects for bias in small sample sizes (Borenstein, Hedges, Higgins, & Rothstein, 2021) and was therefore selected for this meta-analysis. It is the corrected difference in pre-intervention and post-intervention mean outcome measures standardised by SDs. Therefore a g value of 1 means that there is 1 SD difference between interventions, i.e. the difference between control and flavonoid treatment in the current meta-analysis. It is suggested that a g value of 0.2 represents a small effect, 0.5 a medium effect and 0.8 a large effect. Hedge's g and 95% confidence intervals were calculated as an estimate of the summary effect size for each study, using pre-intervention and post-intervention means and SDs and sample sizes for treatment and placebo groups.

Directions of effects were coded, whereby a positive value represented a positive effect of flavonoid, and a negative value represented a negative effect on an outcome measure. This involved reverse coding for some measures, such as reaction times, where a positive value represented a negative effect on cognition. For each study, correlations between pre- and post-intervention scores were estimated by taking a mean average of the within-control and within-treatment correlation coefficients (Pearson r). As this information was generally not available in the published articles, these coefficients were often determined by calculation using the formula below (where pre = pre-intervention, post = post-intervention and diff = change from baseline difference):

$$r = (SD^2_{pre} + SD^2_{post} - SD^2_{diff})/ (2 \times SD_{pre} \times SD_{post})$$

An average of these pre-post intervention correlations was used as an estimate for studies with insufficient data to complete this calculation, in accordance with meta-analysis guidelines described in the Cochrane Handbook (Higgins et al. 2017). In studies where only post-intervention data was reported, means, standard deviations (SD) and sample sizes were collated for treatment and control groups. Additionally, for studies where only change from baseline (CFB) data was available, post-intervention SDs were estimated by calculation using the formula below (where post = post-intervention, diff = change from baseline and r = average pre-post correlation):

$$SD_{post} = SD_{diff}/ \sqrt{(2x(1-r))}$$

The above estimates allowed conversion of multiple types of data for standardization by post-intervention SDs, which enabled direct comparisons of effects in the meta-analyses. Computation of Hedge's g was performed using CMA software. For most studies, data was entered in mean and SD format. For five studies, data was entered directly as effect size values (Cohen's d) where this was the only data available in the paper (Scholey et al., 2012) or where data in a mixture of formats were

obtained and required conversion to a single format for input into the CMA software (Duffy et al. 2003; File et al. 2005; Santos et al., 2003; Whyte, Schafer & Williams, 2017).

Where studies reported multiple experiments with different participant groups, these experiments were treated as separate studies for entry into the meta-analyses (Burns et al., 2006; Elsabagh, Hartley, Ali., et al., 2005; Elsabagh, Hartley, and File 2005; Hartley et al., 2003; Khalid et al., 2017). Where studies reported outcomes for multiple doses or treatment interventions but used the same control group for each comparison, all intervention data were averaged to provide a single entry. In these circumstances, it is considered best practice to combine all relevant experimental intervention groups of the study into a single group, and to combine all relevant control intervention groups into a single control group (Higgins et al. 2017).

Heterogeneity between studies was investigated using a combination of the Q statistic and I^2 statistic according to Borenstein et al. (2021). For clarity, the Q statistic with its associated p-value indicates if existing heterogeneity between effect sizes is statistically significant whilst the I^2 statistic further indicates the extent of heterogeneity present whereby an I^2 value of greater than 50% indicates substantial heterogeneity. Visual inspection of the symmetry of the funnel plot and Fail-safe N were used to assess publication bias.

3.2.7 Moderator Analysis

Further analyses of the included studies were conducted according to the following moderators: flavonoid source, duration of supplementation, age of participant, and cognitive health status of participant. Mixed effects models were used to compare effect sizes of moderator subgroups.

The studies were divided into the following flavonoid source subgroups; berry, citrus, cocoa, ginkgo, pine bark, soya, tea or other; and the extent to which these subgroups explained any heterogeneity in the global analysis was examined. Duration of Supplementation was categorized to correspond with the timeframe of acute studies and significant increases in participant burden into the following categories: up to and including 24 hours; greater than 24 hours, up to and including 6 weeks; greater than 6 weeks, up to and including 3 months; and greater duration than 3 months.

Age was categorized according to key physical, social and cognitive developmental changes across the lifespan (Borland, 1978; Sawyer, Azzopardi, Wickremarathne, & Patton, 2018) into Child (0-12y), Adolescents (13-18y), Young Adult (18-39y), Middle Aged Adult (40-59), and Older Adult (60y+). Where the age ranges of study subjects straddled two or more subgroups, the mean age reported in the paper was used to determine the subgroup classification. For the cognitive health factor analysis, studies were divided into healthy subjects and cognitively unhealthy. Participants with a diagnosis of a condition associated with cognitive deficit which was likely to negatively impact on their performance in cognitive tasks, were classified as cognitively unhealthy. This included participants with mild cognitive impairment, dementia, and Down's Syndrome.

For some moderators, including flavonoid dose, design (acute or chronic), and cognitive ability, individual studies often reported results for multiple subgroups within a moderator category. Separate meta-analyses were therefore performed with data for each sub-group entered individually, rather than a comparative analysis which would lead to the exclusion of these studies.

For the 'Dose meta-analysis', reported flavonoid doses were categorized as Low, Medium or High based on habitual dietary averages. Global patterns of flavonoid consumption (Escobar-Cévoli et al., 2017; Vogiatzoglou et al., 2015; Vogiatzoglou et al., 2014; Zamora-Ros et al., 2013) were used to

create a scale of flavonoid intake according to dietary source against which research doses were mapped. The categories of flavonoid doses according to source are outlined in Table 3.1.

Table 3. 1: Categorisation of dose ranges for each flavonoid source based on typical habitual intake.

	Dose range (mg)		
Flavonoid source	Low	Medium	High
Cocoa	0-349	350-699	700+
Citrus	0-74	75-149	150+
Tea	0-424	425-849	850+
Ginkgo	0-39	40-79	80+
Berry	0-349	350-699	700+
Soya	0-69	70-139	140+
Pine bark	0-249	250-499	500+
Other	N/A	N/A	N/A

Acute and chronic studies were individually assessed. Acute studies were classed as those with a single dose and a testing duration of 24 hours or less. Studies testing at timepoints beyond 24 hours, with repeated daily supplementation, were classed as chronic studies.

Cognitive abilities were divided into subgroups according to CHC classifications (Pase & Stough, 2014); general abilities which are independent of domain (word fluency, fluid reasoning, long-term memory encoding and retrieval, processing speed, working memory/short-term memory, reaction/decision speed), domain-dependent sensory and motor function (visuospatial ability), acquired knowledge (van Aken, van der Heijden, Oomens, Kessels, & Egger, 2019) and unclassified

tasks with multiple parts that straddled many abilities (e.g., Mini mental state examination MMSE) as well as subjective mood (where subjects self-rated how they were feeling).

3.3. Results

Our preliminary literature search yielded 310 studies. Screening against the pre-determined eligibility criteria identified 76 papers for further review comprising 80 experimental studies. A summary of the study selection process is outlined in Figure 3.1.

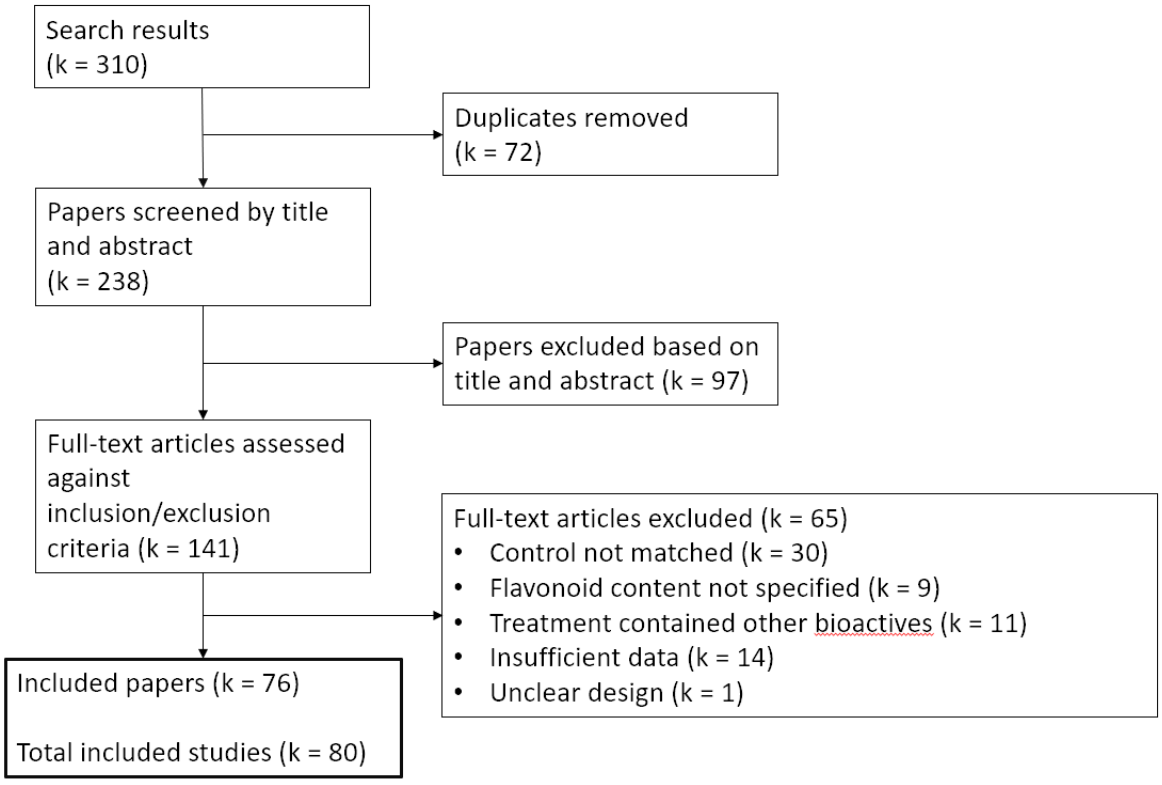


Figure 3. 1: Flow diagram of the literature selection process outlining identification of included studies for meta-analysis of the effect of flavonoids on cognition.

The overall quality of the studies was high when assessed against the Cochrane Risk of Bias Tool (Figure 3.2). Whilst sources of bias were identified in the methods and reporting of results, there was an overall low risk that the true effects of flavonoids on cognitive performance had been

affected by bias from the included studies. For instance, minor sources of bias were identified in relation to blinding of participants and personnel where seven studies were single-blinded, (Barfoot et al., 2019; Dodd et al., 2019; Khalid et al., 2017; Lamport, Pal, et al., 2016; Vanata & Metzger, 2007; Whyte et al., 2019; Whyte & Williams, 2015) incomplete outcome data was reported in four studies, (Burns et al., 2006; Duffy et al., 2003; Lamport, Pal, et al., 2016; Solomon et al., 2002) and non-blinding of outcome assessments was apparent in nine studies (Barfoot et al., 2019; de la Torre et al., 2016; Dodd et al., 2019; Khalid et al., 2017; Lamport, Pal, et al., 2016; Scholey et al., 2010; Vanata & Metzger, 2007; Whyte et al., 2019; Whyte & Williams, 2015) Selective reporting was detected in five studies, including omission of non-significant results, (Hendrickson & Mattes, 2008; Kennedy et al., 2007; Kennedy et al., 2000) reporting of phase 1 only in a crossover study (Howes, Bray, Lorenz, Smerdely, & Howes, 2004) and reporting of mood data excluding cognitive results (Khalid et al., 2017).

Twenty-four studies included 'other' sources of bias mainly in relation to the appropriateness of the placebos administered, typically these included ingredients which were not matched to the treatment with unclear effects on measured outcomes. Eleven studies reported the addition of either natural or artificial colouring, (Boespflug et al., 2018; Bowtell et al., 2017; Chai et al., 2019; Gleason et al., 2009; Gleason et al., 2015; Haskell-Ramsay et al., 2017; Hendrickson & Mattes, 2008; Keane et al., 2016; Lamport, Pal, et al., 2016; Lee et al., 2017; Miller et al., 2018) whilst one study used colouring in both the treatment as well as the placebo (Vanata & Metzger, 2007). One study used a placebo containing fructo-oligosaccharides (Thorp et al., 2009), six studies used placebos which themselves contained flavonoids (Crews Jr et al., 2008; Desideri et al., 2012; Francis et al., 2006; Kean et al., 2015; Mastroiacovo et al., 2015; Scholey et al., 2010). One study did not report final n values for results of dependent variables where subjects had difficulties with the cognitive tasks involved (File et al., 2005). Finally, four studies reported chronic data where measurements were in fact recorded in the immediate postprandial period following the final chronic dose.

(Elsabagh, Hartley, Ali., et al., 2005; Elsabagh, Hartley, and File 2005; Hartley et al., 2003; Moulton et al., 2001) Visual inspection of the funnel plot showed no evidence of publication bias (Figure 3.3). This was confirmed by a Fail-safe N value of 448.

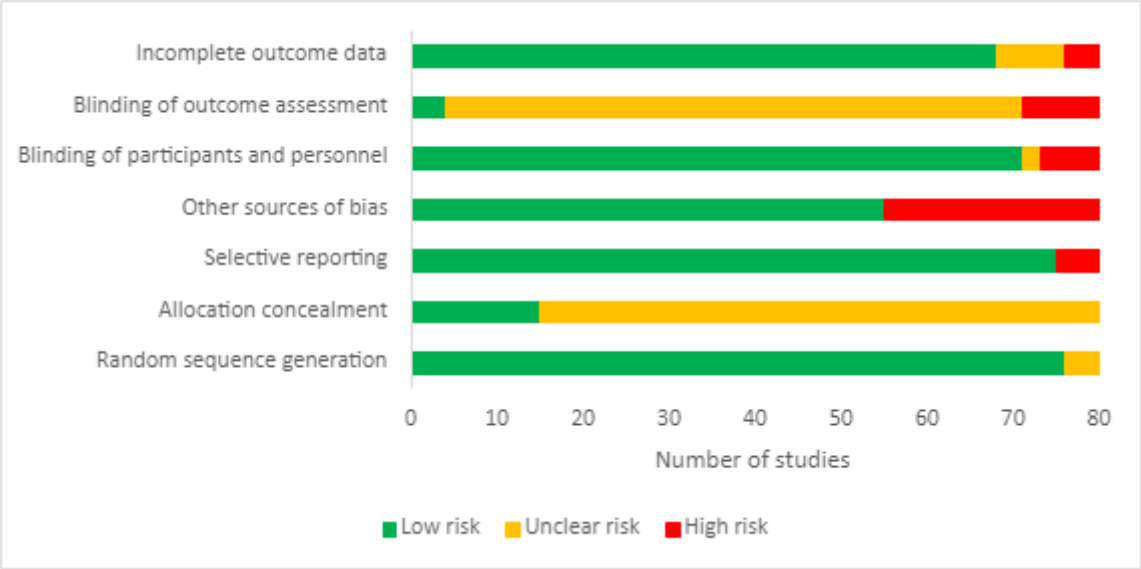


Figure 3. 2: Methodological quality summary: investigator judgments about each methodological quality item from the Cochrane Risk of Bias tool for each study included in the meta-analyses.

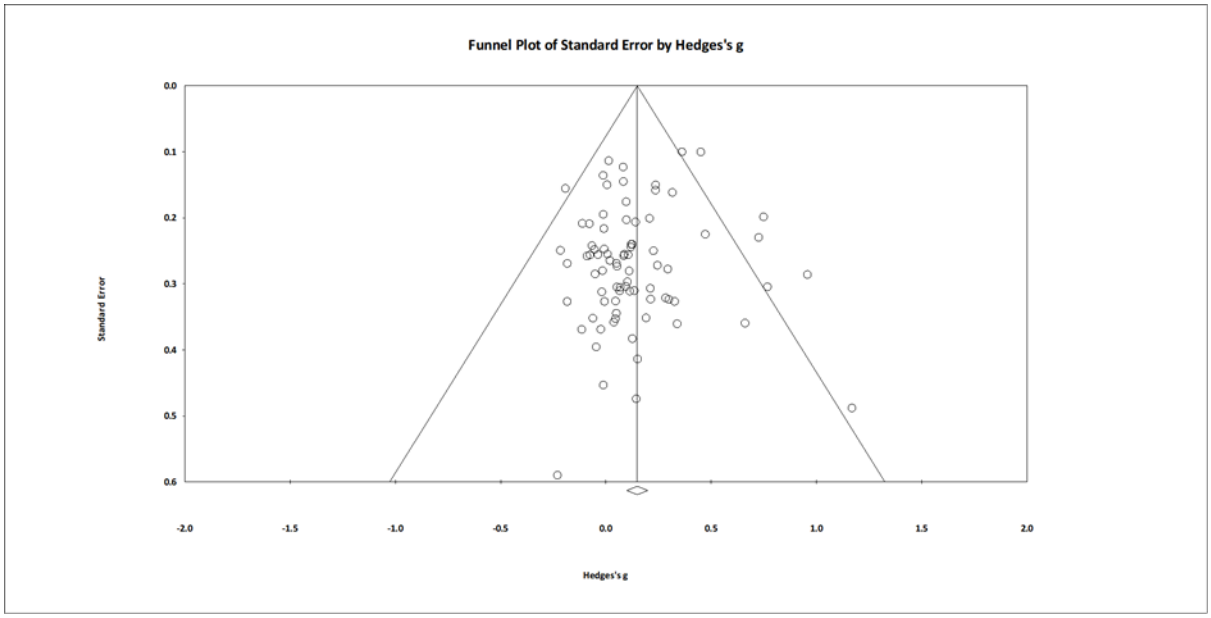


Figure 3. 3: Funnel plot for the cognitive effects of flavonoids showing no publication bias.

3.3.1 Global Effect of Flavonoids on Cognition

Eighty studies were included in this meta-analysis, with sample sizes ranging from n=10 (Watson et al., 2018) to n=410 (Herrschaft et al., 2012). Data from 5519 participants were pooled in our global meta-analysis. Using a random effects model, the computed effect size, Hedge's g, was 0.148 (SE = 0.025, 95% CI 0.098 to 0.198, Z-value = 5.825, $p < 0.001$: Figure 3.4) in favour of flavonoids, without significant heterogeneity (Q = 79.451, df = 79, p = 0.465, I^2 = 0.567%). This indicates that flavonoid supplementation has a significant positive effect on measures of cognitive performance. Despite mixed findings from individual studies, the reported effect sizes across the literature are not significantly different from each other.

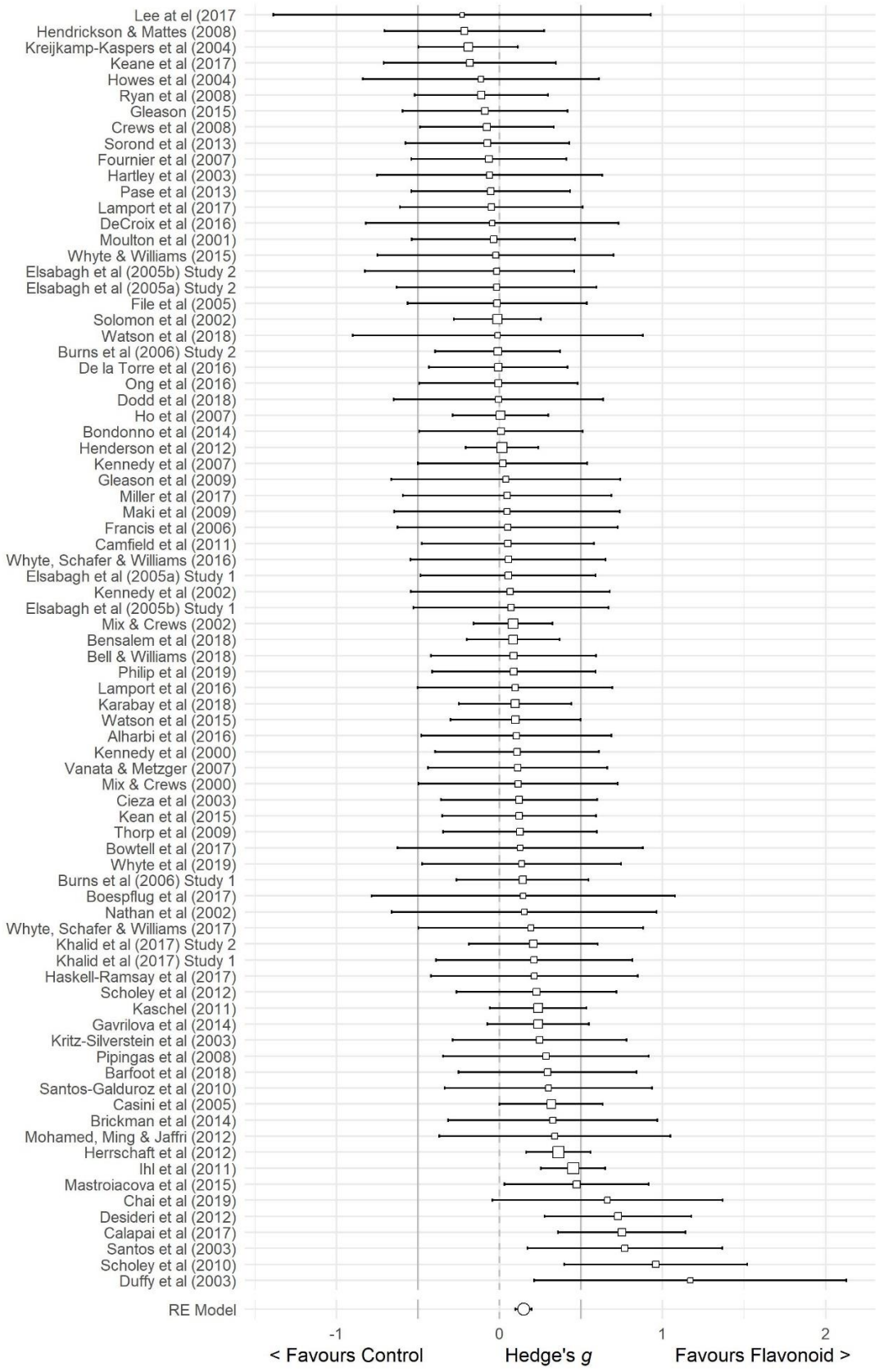


Figure 3. 4: Forest Plot of studies investigating the effects of flavonoid intervention on cognition, depicting Hedge's g with associated 95% confidence intervals

3.3.2 Moderator Analyses

The findings of the moderator analyses are summarised below.

3.2.1 Comparative Analyses

Flavonoid source: The results of the mixed-effects model for flavonoid source are summarised in Table 3.2. These indicated significant benefits to cognition in favour of berries, ginkgo and cocoa, with the largest effects seen following cocoa interventions (k = 11, g = 0.224. 95% CI = 0.014-0.434, p = 0.036), followed by ginkgo (k = 22, g = 0.187, 95% CI = 0.103-0.271, p = <0.001) and then berries (k = 23, g = 0.149, 95% CI = 0.038-0.261, p = 0.009). Berries, ginkgo and cocoa studies represent most of the included studies. Collectively these three subgroups comprise 56 of the 80 studies. In general, the smaller subgroups, citrus (k = 3), pine bark (k = 2), soya, tea (k = 2) and other (k = 2) subgroups did not yield significant benefits to cognition although Hedge's g was positive in all cases (favouring flavonoid intervention). Soya was one of the larger sub-groups (k = 15), but studies were notably focussed on post-menopausal women. The overall difference between subgroups of flavonoid sources was not significant, Q(7) = 5.109, p = 0.647. Figure 3.5 depicts the results of the flavonoid source analysis in a forest plot. The largest effect size was observed for the cocoa subgroup, but this was still small in overall magnitude.

Table 3. 2: Effect of flavonoids on cognition by flavonoid source

Flavonoid Source	k	Mean weighted g	95% CI (lower limit, upper limit)	p
Berry	23	0.149	0.038, 0.261	**0.009**
Citrus	3	0.065	-0.242, 0.371	0.679
Cocoa	11	0.224	0.014, 0.434	**0.036**
Ginkgo	22	0.187	0.103, 0.271	**<0.001**
Pine Bark	2	0.011	-0.347, 0.370	0.950
Soya	15	0.054	-0.055, 0.162	0.331
Tea	2	0.093	-0.228, 0.414	0.571
Other	2	0.119	-0.289, 0.527	0.568

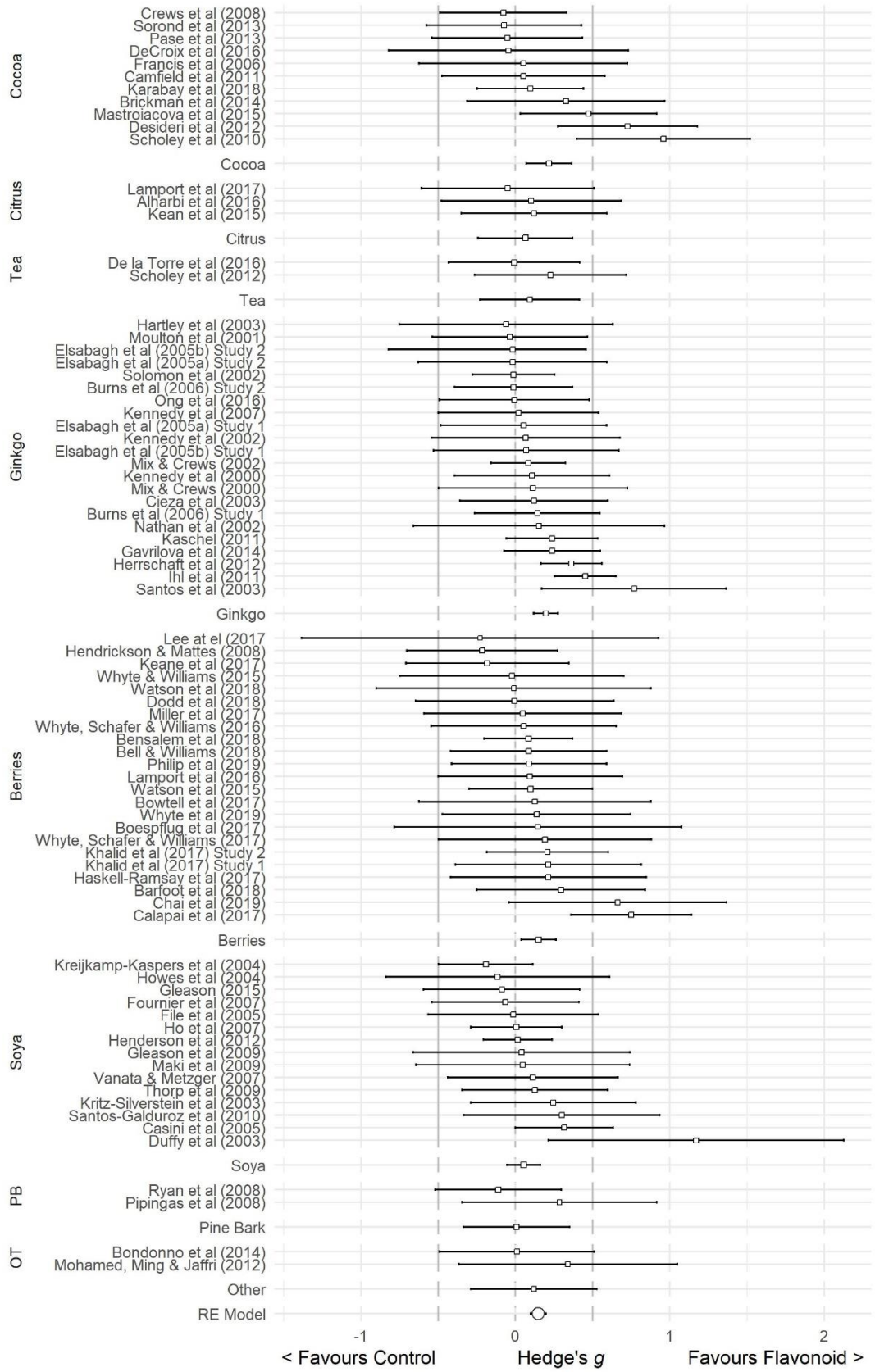


Figure 3. 5: Forest Plot of the effects of flavonoid intervention on cognition according to flavonoid source, depicting Hedge's g with associated 95% confidence intervals

Duration of Supplementation: The results of the mixed-effects model for duration of flavonoid supplementation are presented in Table 3.3. Acute studies were categorized in the duration of supplementation analysis as 'up to and including 24 hours.' As per the acute/chronic analysis, positive effects on cognition that were near to significance were demonstrated here (k = 31, g = 0.094, 95% CI = -0.002-0.190, p = 0.055). The analysis of supplementation duration further revealed that chronic treatment with flavonoids for less than 6 weeks did not produce a significant effect on cognition (p = 0.912). However, only 3 studies fell into this category and so this analysis may lack power. Treatment for greater than 6 weeks up to and including 3 months (k = 31) resulted in significant effects on cognition (p = 0.001). Increasing the duration of the intervention to greater than 3 months (k = 19) yielded a similar significant effect (p = 0.008). Heterogeneity between the supplementation duration subgroups was not significant Q(3) = 1.604, p = 0.659.

Table 3. 3: Effect of flavonoids on cognition by duration of supplementation

Duration of flavonoid supplementation	k	Mean weighted g	95% CI (lower limit, upper limit)	p
≤ 24 hours	31	0.094	-0.002, 0.190	0.055
> 24h ≤ 6 weeks	3	-0.020	-0.367, 0.328	0.912
> 6 weeks ≤ 3 months	31	0.150	0.058, 0.243	**0.001**
> 3 months	19	0.154	0.040, 0.267	**0.008**

Age: The results of the age subgroup analysis are presented in Table 3.4. These showed a small, yet significant benefit to cognition, in middle-aged (k = 22, p = 0.037) and older adults (k = 29, p = 0.001). Treatment with flavonoids did not show significant benefits in children (k = 5, p = 0.172) and young adults (k = 24, p = 0.105). The number of studies in children is notably sparse and it is noteworthy that, of the 5 studies involving children as participants that were analysed here, all were acute design and investigated blueberry supplementation. Further to this, there is an absence of studies conducted in the adolescent age group. However, young adults are well-represented by 24 studies in this meta-analysis. Heterogeneity of the effects between age subgroups was not statistically significant Q(3) = 1.605, p = 0.658.

Table 3. 4: Effect of flavonoids on cognition by participant age group

Age group	k	Mean weighted g	95% CI (lower limit, upper limit)	p
Children	5	0.171	-0.074, 0.416	0.172
Young Adults	24	0.088	-0.018, 0.194	0.105
Middle-aged Adults	22	0.112	0.007, 0.217	**0.037**
Older Adults	29	0.176	0.074, 0.278	**0.001**

Cognitive Health Factor: The results of the cognitive health factor subgroup analysis are presented in Table 3.5. Subgroup analysis revealed significant benefits of flavonoids on cognition in both healthy (p = <0.001) and cognitively unhealthy subjects (p = <0.001). These effects were found to be significantly heterogenous Q(1) =5.319, p = 0.021, with larger effect sizes observed in cognitively unhealthy participants versus healthy participants (g = 0.306 & 0.103, respectively). The positive effects in the cognitively unhealthy subgroup were observed despite the small number of studies

analysed (k = 8). Interestingly, of the eight studies in the cognitively unhealthy subgroup, 7 of these involved older adult participants (Ihl et al. 2011, Herrschaft et al. 2012, Gavrilova et al. 2014, Desideri et al. 2012, Gleason et al. 2015, Lee et al. 2017 and Boespflug et al. 2017). The one exception was a study in young adults with Down's Syndrome (de la Torre et al. 2016). Four of the older adult studies were conducted in MCI patients (Gavrilova et al. 2014, Desideri et al. 2012, Lee et al. 2017 and Boespflug et al. 2017). the remaining 3 studies investigated flavonoid effects in patients with Alzheimer's disease or vascular dementia (Ihl et al. 2011, Herrschaft et al. 2012 and Gleason et al. 2015).

Table 3. 5: Effect of flavonoids on cognition by participant cognitive health status

Cognitive Health Status	k	Mean weighted g	95% CI (lower limit, upper limit)	p
Healthy	72	0.103	0.048, 0.158	**<0.001**
Cognitively Unhealthy	8	0.306	0.143, 0.469	**<0.001**

Parallel or Crossover Group Design: The effect sizes were analysed as a function of the study design (parallel or crossover). Both designs showed significant positive effects of flavonoids on cognition. The difference in effect sizes between the two study design types was non-significant, Q(1) = 0.075, p = 0.785. This clearly shows that the observed benefits to cognitive performance following flavonoid intervention occur irrespective of the study design employed.

3.2.2 Individual Analyses

For moderators where some or all studies reported results for multiple subgroups, comparator analyses were not possible. Results of the analyses of individual subgroups are presented here.

Acute or Chronic Design: The acute/chronic design meta-analyses revealed studies employing chronic flavonoid interventions showed a small but significant benefit to cognition ($p = <0.001$) and studies which involved acute flavonoid supplementation showed an effect approaching significance ($p = 0.055$). Despite reasons of financial burden and patient commitment given as constraints to the running of longer-term trials, a larger proportion of the analysed studies were chronic in design. Thirty-one acute studies and 51 chronic studies were analysed here.

Dose of flavonoid: Many of the reviewed studies investigated the effects of a low (46 studies) or medium flavonoid dose (31 studies). Only 13 studies used a high-dose flavonoid intervention, representing 561 subjects. Results of the Dose meta-analysis are presented in table 3.6. These revealed studies employing low and medium doses of flavonoids showed significant benefits on cognition, ($p = 0.013$ and $p = <0.001$ respectively). Studies which tested high flavonoid doses showed no significant effects overall ($p = 0.181$).

Table 3. 6: Effect of flavonoids on cognition by flavonoid dose

Flavonoid Dose	k	Mean weighted g	95% CI (lower limit, upper limit)	p
Low	46	0.094	0.020, 0.168	**0.013**
Medium	31	0.162	0.092, 0.232	**<0.001**
High	13	0.100	-0.046, 0.245	0.181

Cognitive Ability: The Cognitive Ability analyses showed significant effects of flavonoid intervention in three areas of cognitive function. These were long-term memory (p = 0.014), processing speed (p = 0.008) and subjective mood (p = 0.006) which are amongst the largest subgroups that were analysed (k = 55, 52 and 34 respectively). All other cognitive abilities; word fluency (k = 24), acquired knowledge (k = 14), fluid reasoning (k = 19), working memory/short-term memory (k = 46), visuospatial ability (k = 16) and reaction/decision speed (k = 24), failed to yield significant effects. The potential impact of the number of studies (k) included in the sub-group analysis for each cognitive ability is explored in the discussion.

3.4. Discussion

To our knowledge these are the first meta-analyses to assess data obtained from randomized control trials covering a range of dietary flavonoids across multiple populations, and their effects on cognitive function, encompassing a variety of cognitive abilities across the human lifespan. The results of our global analysis showed that flavonoid supplementation has a significant (though small) positive effect on cognition. Funder and Ozer (2019) suggest that small effect sizes in psychological measures may have important impact in the longer term, particularly when considering health

outcomes where effects are likely to be cumulative, such as cognitive health (Cigolle, Langa, Kabeto, Tian, & Blaum, 2007) as observed here. Thus, the small global effect of flavonoids on cognition (g = 0.148) has potential significance. Importantly, the overall assessment of a low risk of bias in the included studies and minimal publication bias gives confidence in our results.

The analyses of moderators also revealed several interesting findings. The effect of flavonoids on cognitive measures was shown to be influenced by flavonoid source, where significant positive effects were observed with berries, ginkgo and cocoa. Other intervention characteristics which demonstrated moderating effects were dose and duration of supplementation. Here, low and medium flavonoid doses showed significant benefits to cognition with chronic treatments of 6 weeks and longer showing significant effects on cognitive performance and acute interventions yielding near significant effects. Additionally, with respect to participant factors, both age and cognitive health status were found to moderate the cognitive response to flavonoid supplementation. Significant benefits were measured in middle-aged and older adults, which is likely to be driven by the influence of cognitive health status, whereby more pronounced effects were seen in cognitively unhealthy compared with healthy participants. Our results also revealed the cognitive abilities of long-term memory, mood and processing speed to be sensitive to flavonoid treatment.

3.4.1 Flavonoids and Cognition

The positive result of our global analysis is consistent with meta-analyses by Poti et al. (2019) and Ammar, Trabelsi, Boukhris, et al. (2020). Poti et al. (2019) showed significant effects of polyphenol supplementation on WAIS – Block design, Rey's auditory verbal learning task (RAVLT) immediate recall and Trail-Making Task B, reported in test score metrics, in older adults. Ammar, Trabelsi, Boukhris, et al. (2020) observed improvements in Simple Reaction Time and Serial 7s with moderate effect sizes (SMD = -0.926 and 1.467 respectively) and Mental Fatigue (very large effect size of -

3.521) in young and middle-aged adults. The specificity of the outcome measures included in these meta-analyses could explain the larger effect sizes seen in comparison to our global analysis. Other differences in methodology, namely inclusion of non-flavonoid polyphenols, such as resveratrol, and different populations of interest, may also have contributed to the larger effect size estimates seen by Ammar, Trabelsi, Boukhris, et al. (2020). In contrast, another recent meta-analysis by Ammar, Trabelsi, Müller, et al. (2020) did not find any significant effects of polyphenols on cognition in older adults, which reviewed performance in TMT-A and TMT-B only. Both Poti et al. (2019) and Ammar, Trabelsi, Müller, et al. (2020) assessed performance in TMT-B in older adults. However, Poti et al. (2019) investigated the effects of polyphenols in healthy individuals as well as MCI patients, whereas Ammar, Trabelsi, Müller, et al.,. (2020) limited their meta-analysis to healthy subjects. In general, faster TMT-B completion times were reported for healthy older adults compared with older adults including those with MCI (Potì et al., 2019). Although clear ceiling effects were not observed, TMT may place insufficient cognitive demand on healthy subjects to detect cognitive changes following polyphenol supplementation. This is in keeping with evidence indicating flavonoids are most effective at improving cognition in more cognitively challenging situations (Miller et al., 2018; Whyte et al., 2016). This difference in population characteristic is likely to explain the discrepancy in review outcomes. Indeed, cognitive health status was examined in our cognitive health subgroup analysis and suggests that the presence of cognitive deficits provide greater potential for measurable improvements in cognitive performance.

The small effect size observed in our global meta-analysis is in keeping with the magnitude of effect sizes demonstrated by other nutrients and food components. For example, in a meta-analysis by Suh et al. (2020) over 3 months of vitamin B (folate, vitamin B6 and vitamin B12) was associated with improved global cognition (SMD = 0.18) and episodic memory (SMD = 0.09) in middle-aged and older adults. Vitamin B is thought to reduce homocysteine levels, beyond which multiple biological mechanisms of action could explain the connection it has with improved cognitive performance,

including vascular mechanisms, prevention of neuronal apoptosis and epi-genetic modifications (Olaso-Gonzalez et al., 2021) Similarly, a range of vascular, metabolic, and neurochemical mechanisms of action have been proposed for flavonoids and their benefits to cognition.

These findings have important implications for the interpretation of existing flavonoid research and for the design of future clinical trials investigating the impact of flavonoids on cognition. The common practice of using medium to large effect size estimates to establish subject sample size is likely to lead to underpowering of trials. Appropriate sampling should in fact consider a small expected effect size. Indeed, study sample size and other research best practices in the wider field of nutrition and cognition have received commentary, including by Brydges and Gaeta (2020) who found publication bias in the blueberry and cognition studies reviewed by Hein, Whyte, Wood, Rodriguez-Mateos, and Williams (2019). However, it should be noted that the analysis by Brydges and Gaeta used p-values drawn from only the key findings from the individual studies and were not representative of all reported results in the original papers. A subsequent re-analysis by Whyte et al. accounting for all the dependent variables reported in the original source papers found no evidence of publication bias (Whyte, Hein, et al. 2020). Importantly, in this current meta-analysis the computed Fail safe N value was 448, meaning a further 448 non-significant studies would be required for alternative conclusions in relation to our primary research objective to be drawn. Reassuringly, the risk of publication bias affecting the results of our meta-analysis is very low. In addition to the global analysis, our subgroup analyses also revealed some interesting findings with regard to moderators of flavonoid induced benefits to cognition that warrant further discussion.

3.4.1.1. Moderators of flavonoid effects

The subgroup analysis of flavonoid source showed significant effects for berries, ginkgo, and cocoa, whereas citrus, soya, pine bark and tea revealed no benefit to cognition. It is widely accepted that

the bioavailability of flavonoids and their metabolites is a key determinant of their efficacy in relation to pharmacological effects. It is therefore reasonable to expect the bioavailability of the flavonoid sub-classes (reviewed by Di Lorenzo et al. 2021), to contribute to their effectiveness in relation to cognitive measures. In light of this, variations in the cognitive effects according to dietary flavonoid source could be attributed to their flavonoid profile and predominant flavonoid sub-class(es); such as flavan-3-ols in cocoa, anthocyanins in berries and flavanones in citrus fruit (Haytowitz, Wu, & Bhagwat, 2018). Indeed, Ammar, Trabelsi, Boukhris, et al. (2020) suggested differences in individual study results observed with soya and blueberry were linked to the bioavailability of isoflavones and anthocyanins respectively. The non-significant effect of tea demonstrated in the current meta-analysis is in-keeping with the low bioavailability of galloylated tea catechins. However, our subgroup analysis findings cannot solely be explained by flavonoid bioavailability, especially as absorption rates for anthocyanins are reported to be low, but isoflavone absorption rates are high (Manach & Donovan, 2004; Manach et al. 2004). Here, significant cognitive effects for anthocyanin-rich berries were observed, whereas isoflavone-rich soya interventions were not found to be effective. The number of included studies per subgroup is likely to be a relevant factor. Significant effects were seen with the largest subgroups; ginkgo (k = 22), berries (k = 23) and cocoa (k = 11). Apart from soya (k = 15), benefits did not achieve statistical significance for the smallest sub-groups; citrus (k = 3), pine bark (k = 2), tea (k =2) and others (k = 2), possibly suggesting a lack of statistical power. However, in addition to the number of studies, it is also important to consider the mechanisms of action of these flavonoid compounds within the central and peripheral nervous system. As with pharmacological effects in general, some flavonoid types may impact cognitive function more effectively than others through mechanistic differences (as described in Section 3.1 Introduction), or only be effective in specific populations. However, it was beyond the scope of the current meta-analyses to investigate this.

For ginkgo biloba, our meta-analysis revealed a significant benefit to cognition. This may reflect the fact that many of these studies focussed on older or cognitively impaired populations. Indeed, previous meta-analyses which have focussed on dementia and Alzheimer's disease patients have similarly reported benefits to cognition (Birks & Grimley Evans, 2009; Weinmann et al. 2010), particularly in response to supplementation with 240mg of the standardized extract EGb 761 (Gauthier & Schlaefke, 2014; Tan et al., 2015). In healthy subjects, however, Laws et al. (2012) observed no improvements to memory, executive function, nor attention. These inconsistencies are likely due to health status differences in the populations investigated, where cognitively impaired subjects have greater scope for improvement and response to flavonoid treatment. Indeed, this is an outcome demonstrated in the current health factor analysis that revealed much greater effect sizes in cognitively unhealthy subjects.

The lack of a significant effect with soya supplementation observed here is contrary to the findings of two previous meta-analyses which assessed the effects of soya and soy isoflavones on cognition. These found positive effects in post-menopausal women (SMD = 0.08) (Cheng et al., 2015) as well as younger men and women (SMD = 0.19) (Cui et al., 2020) which demonstrated similarly small effect sizes. Discrepancies in our results may be due to differences in study selection criteria. Our strict inclusion criteria meant that some studies included in these two previous meta-analyses, were excluded from our meta-analyses based on the use of controls containing non-flavonoid active ingredients. Cheng et al. (2015) limited their meta-analysis to post-menopausal women, whereas our review included all populations. Cui et al. (2020) excluded red clover as a source of soya isoflavones, whereas our inclusion criteria permitted its inclusion. These methodological differences may therefore account for some of the differences in findings.

The analyses of acute and chronic designs showed significant effects of chronic flavonoid intervention on cognitive performance, whilst the acute effect only approached significance. Further

to this, our Duration of Supplementation subgroup analysis showed significant effects of flavonoid supplementation for periods greater than 6 weeks up to 3 months, and for periods longer than 3 months. This result suggests that for chronic supplementation, at least 6 weeks of flavonoid treatment is required for cognitive benefits to manifest and further corroborates the cumulative nature of flavonoid effects. Indeed, whilst much focus has been placed on the metabolism of flavonoids in the 24 hours immediately after consumption, with identification of associated peak plasma levels (see review DiLorenzo 2021), our findings point to the importance of assessing flavonoid metabolism over an extended period. Although longer trial durations have financial and recruitment implications, when planning trials researchers should consider a minimum of 6 weeks of supplementation, to identify extended time course effects and to allow sufficient time for detectable cognitive changes to occur.

Our observation of stronger improvements to cognition following chronic relative to acute supplementation may be partly explained by the rate at which bidirectional effects between flavonoids and the gut microbiota occur. Flavonoids can act as prebiotics to enhance the growth and establishment of beneficial strains of bacteria present in the gut including bacteria from the Bifidobacteriaceae and Lactobacillaceae families (Alves-Santos et al., 2020; Filosa et al., 2018). In turn, biotransformation by metabolism, depolymerisation and deconjugation of unabsorbed flavonoid molecules by the gut microbiota occurs in the colon to promote bioavailability of active metabolites known to elicit both general and cognitive health effects (see reviews for proposed flavonoid mechanisms of action - Cichon et al. 2020; Flanagan et al. 2018). The gut microbiota further interacts with the central nervous system through the production of neurotransmitters, for example γ-amino butyric acid (GABA) by species of Lactobacillus and Bifidobacterium (see detailed review of flavonoid and gut microbiota relationship by Yunes et al., 2016, Aravind et al., 2021). Improved cognitive performance shown for flavonoid treatments of 6 weeks and longer likely reflect the time required for changes to the gut microbiota to take place and for associated increases in

flavonoid metabolites to lead to behavioural changes. Increases in urinary excretion of hippuric acid, a product of gut microbiota metabolism, were measured following 8 weeks of a high-polyphenol diet (Vetrani et al., 2016), whilst increased excretion was detected after only 2 weeks following blueberry treatment in children (Barfoot et al., 2021). In addition to urinary metabolites, improvements to accuracy in a modified attention network task (MANT; a measure of executive function, attention and inhibition) described in detail by Whyte et al. (2016) were also concurrently observed in the same study following 2 and 4 weeks of blueberry treatment (Barfoot et al., 2021). Clarity around the length of supplementation required for metabolic effects is therefore required, particularly across different age groups. Whether cognitive changes consistently coincide with changes in metabolites remains to be established.

In a recent commentary on published reviews of polyphenol effects on cognition, Lamport and Williams (2020) observed the reporting of more pronounced cognitive effects following chronic polyphenol interventions. This was attributed to the greater number of chronic studies reviewed, which is also reflected in the findings here. Additionally, acute effects can be masked by numerous, transient confounds such as recent diet, time of day of testing and recent exercise. A possible interpretation is that acute effects are likely more subtle in nature, rendering them less detectable than chronic effects. The cognitive abilities or tasks that are sensitive to acute treatment may also differ from those of chronic intervention. For example, faster reaction times were recorded in the MANT following acute blueberry supplementation (Barfoot et al., 2019) compared to improved accuracy scores in the MANT following chronic blueberry supplementation (Barfoot et al., 2021).

The subgroup analysis for age of participants suggests flavonoids are particularly beneficial to cognition in middle-aged and older adults. This may reflect the commencement of age-related cognitive decline from middle age onwards, thus increasing the likelihood of flavonoids exerting a detectable effect. Young adults at the peak of their cognitive ability may exhibit ceiling effects during

testing leading to non-significant effects of flavonoid supplementation (Strittmatter et al. 2020). Cognitive testing in future research should therefore account for this through careful selection of cognitive tasks with proven sensitivity in each age group. Significant positive outcomes were seen in a previous meta-analysis of studies investigating young and middle-aged adults (Ammar, Trabelsi, Boukhris, et al., 2020). Our subgroup analysis suggests that this may have been driven by the effects seen in middle-aged adults. As with our observations for middle-aged adults, Cheng et al. (2015) showed soya isoflavones were effective in postmenopausal women younger than 60 years. However, the effects in women older than 60 years were non-significant. This may represent an effect that is specific to isoflavones. Indeed, our findings demonstrate a clear benefit of flavonoids more generally in older adults, which is consistent with previous findings (Poti et al. 2019). Regarding children, only five studies were included in our meta-analysis. All five studies investigated acute blueberry interventions meaning that the findings are not representative of flavonoid sources more generally, and do not inform us on potential chronic flavonoid effects in children. The non-significant outcome for this subgroup may be partially due to the limited number of studies (k = 5) coupled with the acute designs. The effects of flavonoids on cognition in children therefore remains unclear and warrants further research, particularly in foods outside of the berries group.

The subgroup analysis of cognitive health factor showed significant positive effects of flavonoids on cognition in both cognitively healthy and unhealthy subjects, but effect sizes were observed to be significantly greater for the latter population. This is likely due to greater scope for cognitive improvement in the cognitively unhealthy. These results are in keeping with meta-analyses which have shown cognitive benefits of supplementing with Gingko biloba in Alzheimer's disease and cognitive decline (Birks & Grimley Evans, 2009; Gauthier & Schlaefke, 2014; Savaskan et al. 2018; Tan et al., 2015; Weinmann et al., 2010.) Importantly, our findings show flavonoids are beneficial to the cognitive performance of cognitively unhealthy subjects, including patients with MCI and AD. This result is particularly pertinent in light of the increasing financial and social burden of

neurodegenerative diseases in an ageing population. The potential clinical significance of this outcome warrants further investigation. Furthermore, the potential for flavonoid supplementation to support the maintenance of cognition in healthy populations and prevent onset of neurodegenerative disease may in fact have greater impact than attempts to treat such conditions.

Analysis of the individual flavonoid dose categories showed significant positive effects on cognition following low and medium but not high doses. Low and medium dose studies comprised the majority of the analysed studies. Thirteen studies used a high-dose flavonoid intervention, representing 561 subjects. The reason for a lack of significant effects at high doses is unclear at this stage and warrants further investigation. The exact shape of the flavonoid-cognition dose-response curve is yet to be established. Studies which have investigated a dose-response, comparing a range of high, medium, or low doses with a control, have demonstrated some benefits for higher doses (e.g. Bell & Williams, 2019; Camfield et al., 2012; Desideri et al., 2012; Karabay et al., 2018; Kennedy et al., 2000; Mastroiacovo et al., 2015; Ong et al., 2016; Pase et al., 2013; Scholey et al., 2010), indicating that the cognitive effects of flavonoids may increase with increasing dose, however these dose-related effects are mainly restricted to cocoa and are yet to be fully investigated for other flavonoid sources. Indeed, possible reasons for this may be that high intakes are not recommended for some flavonoid sources such as ginkgo biloba, green tea, or soya due to the potential impact on the liver or endocrine system. Berries and citrus fruits may simply be difficult to consume in high amounts due to large volumes or side effects of high acidity and fibre. Methodologically, it should also be noted that while well-designed dose response trials impart valuable information in isolation, the statistical constraints of a meta-analysis preclude them from comparative subgroup analyses when combined with other single-dose investigations. Therefore, as performed here, combined dose findings from such studies can only be investigated at the individual dose level. Irrespective of these issues, our findings have clear implications for potential public health messages in that the positive effects of flavonoids are observed from doses achievable within habitual daily dietary intake ranges.

Analysis of the individual cognitive abilities showed significant beneficial effects of flavonoids on long term memory, processing speed and mood. Cheng et al. (2015) adopted a similar methodology to examine the effects of soya isoflavones on different cognitive abilities and found significant effects on visual memory only. In a comparative subgroup analysis Cui et al. (2020) showed significant effects of soya isoflavones on the memory domain, but also found that effect sizes were not significantly heterogeneous between domains, and therefore evidence of a particular cognitive domain showing greater sensitivity to flavonoid intervention was not demonstrated. Here, most studies included in this meta-analysis adopted a battery of cognitive tasks which assessed a range of cognitive abilities. As with dose, a comparative subgroup analysis to directly compare the effect of flavonoids on the different cognitive abilities was therefore not possible. Further investigations into the relative sensitivity of cognitive abilities to flavonoid intervention are needed. A consensus in the cognitive tasks adopted across intervention studies would help to achieve this by allowing the meta-analysis of data for specific tasks.

With respect to the positive outcomes for long-term memory and processing speed, these are likely to be products of the large number of studies included in the current meta-analysis to assess performance in these abilities. In relation to long-term memory, this is unsurprising given the focus of the majority of cognitive-flavonoid research has been placed on the older adult population, in which preservation or improvement of memory is of particular interest. Although memory appears to consistently respond to flavonoid supplementation, whether it shows greater sensitivity over other abilities remains inconclusive. The lack of positive findings for the cognitive abilities of word fluency, acquired knowledge, fluid reasoning, working memory/short-term memory, visuospatial ability and reaction/decision speed could be due to the relatively low number of studies in each subgroup, providing insufficient power to detect small effects. Additionally, it is also possible that

the tasks employed to assess each of these abilities may not have been sufficiently sensitive to detect subtle cognitive changes.

Finally, for the comparative analysis of crossover and parallel study designs, there was no significant heterogeneity observed between subgroups. Importantly, this addresses potential compatibility issues of including both types of design in a meta-analysis and provides justification for the adopted methodology. This result suggests that any potential underweighting of effect sizes in crossover studies was minimal, with little impact on the overall estimated effect size.

3.4.2 Summary and Conclusions

These meta-analyses provide evidence for the positive effects of flavonoids on cognitive function and demonstrates the moderating influence of a number of factors, including duration of supplementation and dietary source of flavonoid. It appears that for chronic effects, at least 6 weeks of supplementation may be required for benefits to manifest, and improvements may be more likely following either blueberry, cocoa or ginkgo supplementation. The potential of these and other flavonoid-rich foods to mitigate pathological cognitive decline in healthy individuals requires clarification in future research. Of the four comparative analyses performed, the only factor to demonstrate significant heterogeneity between subgroups was cognitive health status. Indeed, cognitive health status emerged as an important determinant of the magnitude of cognitive effects. Whilst significant benefits were seen across both cognitively healthy and unhealthy populations, they were most apparent in the cognitively unhealthy, with pre-existing cognitive deficits likely to facilitate the subsequent improvements seen in middle-aged and older adults. Absence of heterogeneity of effect sizes between flavonoid sources, duration of supplementation and age of participants subgroups could indicate a genuine absence of significant difference, or more likely, certain sub-groups are under-represented and, therefore, further high quality RCTs are required.

A strength of these meta-analyses is the extensive coverage of the existing literature, incorporating a wide range of flavonoid sources and populations, and allowing a detailed examination of a host of contributing variables. Reassuringly, there were few potential issues with bias in the literature, and the fail-safe N for the global analysis was high allowing confidence in the findings. Some limitations to this review include; studies were restricted to those published in English. The cognitive effects observed have been interpreted in relation to the flavonoid content of the interventions described. However, it is acknowledged that non-flavonoid polyphenols may also have contributed to these outcomes.

There were also a low number of studies which investigated citrus, soya, pine bark and tea, or that investigated the effects of flavonoids on cognition in children outside of blueberry supplementation. Therefore, there is scope for further examination in these areas with appropriate controls. Indeed, these meta-analyses highlight several areas where the number of quality studies are limited. It remains unclear whether flavonoids exert their effects on specific cognitive abilities or domains, and so future research should aim for a consensus on the cognitive tasks used, facilitating consistent investigation across a broad spectrum of cognitive abilities and allowing easy comparison between studies in meta-analysis or systematic review. Establishing effects specific to particular cognitive abilities would support better targeting of potential patient or consumer recommendations, for example, where cognitive health priorities may vary between age groups. Importantly, the need for well-designed dose-response studies to establish optimal flavonoid doses to better inform public health messages relating to flavonoid intake is apparent. Overall, the positive impact of flavonoid supplementation on cognitive health observed here presents the potential for a highly accessible, safe and cost-effective intervention programme to tackle the burden of cognitive decline. As outlined above particularly significant effects were ascribed to berry interventions, in healthy populations, older adults and at low doses. Therefore, in-keeping with the findings and recommendations of these meta-analyses, a dose and time response study investigating the acute

effects of an anthocyanin-rich blueberry extract in older adults was conducted. The outcomes of which are reported in Chapter 4.

Chapter 4: Randomised Older Adult Blueberry Trial – ROAB

4.1 Introduction

As discussed in Chapter 1, there is a growing body of evidence demonstrating the cognitive and mood effects of anthocyanin-rich berry fruits across the human lifespan in healthy and cognitively impaired patient populations, with blueberries receiving significant attention. For reviews see Bell & Williams (2021) and Lamport & Williams (2020). Findings from the existing research suggest that the cognitive domains showing sensitivity to berry flavonoid treatment vary with participant age (Bell et al. 2015). For example, in children, berry flavonoids have demonstrated positive effects on executive function (EF) and episodic memory (EM) measures (Barfoot et al. 2019, Barfoot et al. 2021, Whyte et al. 2015, Whyte et al. 2016 and Whyte et al. 2017) as well as mood (Khalid et al. 2017), whilst benefits to attention, working memory, executive function (Bell and Williams. 2020, Haskell-Ramsey et al. 2017, Hendrickson and Mattes. 2008, Philip et al. 2019, Watson et al. 2015, Watson et al. 2018 and Whyte et al. 2019) and mood (Khalid et al. 2017) have been evidenced in young adults. In comparison, in middle-aged adults, improvements to working memory, executive function and episodic memory have been reported (Lamport, Pal, et al., 2016 and Whyte et al., 2021). In older adults the majority of positive cognitive effects have been revealed for measures of EM (Bell & Willams. 2018; Dodd et a. 2019; Bensalem et al. 2018, Calapai et al., 2019, Chai et al. 2019, Miller at al. 2018, Whyte et al. 2018). The differences in observed cognitive effects between age groups may correspond to changes in cognitive development throughout the human lifespan. For example improvements to EM may be detectible in children when EM is evolving and in middle aged and older adults when memory is known to decline. A wider range of domains appear to be sensitive to berry flavonoid treatment in young adults compared to other age groups. However, this likely due to the more extensive testing that has been completed in this population.

Another key observation is that conditions which place increased cognitive demand on study subjects have elicited greater sensitivity to berry flavonoid intervention. This includes the more cognitively challenging trials of EF tasks, as demonstrated with incongruent and high load trials of the modified attention network task (Whyte et al, 2017, Whyte et al. 2019 and Barfoot et al. 2021). The more cognitively demanding aspects of the modified Flanker task (Whyte et al. 2016), picture matching task (Whyte et al. 2016) and Go/no-go task (Whyte et al. 2021) have also shown particular sensitivity to berry supplementation. Additionally, time-course studies have shown cognitive performance declines during the course of the day. The worsening of performance has been attributed to the development of cognitive fatigue, when cognitive reserves are reduced, as the day progresses. Berry treatments have shown to be effective at alleviating this temporal decline in cognition in children (Whyte et al. 2016), young adults (Whyte et al. 2019) and middle-aged adults (Whyte et al. 2021).

In older adults, randomised controlled trials have predominantly investigated chronic berry supplementation in both healthy older subjects and those affected by cognitive decline. Improvements to EM have been demonstrated following daily consumption of grape juice in healthy older adults (Bensalem et al., 2019 and Calapai et al., 2019) as well as subjects with mild cognitive impairment (MCI) (Krikorian 2010). Similarly, regular cherry juice consumption has also yielded positive effects on EM in older adults. In healthy older subjects, Chai (2019) showed chronic tart cherry juice supplementation was associated with improved episodic visual memory as well as visual sustained attention and spatial working memory. Further to this, Kent et al. (2017) found sweet cherry juice was effective at improving verbal fluency as well as short and long-term memory in older adults diagnosed with mild-to-moderate dementia. However, despite promising evidence for benefits of berry flavonoids to mood in children, adolescents and young adults (Khalid et al., 2017, Fisk et al., 2020 and Barfoot et al., 2018), investigations into the effects of chronic flavonoid-rich berry interventions have not yet shown positive effects on mood in older adults (Miller et al., 2018

and Whyte et al., 2018). More specifically, positive affect measured by PANAS was improved following acute blueberry supplementation (Khalid et al., 2017 and Barfoot et al., 2018) but not following chronic blueberry intervention (Whyte et al., 2018; Fisk et al., 2020). This suggests that in the longer term positive mood may be more stable to berry effects than short-term fluctuations during the course of a day. Following chronic blueberry supplementation, improvements to symptoms of depression were found in adolescents (Fisk et al., 2020) but not in older adults (Miller et al., 2018, Boespflug et al., 2018). Whether a true differential for the effects of berry treatment on depression between age groups exists remains unclear.

With regards to blueberry supplementation in particular, there is an expanding body of evidence in support of their cognitive benefits in older adults. In a small study of MCI patients, Krikorian et al. (2010) showed 12 weeks of daily blueberry juice improved memory function, measured using Verbal Paired Associate Learning Test and California Verbal Learning Test. However, this study compared blueberry effects with a placebo employed in a companion grape juice study and was not completely matched for non-polyphenol content to the blueberry juice. In a more recent study, Krikorian et al. (2020) again investigated the effects of blueberry supplementation in a small sample of older adults with MCI. This time extending the intervention period to 16 weeks, resulting in improvements to semantic access and visuospatial memory.

Miller et al. (2018) investigated the same daily dose of freeze-dried blueberry powder, 12g twice a day, equivalent to one cup of whole fruit per day, in a parallel-designed study. Thirty-seven healthy subjects, aged between 60 and 75 years completed the 90-day trial. The blueberry group exhibited fewer repetition errors in the CVLT and improvements to switch cost on a Switching task. Importantly, this study showed emerging evidence for beneficial effects of BB on aspects of executive function, extending previously observed effects in episodic memory. In keeping with

findings from berry trials in children, young adults and middle-aged adults, measures of EF were improved in the most challenging tasks.

To date, only one published trial has attempted to elucidate the effects of a short-term blueberry intervention on cognitive measures in older adults (Dodd et al., 2019). A relatively small sample of 18 healthy subjects aged between 60 and 75 years were included in a cross-over study. In addition to effects on cognition, cardiovascular outcomes including BP, arterial stiffness (digital volume pulse) as well as plasma brain-derived neurotrophic factor (BDNF), were measured. Cognitive function was recorded at baseline, 2h and 5h post-intervention and cardiovascular measurements were taken at baseline and 1h post-intervention. Supplementation with a blueberry beverage comprised of 30g freeze-dried blueberry powder mixed with milk, alleviated the decline in global cognitive scores, measured from baseline to 2 hours, seen with placebo. Also at 2 hours, the BB beverage produced improvements to immediate word recognition compared to placebo. With respect to cardiovascular effects, increases in blood pressure were seen with placebo across testing timepoints compared to baseline. Peak blueberry polyphenol metabolites have been measured between 1 – 2 hours and 6 hours post-intervention in plasma (Rodriguez-Mateos et al., 2013). Dodd and colleagues commented that the testing timepoint at 5h may therefore have been too early to detect cognitive changes resulting from the second metabolite peak. Indeed, work from our laboratory has shown cognitive effects of blueberry treatment in children (Whyte et al., 2015, Whyte et al., 2016, Whyte et al., 2017 and Barfoot et al., 2019) and middle-aged adults (Whyte et al., 2021) coincide with polyphenol metabolite peaks in plasma at between 1-2h and 6h post-consumption.

Further, whilst existing investigations into the acute cognitive and cardiovascular effects of blueberry supplementation have not shown a clear interplay between the two measures, Whyte et al. (2018) demonstrated benefits to both following chronic BB supplementation in older adults. Healthy older adults, aged between 65 and 80 years with self-reported memory complaints, were recruited to the

study by Whyte et al. (2018) and randomised to one of the four conditions; WBE 111, WBP 500, WBP 1000 or placebo. The interventions were formulated as capsules for consumption with dosage instructions to take two capsules with water every morning at breakfast for 6 months, making this one of the longer chronic trials ever completed. Cognitive, mood and cardiovascular outcomes (blood pressure and pulse rate) were measured at baseline, 3 months and 6 months. A cognitive task battery was employed to detect cognitive changes. This was comprised of Rey's Auditory Verbal Learning task (RAVLT), an object recognition task and the Corsi Blocks task, to measure effects on episodic memory. Outcome measures recorded for serial subtractions and Sternberg memory scanning were used to assess working memory. Executive function was evaluated using the Modified Attention Network Task (MANT) and Stroop task. Finally, subjective mood was assessed by the Positive and Negative Affect Schedule—NOW (PANAS-NOW). The results showed significant improvements to delayed word recognition in RAVLT and a trend towards significance for total number of sequences correctly recalled in the Corsi Blocks task at 3 months following WBE111. No other significant treatment-related effects were observed with respect to cognitive measures at 3 months or 6 months for WBE111. These findings add to literature that suggests episodic memory is the cognitive domain most sensitive to flavonoid supplementation in older adults (Bell et al. 2015). Significant reductions to systolic blood pressure (SBP) at 3 months, which were sustained at 6 months, were also recorded following WBE111 treatment.

Neither dose of whole blueberry powder (WBP 500 and WBP 1000) yielded any advantages to cognitive performance nor cardiovascular measures following either 3- or 6-months' treatment. It is plausible that the lack of positive results following WBP 500 and WBP 1000 are due to a dose-dependent relationship between blueberry supplementation and cognitive measures, as already demonstrated by Whyte et al. (2016) and associate FMD findings (Rodriguez-Mateos et al., 2013). The WBP500 and WBP1000 interventions provided 1.35mg and 2.7mg of anthocyanins respectively. WBE111 provided a relatively larger dose of 7mg anthocyanins. However, this is still a small dose,

particularly when compared with quantities of anthocyanins investigated in other blueberry and cognition research which have typically ranged from 143mg to 579mg (Whyte et al., 2015, 2016 and 2017, Barfoot et al., 2018, Boespflug et al., 2017, Bowtell et al., 2017, Dodd et al., 2019, Krikorian et al., 2010 and Miller et al., 2018).

The primary objective of this randomised, double-blinded, placebo-controlled study is to investigate the efficacy of wild blueberry extract in maintaining episodic memory throughout the day in older adults (68 -75 years). The cognitive effects of flavonoids may be most pronounced in older adult populations as revealed by meta-analysis (Chapter 3). Although there is a growing body of evidence for the positive effects of short-term berry flavonoid treatment on cognition, there is a paucity of acute data in ageing adults. Importantly, cognitive function measured by one-off neurocognitive testing is related to the ability of older adults to complete everyday tasks and therefore to live independently (Salthouse, 2012) and in this regard, may be indicative of overall wellbeing. The trajectory of healthy ageing shows a linear decline in memory and reasoning, often commencing during the 30s which then accelerates from 65 years onwards (Salthouse, 2019). Further, positive cognitive effects of anthocyanins have been demonstrated in adults aged over 68 years with MCI (Krikorian et al., 2012). Cognitive decline from baseline has been seen in 18.2% of older adults followed over three years (Comijs, Dik, Deeg, & Jonker, 2004; Weaver Cargin, Collie, Masters, & Maruff, 2008). Therefore, in order to achieve a relatively homogenous sample in relation to level of age-related cognitive decline the age range 68 – 75 years was chosen for this study.

Berry treatment may be particularly beneficial for cognitive function, as indicated by meta-analysis (Chapter 3). In particular, blueberries are a rich source of anthocyanins, the flavonoid group to which the cognitive effects of berries are attributed to and were selected for investigation in older adults based on the strong evidence of the cognitive benefits which already exists in young children. Specifically, wild blueberry extract (WBE) was selected for investigation due to the existing evidence

that supplementation with a dose of 111mg can improve episodic memory in RAVLT and Corsi Blocks task performance following chronic consumption (Whyte et al., 2018). It is therefore reasonable to examine the acute effects of this dose on EM.

The secondary objective of this study is to investigate the effect of the oral intake of wild blueberry extract in maintaining executive function and mood in older adults. Finally, the tertiary objective of this study is to determine the cardiovascular effects following wild blueberry extract in healthy older adults through measurement of systolic and diastolic blood pressure and heart rate. Whyte and colleagues showed beneficial effects of 3 months supplementation with WBE 111mg on cognitive outcomes and cardiovascular measures (Whyte et al., 2018). However, dose response relationships between WBE and its acute cognitive and cardiovascular effects have not been previously examined. Thus a range of WBE doses including 111mg tested by Whyte et al. (2018) will be investigated in order to identify the optimal dose at which cognitive and cardiovascular effects occur and to establish whether these effects are dose-dependent in nature (111mg, 222mg, 444mg and 888mg). Indeed, in young adults, plasma concentrations of blueberry flavonoids were shown to be time and dose-dependent (Rodriguez-Mateos et al., 2013). These were observed at between 1 and 2 hours, with a second peak at 6 hours following oral administration. Corresponding increases in FMD were found in a similar time-dependent manner post-intervention. The vasodilatory effects of anthocyanins which may underpin both cardiovascular and cognitive mechanisms (as reviewed in Chapter 1) indicate cognitive benefits may be most likely detected between 1-2 hours and 6 hours. Indeed, this has been found following wild blueberry supplementation in children (Whyte et al., 2015, Whyte et al., 2016, Whyte et al., 2020 and Barfoot et al., 2019). Although a similar study in older adults has not been completed, the findings by Rodriguez-Mateos et al. (2013) give some indication of the metabolites which may be present in the circulatory system following blueberry treatment in an older adult sample. A test day schedule to encompass the timings of these observed

peak plasma concentrations of blueberry flavonoids and FMD was therefore adopted with cognitive assessment and cardiovascular measures at 2 hours, 4 hours and 6 hours.

Age-related changes in cognitive performance are particularly pronounced in the cognitive domains of memory, executive function, processing speed and reasoning (Deary et al., 2009). Testing of the effects of anthocyanins on episodic memory (EM), working memory (WM) and executive function (EF) is therefore of particular focus here. A cognitive battery was developed to assess these domains of cognitive function according to demonstration of sensitivity to flavonoid effects. Specifically, WBE 111 supplementation was shown to improve performance in Rey's Auditory Verbal Learning task (RAVLT) and Corsi Blocks task (Whyte et al., 2018) therefore episodic memory will be assessed by RAVLT (verbal episodic memory) and Corsi Blocks task (spatial episodic memory).
Executive function will be measured using Switching task, and Trail Making Tasks A and B. Previously, consumption of a blueberry supplement was associated with reduced switch cost in the Switching task by Miller et al. (2018). TMT-A and TMT-B has shown sensitivity to flavonoid intake in both healthy older adults and MCI patients (Gavrilova et al., 2014, Desideri et al., 2012 and Mastroiacovo et al., 2015). Choice Reaction Time performance has been found to improve following acute flavonoid supplementation (Kennedy et al. 2007, Field et al. 2011). Therefore alertness and motor speed will be assessed using Choice Reaction Time. Mood will be assessed by the Positive and Negative Affect Schedule (PANAS) questionnaire as per methods by Khalid et al. (2017) and Fisk et al. (2020).

It is predicted that improved or maintained performance will be detected in measures of episodic memory and executive function after WBE consumption, compared with placebo. The acute effects of blueberry supplementation on mood in older adults is yet to be established. This study seeks to elucidate the impact of WBE on negative and/or positive affect and to identify potential benefits to mood throughout the day. Systolic blood pressure, diastolic blood pressure and heart rate are

expected to be reduced following WBE compared with placebo, specifically between-treatment differences will be most evident at 2h and 6h post-intervention when vasodilatory effects have shown to peak (Rodriguez-Mateos et al., 2013).

4.2 Methods

4.2.1 Participants

This study was reviewed and given a favourable ethical opinion for conduct by the University of Reading Research Ethics Committee (UREC 18/04).

4.2.1.1 Determination of Sample Size

To determine the minimum number of participants needed to achieve a power of 0.8, G*Power 3.1 software was used to conduct an a-priori analysis. Whyte et al. (2018) showed an effect of d = 0.3 on RAVLT delayed word recognition following chronic supplementation with 111mg WBE, therefore an effect size of d = 0.3 was used with an alpha level of 0.05 (Cohen, 1992) in the power analysis. This indicated that 27 participants would be required to detect an improvement in cognitive performance following WBE compared with placebo.

4.2.1.2 Recruitment

Healthy older adults aged 68 – 75 years from Reading, UK and the local area were recruited via the Psychology Department's Older Adult Panel, local community noticeboards, the University of the Third Age and other older adult activity groups. Forty volunteers attended a familiarisation visit, after which twelve withdrew because of the demands of the task battery and length of the test day.

Twenty-eight participants progressed to randomisation and completed all stages testing. Participants received an expenses payment of £70 on completion of testing. See figure 4.1 for consort diagram of the recruitment stages.

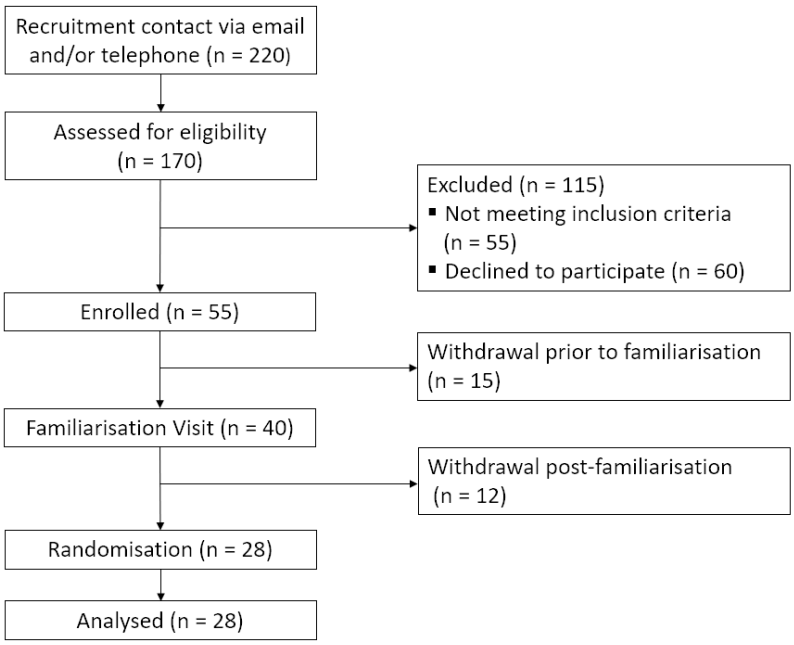


Figure 4. 1: Consort diagram showing subject numbers at each stage of recruitment and participation in the study

4.2.1.3 Subject Information

4.2.1.3.1 Screening at recruitment

Screening for suitability of participants was completed prior to enrolment to the experiment according to the inclusion and exclusion criteria outlined in Table 4.1. Weight and height measurements were taken for each participant at the beginning of the practice visit. Weight was recorded using Salter digital scales in kilograms. To avoid overestimate of weight measurements, participants were asked to remove their shoes, jackets or coats and to empty their pockets of

objects, such as keys and mobile phones. Height in metres, was recorded using a stadiometer. These were used to calculate BMI using the formula: BMI = Weight (kg)/Height $(m)^2$.

Table 4. 1: Inclusion and exclusion criteria used to screen subjects prior to enrolment to the study.

Inclusion Criteria	Exclusion Criteria
Overall healthy volunteers of all ethnicities	Use of complementary and alternative medicine for memory or cognitive performance within 1 month prior to study participation
Age: 68-75 years	History of metabolic disorder, diabetes, substance abuse or diagnosis of psychiatric or neurological conditions
Willing to maintain their normal dietary and exercise habits to avoid changes in body weight over the course of the study	Allergic reactions to compounds similar to those in the investigational product
Able to understand the nature of the study and able to give signed written informed consent.	Drinking more than 2 alcoholic beverages per day on average in a week
	Smokers
	BMI greater or equal to 30
	Vegetarians
	Participation in other clinical trials within the previous month or other cognitive trial within the previous 6 months

A copy of the health questionnaire used for screening can be found in Appendix B.

4.2.1.3.2 Demographics

Demographic data for all 28 participants included in the analyses are summarised in Table 4.2.

Table 4. 2: Subject demographic information

Baseline Characteristics	**Mean**	**SD**	**Range**
Age at enrolment (y)	70.71	2.14	68-75
Gender	11M/17F	N/A	N/A
BMI (kg/m^2)	24.51	3.30	19.94-30.95
MMSE	27.9	1.58	24-30
NART (number of errors)	9.0	5.11	2-20
Frequency of Forgetting 1	5.01	0.75	3.8-6.5
Frequency of Forgetting 2	5.22	1.10	3.0-7.0
Raven's Progressive Matrices	50.21	5.86	39-60
YALE physical activity survey – energy expenditure (kcal/week)	5519	3798.36	1345-17700
Energy consumption (kcal/day)	1772	632.85	765-4005
Fruit consumption (g/day)	291	172.61	50-789
Vegetable consumption (g/day)	299	179.15	53-1028

4.2.1.4 Informed Consent

All prospective participants were provided with the study information sheet via e-mail or post, prior to screening. This gave detailed information about the procedures involved in the experiment (see Appendix C). Individuals who then opted to take part in the experiment were required to give signed consent at the start of their first familiarisation visit. See Appendix D for a copy of the consent form.

4.2.1.5 Low Polyphenol Diet

During the 24 hours prior to each test visit, participants were required to adhere to a low polyphenol diet and to abstain from consuming caffeinated beverages and alcohol. Participants also fasted for 12 hours before attending each test visit. On arrival at 08.00h subjects received a standardised low polyphenol breakfast of two croissants (Total Energy 338kcal, Fat 18.2g, Carbohydrate 38g, Protein 5.2g), with water to drink. This procedure was adopted to limit the effects of prior polyphenol consumption on the cognitive and cardiovascular outcome measures. A copy of the guidance on a low polyphenol diet provided to participants can be found in Appendix E.

4.2.2 Study Design

4.2.2.1 Overall Study Design and Plan

The study employed a randomized, double-blinded, placebo-controlled, cross-over design comprised of seven visits, including two screening/familiarisation visits (one and two weeks prior to test day 1). A cross-over design was employed to reduce the impact of individual variation when comparing the WBE with the placebo. In order to control for confounding due to practice effects, participants attended familiarisation visits to acquaint themselves with the cognitive task battery prior to

receiving the interventions and testing. Randomisation of the order in which interventions were consumed further minimised practice effects. Baseline measurements were taken to allow consideration of any variance in cognitive performance or cardiovascular outcomes at baseline between conditions. Additionally, a suitable control condition matched for appearance and taste was employed.

At the screening/familiarisation visits all participants completed a number of questionnaires (as below) to check for the inclusion/exclusion criteria.

- Mini-mental state examination (MMSE): to give a measure of global cognitive function (Folstein, Folstein, & McHugh, 1975)
- National Adult Reading Test (NART): to give a measure of premorbid intellectual functioning (Nelson & Willison, 1991)
- Frequency of Forgetting Questionnaire (Zelinski & Gilewski, 2004)
- Ravens Progressive Matrices questionnaire (Raven & Court, 1998)
- YALE physical activity questionnaire (Dipietro, Caspersen, Ostfeld, & Nadel, 1993)

Subjects attended the lab on seven separate occasions each separated by a 7-day washout period. Exceptions were permitted to allow for unplanned events.

4.2.2.2 Treatments

All treatments were administered as three beige opaque size 1 capsules. Four different doses (111 mg, 222 mg, 444 mg and 888 mg) of a patented formulation consisting of wild blueberry powder (89% extract), L-Cysteine (10%) and L-Glutathione (1%) were used. The wild blueberry extract was obtained from the Vaccinium angustifolium cultivar using a proprietary process. The contents of the placebo capsules consisted of the inert components of the wild blueberry formulation, namely

artificially coloured maltodextrin. All capsules were provided by Naturex and stored under dry conditions at room temperature.

The antioxidants, L-cysteine and L-glutathione, were included in the formulation of the WBE to enhance the stability of the blueberry anthocyanins and therefore increase anthocyanin bioavailability. This is important because anthocyanins are inherently unstable compounds which undergo significant breakdown during manufacturing processes and whilst in storage. For example, baking at 180^{o}C for 12 minutes was found to reduce anthocyanin content of freeze-dried blueberry powder by 42% (Rodriguez-Mateos, Heiss, Borges, & Crozier, 2014). The process of cabinet drying involving exposures to temperatures between 50^{o}C and 90^{o}C for 4.5 hours resulted in 41% anthocyanin degradation in blueberries compared to fresh fruit (Lohachoompol, Srzednicki, & Craske, 2004). Even the process of freeze-drying, which is employed to maintain anthocyanin content, was shown to reduce anthocyanin concentration by between 11% and 27% compared to blueberry juice (Darniadi, Ifie, Ho, & Murray, 2019). Anthocyanin degradation was found to range from 3% after storage at room temperature (25^{o}C) for 2 weeks to 85% loss at 80^{o}C for 3 days (Fracassetti et al., 2013). Anthocyanins are particularly susceptible to catabolism in aqueous forms and under increasing pH environments, such as those found along the gastrointestinal tract. Therefore, stabilisation of anthocyanins at neutral pH is likely to increase bioavailability by reducing their breakdown during transit through the intestine (pH 4-7) to enable higher anthocyanin levels arriving at the colon for microbial metabolism. Anthocyanins are also prone to thermal and oxidative degradation. The addition of thiol-containing antioxidants, such as L-cysteine and L-glutathione, are purported to protect anthocyanins from oxidation through interactions between the thiol group and the anthocyanins at position 4 of the C-ring. Secondly, the carboxylic acid functional group of L-cysteine and L-glutathione protect the cation part of the anthocyanin. See figure 4.2 for diagrammatic depiction of the interaction between anthocyanins and L-cysteine.

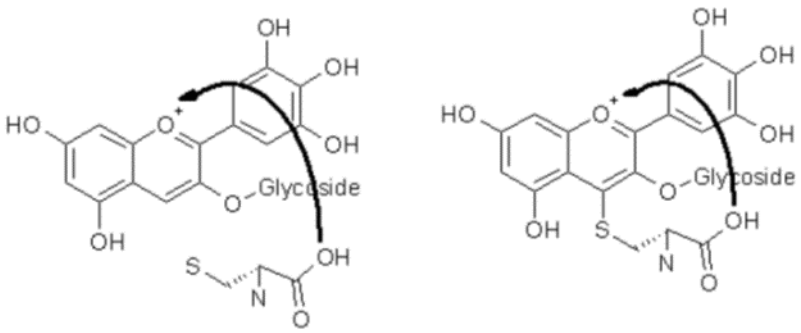


Figure 4.2: Schematic representation of the putative interaction between anthocyanins and L-cysteine adapted from Eisenberger 2014.

Indeed, glutathione has been shown to effectively stabilise blackberry anthocyanins (Stebbins, Howard, Prior, Brownmiller, & Mauromoustakos, 2017) and anthocyanins from extracts of grape, black carrot, red cabbage, blackberry, blackcurrant and cranberry; specifically these were shown to be successfully stabilised by L-cysteine at 37^{o}C at pH 7, with over 50% of the initial anthocyanin content maintained at 4 hours (Cortez, Luna-Vital, Margulis, & Gonzalez de Mejia, 2017). Evidence from Rodriguez-Mateos et al. (2013) showed that peak plasma concentrations of blueberry polyphenol metabolites coincided with the vasodilatory response in a dose and time-dependent manner at between 1-2 hours and 6 hours post-intervention. It would be reasonable to expect the amplitude of these peaks to be increased by stabilisation of anthocyanins in WBE.

Table 4. 3: Intervention Arms Included in the Study

Treatment Group	Intervention
Placebo	**Formulation containing inert artificially coloured maltodextrin, once daily, in a 3-hard capsule regimen**
WBE- 111 mg	**Formulation containing 100 mg wild blueberry extract + 11 mg L-Cysteine + 1 mg L-Glutathione, once daily, in a 3-hard capsule regimen**
WBE- 222 mg	**Formulation containing 200 mg wild blueberry extract + 22 mg L-Cysteine + 2 mg L-Glutathione, once daily, in a 3-hard capsule regimen**
WBE - 444 mg	**Formulation containing 400 mg wild blueberry extract + 44 mg L-Cysteine + 4 mg L-Glutathione, once daily, in a 3-hard capsule regimen**
WBE- 888 mg	**Formulation containing 800 mg wild blueberry extract + 88 mg L-Cysteine + 8 mg L-Glutathione, once daily, in a 3-hard capsule regimen**

4.2.2.3 Method of Assigning Subjects to Treatment Order

Following the two practice visits, participants were randomised to receive either placebo, wild blueberry extract 111 mg, 222 mg, 444 mg, or 888 mg according to participant number. The randomisation was carried out using a computerised random number generator (www.random.org) by NATUREX-DBS. The investigators and the participants remained unaware of the relationship between participant number and the order of the doses. The study was therefore double-blinded.

4.2.3 Cognitive Tests

Alternate versions of each of the cognitive tasks were created. Latin square counterbalancing was used to generate the order of presentation across test sessions to limit learning.

4.2.3.1 Primary Endpoints: Episodic Memory Tests

4.2.3.1.1 Rey's Auditory Verbal Learning task (RAVLT).

This task targeted verbal episodic memory (Lezak, Howieson, Loring, & Fischer, 2004). Participants were played a pre-recorded audio of a list of 15 words (list A), read with one second intervals between each word, proceeded by an immediate free recall. List A was presented on five consecutive occasions (recalls 1-5). An interference list consisting of 15 new words (list B) was then played to the participants followed by an immediate free recall (recall B). After a short delay of 2 minutes and long delay of 25 minutes (after completion of the further tasks described below) participants recalled list A (recalls 6 & 7). To conclude, participants were visually presented with all the words from lists A and B, plus 20 additional filler words. Participants were asked to identify words from list A only (word recognition). Separate word lists were generated for presentation across different test sessions to account for the crossover study design. The word lists consisted of fifteen nouns, made up of nine one-syllable and six two-syllable words. All word lists were matched for familiarity (scores between 525 and 599) and concreteness (scores between 549 and 599). Each set of filler words were chosen to match 15 words from list A in terms of number of syllables, eight of which matched semantically and seven matched phonetically. Five words matched list B for syllables of which three matched semantically and two phonetically. The different versions of the word lists were presented in a counterbalanced order across test sessions. The following outcome measures were assessed according to Lezak et al. (2004): total acquisition (sum recalls 1 through to 5), immediate word span (recall 1), proactive interference

(recall 1 minus recall B), retroactive interference (recall 5 minus recall 6), delayed recall (recall 7) plus accuracy and speed of word recognition (number of words correctly identified).

4.2.3.1.2 Corsi Blocks task.

This task targeted spatial episodic memory (Berch et al., 1998; Busch et al., 2005). Nine stationary square white blocks were presented to participants on the computer screen with a black background. Participants were required to observe a sequence in which the blocks changed in colour from white to red at a rate of one block per second. At the end of the sequence, the instruction 'Go' on the screen instructed the participants to start repeating the sequence back in the same order. The left button on the computer mouse was used for block selection. Sequence length ranged from two to nine blocks, giving eight levels of difficulty. Subjects received 4 trials per level which were randomised across the session with 32 trials in total. In accordance with Whyte et al. (2018), measures of the number of correctly reproduced sequences, the longest sequence correctly remembered and first block latency (time taken to commence repeating of the sequence) were recorded.

4.2.3.2 Secondary Endpoints: Executive Function Tests

4.2.3.2.1 Switching Task (Miller et al., 2018).

Participants viewed a circle divided into eight equal segments, with one bold line across the diameter bisecting the circle. Numbers from 1,2,3,4,6,7,8 and 9 were chosen randomly and presented in a clockwise direction in the circle segments for 3000ms with an inter-stimulus interval of 500ms. For trials below the bold line participants were required to identify numbers lower than 5 using the left arrow key, and numbers higher than five using the right arrow key on the computer keyboard (See Figure 4.3a-b for examples of below the bold line trials). On presentation of the number above the

line, participants were required to identify odd numbers using the left arrow key and even numbers using the right arrow key (See figure 4.3c-d for examples of above the bold line trials). The task switched every four trials, with the switch sequence denoted as S1, S2, S3 and S4. One cycle was comprised of eight trials with 48 cycles presented to participants. Measures of accuracy (correct responses out of total trials as quotient) and response time for correct responses (ms) for task and switch sequence were acquired separately according to Whyte et al. (2019).

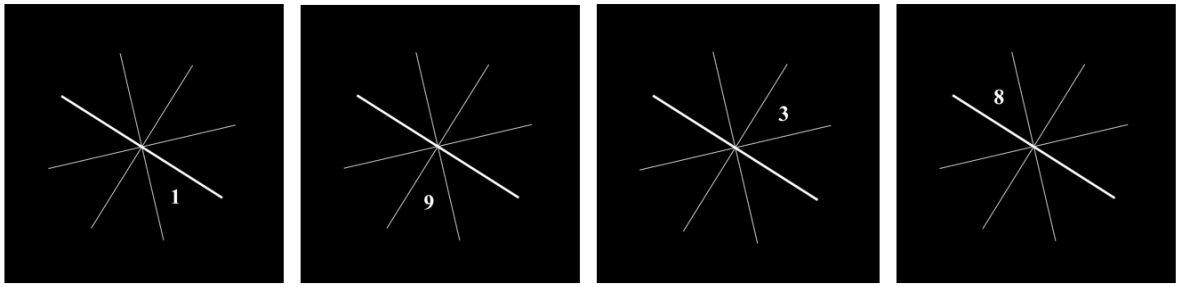


Figure 4. 3: Screen captures of Switching Task display.

Switching task trials below the bold line require identification of numbers a. lower than 5 or b. higher than 5.

Switching task trials above the bold line require identification of numbers c. odd or d. even

4.2.3.2.2 Trail Making Tasks A & B

Twenty-five circles were presented on the computer screen in a mock random arrangement. In TMT-A, the circles contained the numbers 1 – 25. Participants were required to click, using the computer mouse, on the circles in ascending order. In TMT-B, 13 circles contained the numbers 1-13 and 12 circles contained the letters A-L. Participants were asked to click the cursor on circles, alternately on numbers and letters, with numbers in ascending order and letters in alphabetical order. Time taken to complete TMT-A was recorded (ms), as a measure of visual search and motor speed. Time to complete TMT-B (ms) was taken as a measurement of mental flexibility. Both TMT-A and TMT-B were not time restricted. (Lezak, 1995; Mitrushina et al., 2005; Strauss et al., 2006).

4.2.3.2.3 Choice Reaction Time.

A fixation 'X' appeared on the screen for 1000ms between presentations of the stimulus 'X' on the right or left-hand side of the screen. Interstimulus intervals of 250ms, 500ms, 750ms, 1000ms and 1500ms, were randomised across the session comprised of 60 trials. Participants were required to respond as rapidly as possible by pressing the z key for stimuli appearing on the left or the m key for stimuli appearing on the right of the screen. The response time for correct responses (ms) and accuracy (correct responses out of total trials as quotient) were taken giving measures of general alertness and motor speed (Posner, Klein, Summers, & Buggie, 1973).

4.2.3.2.4 Composite Scores

Cognitive composite scores are used to combine cognitive tests, mainly in the assessment of onset or progression of dementias in older adults such as Alzheimer's disease, ADCOMS, Alzheimer's Disease Composite Score (Wang et al. 2016) and ZAVEN, Z-scores of Attention, Verbal fluency, and Episodic memory for Nondemented older adults composite (Lim et al. 2016) and have shown sensitivity to detect pre-clinical cognitive decline (Donohue et al. 2014). While most cognitive composite scores include multiple domains, they have also been used to target specific cognitive domains or constructs, for example AIBL-EM (The Australian Imaging, Biomarkers and Lifestyle study episodic memory composite score) (Lim et al. 2014).

In the current study, by combining test results from a number of neuropsychological assessments, the risk of type I error is reduced whilst increasing the power to detect a change in behaviour in a relatively small sample. The Global Composite score was calculated based on a combination of all the above measures. The effects on specific cognitive domains were measured by grouping outcomes according to either episodic memory or executive function. Composite Episodic Memory

combined scores for CORSI longest sequence remembered, CORSI total number of correct sequences remembered, CORSI latency, RAVLT word recognition accuracy, RAVLT delayed word recall accuracy, foil words rejected, RAVLT words learned, RAVLT total recall, RAVLT PI, RAVLT RI and RAVLT word recognition reaction time, CRT accuracy, Switching Task accuracy, TMT B-A, TMT A, TMT- B, CRT, Switching Task reaction time were used to calculate the Composite Executive Function score.

The effects on accuracy and reaction times were assessed separately. The Composite Accuracy score comprised of measurements of CORSI longest sequence remembered, CORSI total correct number of sequences remembered, RAVLT word recognition accuracy, RAVLT delayed word recall, foil words rejected, RAVLT words learned, RAVLT total recall, RAVLT PI, RAVLT RI, CRT accuracy and Switching Task accuracy. The Composite Reaction Time score was made up of Switching Task reaction time, TMT B-A, CRT reaction time, RAVLT word recognition reaction time, CORSI latency, TMT- A and TMT-B.

The composite scores were calculated as described by Andrade (2021). The raw data for each of the cognitive outcomes was transformed by standardising according to the standard deviation from the mean of the current study sample, giving Z scores per subject for each dependent variable. For tests whereby a positive score represents a negative effect on cognition, such as reaction times, Z scores were multiplied by -1 to reflect the direction of change. Equal weighting was given to each of the component outcomes and the mean average of the Z scores was calculated to give the combined score.

4.2.3.2.5 Mood

4.2.3.2.5.1 PANAS

The PANAS questionnaire was administered at the end of each cognitive test session to assess any changes in mood (Watson et al. 1988). The questionnaire consists of twenty mood-related items, 10 which measure positive affect and 10 which measure negative affect. For each item, participants were asked to rate their present mood against a 5-point Likert scale marked from '1 - Very slightly or not at all' at the far left to '5 - extremely' at the far-right end. A Positive Affect Score and a Negative Affect Score were generated by summing the ratings for positive and negative mood items, respectively. The possible scores ranged between 10 and 50, with a higher score corresponding to a greater level of positive or negative affect.

4.2.4. Cardiovascular Measures

4.2.4.1 Tertiary Endpoints: Blood Pressure and Heart Rate

Blood pressure and heart rate readings were measured using an Omron M6 Comfort automatic digital blood pressure monitor (Omron Healthcare UK Ltd). Measurements were taken as per the manufacturer's instructions. All readings were taken with the cuff applied to the left upper arm between 1 and 2 cm above the elbow. Participants remained in a seated position with their feet flat on the ground and their arm supported by resting on an adjacent desk. A mean average of three consecutive readings separated by a 2-minute interval was recorded. Blood pressure and heart rate were measured at the end of the test battery when participants had been seated for 45 - 60 minutes.

4.2.5. Procedure

The experiment was conducted at the Nutritional Psychology Unit at the University of Reading. Participants completed testing while isolated in individual booths. On all visits to the laboratory including practice visits, on arrival at 08.00h subjects received a standardised low polyphenol breakfast of two croissants. At 08.30h subjects then completed a baseline test battery consisting of cognition, mood, and blood pressure measures. Testing took place in the following order: RAVLT (immediate recall), Choice Reaction Time, Corsi Blocks task, Trail Making tasks A and B, Switching Task and PANAS, blood pressure and heart rate. The intervention, or placebo on practice visits, was administered in the form of three oral capsules immediately after completion of the baseline test battery. Participants repeated the test battery at 2 hours, 4 hours and 6 hours post-intervention. The two practice visits were structured to replicate test visits to allow familiarisation of participants with test day timeline and tests.

A standardised low polyphenol lunch consisting of a sliced white bread cheese sandwich and a packet of ready salted crisps was consumed immediately after the 2-hour post-intervention test battery. See figure 4.4 for study design and intervention day schedule.

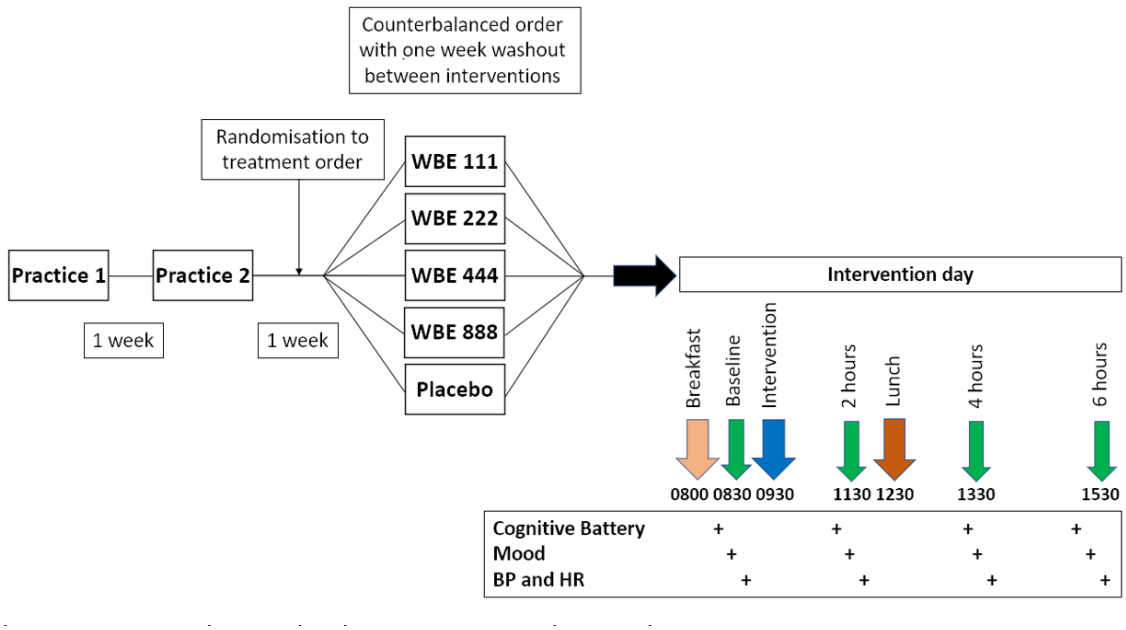


Figure 4. 4: Study design and timeline of procedure on intervention days

4.2.6 Statistical and Analytical Plan

All data were analysed using IBM SPSS statistics version 24. Z score analysis was used to identify outliers; datapoints with z score>3.29 were removed prior to statistical analysis (Tabaschnick & Fidell, 2013). A linear mixed model (LMM), using an unstructured covariance matrix to model successive repeat test sessions, was used to analyse data for all measures. This statistical approach has been adopted in previous berry cognitive studies (Barfoot et al., 2019 and Bell and Williams. 2019). Baseline performance was included as a repeated covariate. Visit, Dose and Session (time post intervention) were included as fixed factors in the model. Visit was included in the model to minimise any residual practice effects(Bell, Lamport, Field, Butler, & Williams, 2018). The aim of the analysis plan was to determine whether dose of Wild Blueberry extract was a significant predictor of cognitive performance, mood or blood pressure. Post hoc pairwise comparisons were used to investigate any significant effects of Dose. A Sidak correction was applied to all multiple comparisons. Significant comparisons are reported ($p<0.05$). For the Switching task only, the additional factors of task and

switch sequence, their respective dose interactions and dose by session interactions, were added to the LMM analysis.

4.3. Results

4.3.1 Episodic Memory

4.3.1.1 RAVLT

Dose

Dose was a significant predictor for Retroactive Interference (RI: recall 5 minus 6) [$F(4,211.113) = 3.001$, $p = 0.019$] but not Proactive Interference (PI: recall 1 minus B). Pairwise comparisons revealed RI was significantly lower with WBE 444 than WBE 111 ($p = 0.015$) although there were no significant differences in RI between any of the wild blueberry extract doses compared with placebo. Dose was a significant predictor for Word Recognition Reaction Time [$F(4,304.262) = 5.793$, $p < 0.001$]. Pairwise comparisons showed Word recognition RT with WBE 444 was significantly faster than placebo ($p = 0.001$), WBE 111 ($p= 0.001$) and WBE 888 ($p = 0.004$). The results are presented in figure 4.5.

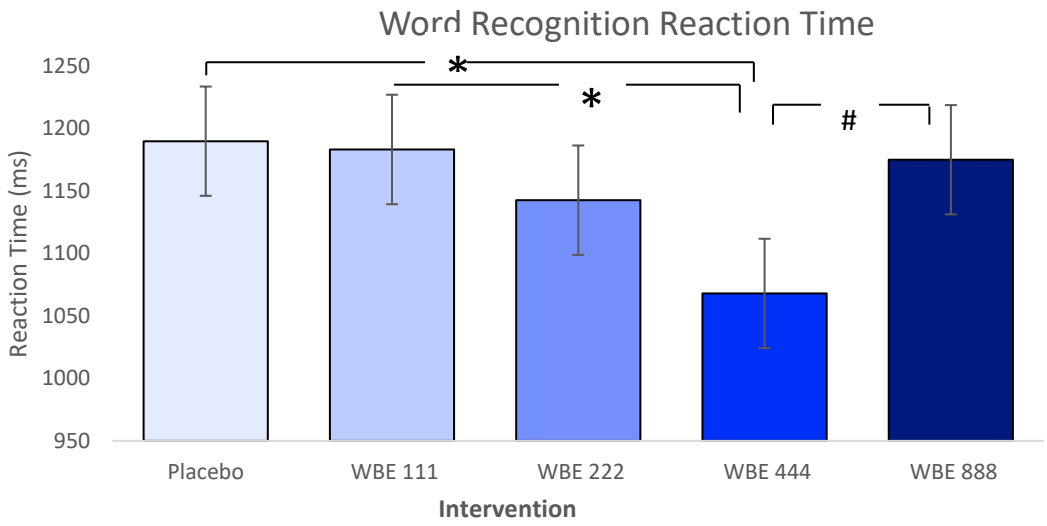


Figure 4. 5: Mean reaction time performance (± SE) for AVLT Word Recognition as a function of intervention, showing significantly faster reaction times with WBE 444 than placebo, WBE 111 and WBE 444. (* p = 0.001, # p = 0.004)

Session

Session (time post intervention) was a significant predictor for Delayed Word Recall [$F(2,52.282) = 13.944$, $p < 0.001$]. Pairwise comparisons revealed significantly more words were recalled at 2 hours post-intervention than at 4 hours ($p < 0.001$) and 6 hours ($p = 0.001$). Session was a significant predictor of Foil Words rejected [$F(2,53.674) = 5.729$, $p = 0.006$], where pairwise comparisons showed significantly fewer foil words were rejected at 4 hours post-intervention than at 2 hours ($p = 0.004$). Session was a significant predictor of Total Recall [$F(2,54.989) = 4.097$, $p = 0.022$]. Pairwise comparisons showed that performance at 2 hours was significantly better than at 4 hours ($p = 0.040$). Session was also a significant predictor of RI [$F(2,53.634) = 3.410$, $p = 0.040$]. Pairwise comparisons showed significantly greater interference was seen at 4 hours than at 2 hours ($p = 0.035$). The effects of session indicate a decline in cognitive performance during the course of the day, particularly at the 4-hour post-intervention timepoint.

Dose*Session

There were no significant Dose by Session interactions with RAVLT.

Visit

Visit was a significant predictor of Delayed Word Recall [F(4,75.867) = 3.163, p = 0.018] and Foil Words rejected [F(4,76.161) = 3.328, p = 0.014]. Pairwise comparisons showed no significant differences between visits for Delayed Word Recall and significantly improved performance in correctly rejecting Foil Words during Visit 4 when compared with Visit 3 (p = 0.018).

4.3.1.2 Corsi Blocks

Dose and Session were not significant predictors for the CORSI Blocks task. There were no Dose by Session interactions for CORSI.

Visit

Visit significantly predicted Longest sequence [F(4,79.691) = 4.465, p = 0.003], where pairwise comparisons showed performance during Visit 1 was significantly lower than each of Visit 2 (p = 0.043), Visit 3 (p = 0.036), Visit 4 (p = 0.017) and Visit 5 (p = 0.002). Total Correct sequences [F(4,81.592) = 8.892, p < 0.001] were significantly predicted by Visit. Pairwise comparisons showed performance was significantly improved during Visit 3 (p = 0.002), Visit 4 (p < 0.001) and Visit 5 (p < 0.001) over performance during Visit 1. Performance during Visit 5 was also significantly better than for Visit 2 (p = 0.015). Visit was a significant predictor of CORSI – Latency [F(4,82.212) = 2.716, p = 0.035]. Pairwise comparisons showed significantly slower reaction times for Visit 1 than Visit 3 (p =

0.025) and Visit 4 (p = 0.033). Here, performance was generally improved with later visit, particularly when compared with Visit 1, which suggests practice effects are being demonstrated for CORSI in the absence of treatment effects.

4.3.2 Executive Function

4.3.2.1 TMT A and B

Dose and Session were not predictors for TMT A and TMT B. There were no Dose by Session for interactions for TMT A, TMT B or TMT B-A.

Visit

Visit was a significant predictor of TMT B-A - [F(4,76.890) = 9.359, p < 0.001]. Pairwise comparisons showed reaction times were significantly faster for Visit 5 than for Visit 1 (p < 0.001). TMT B was also significantly predicted by Visit [F(4,80.425 = 13.838, p < 0.001]. Here, pairwise comparisons showed significantly slower reaction times at Visit 1 than Visit 4 and 5 (ps < 0.001). Much like with CORSI, practice effects are evident in the absence of treatment effects with better performance in TMT- B and TMT B-A in the later visits.

4.3.2.2. Choice Reaction Time

Dose

CRT RT was significantly predicted by Dose [F(4,1365.920) = 2.745, p = 0.027]. Pairwise comparisons showed significantly faster RT was seen with WBE 888 than WBE 444 (p = 0.029) but there were no

significant differences between any of the wild blueberry extract doses compared with placebo. See figure 4.6 for representation of results.

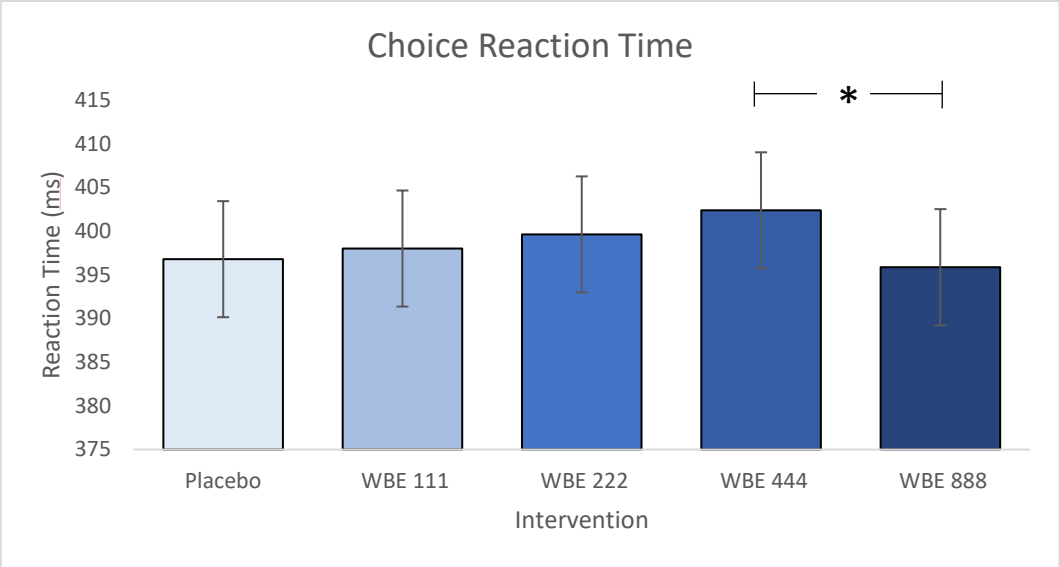


Figure 4. 6: Mean reaction time performance (± SE) for Choice Reaction Time as a function of intervention, showing significantly faster reaction time with WBE 888 than WBE 444. (* p = 0.029). No significant differences between WBE doses and placebo.

Session

Session (time post-intervention) was a significant predictor for CRT Accuracy [F(2,474.828 = 3.284, p = 0.038]. Accuracy was significantly higher at 2 hours than 6 hours (p = 0.039). No Dose by Session Interactions nor Visit Order effects were observed.

4.3.2.3 Switching Task

Dose

Dose was a significant predictor of Switching task Accuracy [F(4,1832.846) = 2.501, p = 0.041] and Switching task RT [F(4,1673.441) = 8.757, p < 0.001]. Pairwise comparisons showed no significant differences between doses for accuracy. However, significantly faster reaction times were measured for WBE 111 than for placebo (p = 0.018), as well as WBE 222 (p < 0.001), WBE 444 (p < 0.001) and WBE 888 (p = 0.033). See figure 4.7 for results.

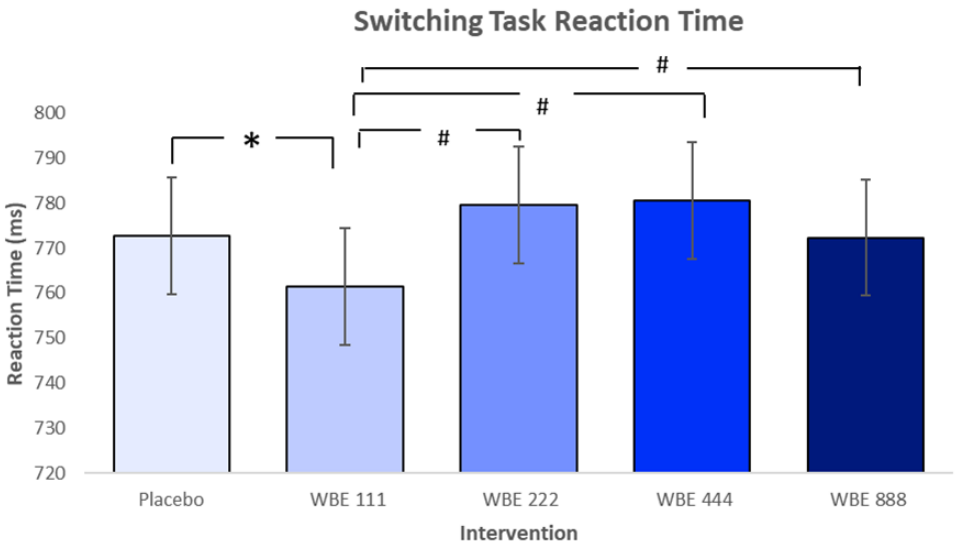


Figure 4. 7: Mean reaction time performance (± SE) for Switching Task as a function of intervention, showing significantly faster reaction times with WBE 111 than placebo, WBE 222, WBE 444 and WBE 888. (* p = 0.018, # p < 0.001, • p = 0.033)

Session

Switching task Accuracy [$F(2599.670) = 9.819$, $p < 0.001$] and Switching task RT [$F(2,439.536) = 5.115$, $p = 0.006$] were significantly predicted by Session. Pairwise comparisons showed accuracy was significantly lower at 4 hours than at 2 hours ($p < 0.001$) and 6 hours ($p = 0.015$). The effects of session are similar to those found with RAVLT, where performance was particularly reduced at the 4 hour post-intervention timepoint, in line with known periods of cognitive fatigue during the time-course of the day. There were no significant differences between sessions for RT.

Dose by Session

There was a significant dose by session interaction for Switching task RT [$F(8,1688.015) = 3.820$, $p < 0.001$]. Pairwise comparisons showed at 2 hours reaction times with WBE 111 were significantly faster than for WBE 888 ($p = 0.002$). At 4 hours reaction times were significantly faster with WBE 888 than placebo ($p = 0.008$), WBE 444 ($p = 0.001$) and WBE 222 ($p < 0.001$). RTs were also significantly faster with WBE 111 than WBE 222 ($p = 0.024$). At 6 hours reaction times with WBE 111 were significantly faster than with WBE 444 ($p = 0.001$) and WBE 222 ($p < 0.001$). See figure 4.8 for representation of results.

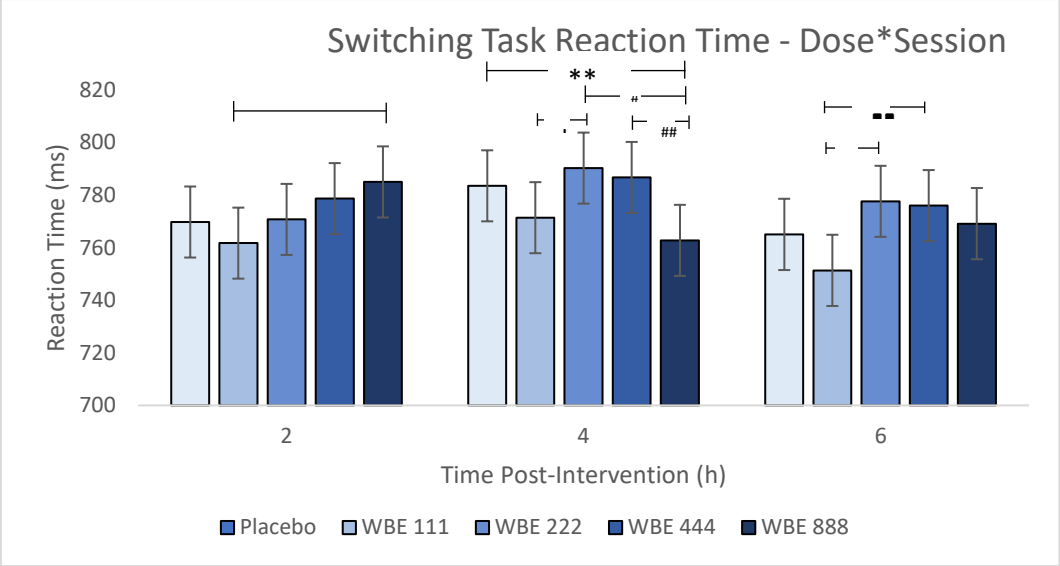


Figure 4. 8: Mean (± SE) Switching Task Reaction Time as a function of Dose by Session showing at 2 hours faster RT with WBE 111 than WBE 888 (* p = 0.002). At 4 hours faster RT with WBE 888 than placebo (**p = 0.008), WBE 222 (# p < 0.001) and WBE 444 (## p = 0.001), faster RT with WBE 111 than WBE 222 (+ p = 0.024). At 6 hours faster RT with WBE 111 than WBE 222 (▪ p < 0.001) and WBE 444 (▪▪ p = 0.001)

Visit

Visit was a significant predictor of Switching task RT [F(4,635.547) = 4.296, p = 0.002]. Pairwise comparisons revealed reaction times were significantly slower at Visit 3 than Visit 1 (p = 0.028), Visit 4 (p = 0.032) and Visit 5 (p = 0.038).

4.3.3 Composite Scores

Session

Session was a significant predictor of the Composite Accuracy Score [F(2,55.339) =3.594, p = 0.034]. Pairwise comparisons showed a significant decline from 2 hours to 4 hours (p = 0.034). Session also

significantly predicted the Composite Executive Function Score [F(2,55.174) = 3.315, p = 0.044] Pairwise comparisons revealed poorer performance at 4 hours was approaching significance when compared with 2 hours post-intervention (p = 0.075). Consistent with aspects of RAVLT (delayed word recall, foil words rejected, total recall and retroactive interference) and TST Accuracy, Composite Executive Function Score and Composite Accuracy Score show a decline in cognitive performance during the day, particularly at 4 hours post-intervention which may be attributed to cognitive fatigue.

Dose by Session

There was a significant Dose by Session interaction for the Composite Executive Function Score [F(8,206.019) = 2.057, p = 0.042]. Pairwise comparisons showed with the placebo, performance was significantly weaker at 4 hours than at 2 hours (p= 0.004) and 6 hours (p = 0.042). See results represented in figure 4.9 and 4.10.

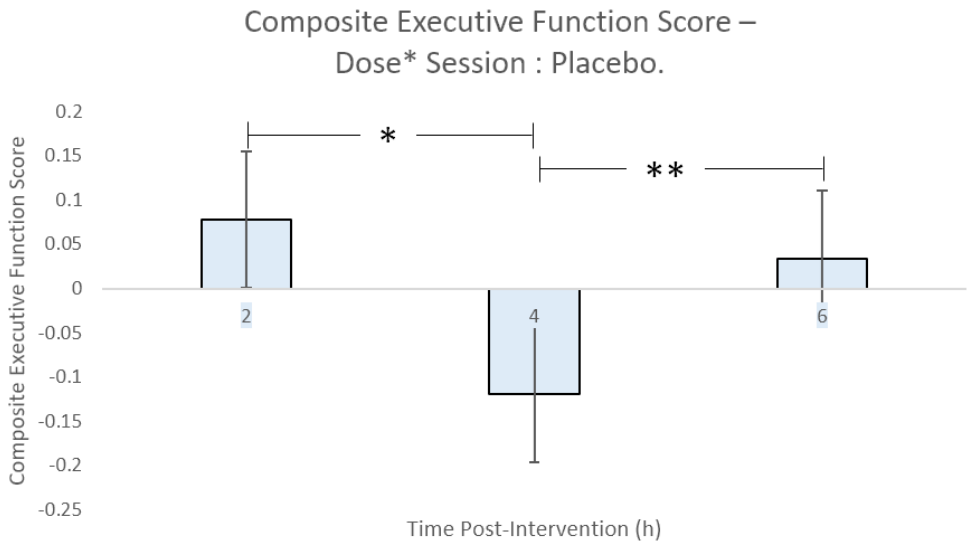


Figure 4. 9:Composite Executive Function Score for placebo across the duration of the test day, showing significantly poorer performance at 4 hours post-intervention compared with 2 hours (* p = 0.004) and 6 hours (** p = 0.042).

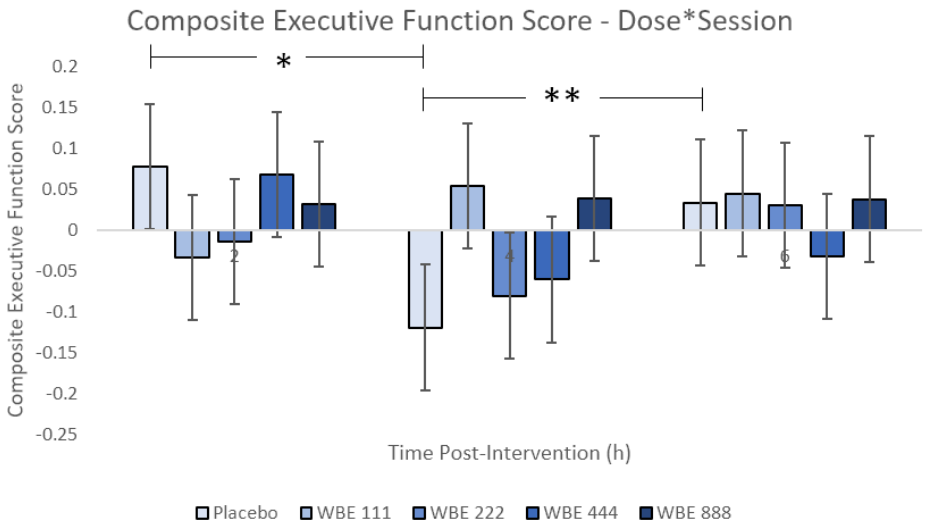


Figure 4. 10:Composite Executive Function Score as a function of Dose by Session, showing the significant dip in performance at 4 hours post intervention is seen with placebo only but not with WBE supplementation.

Visit

In keeping with results for Corsi (longest sequence, total correct sequences and first block latency) and TMT B and TMT B-A, in the absence of treatment effects, all composite cognitive scores were significantly predicted by Visit, whereby later performance was better or faster for later visits compared with earlier visits of the study. These results indicate considerable practice effects as subjects progressed through the stages of the study. Specifically, visit was a significant predictor of Composite Accuracy Score [$F(4,79.433) = 4.717$, $p = 0.002$], Pairwise comparisons revealed Composite Accuracy was significantly improved at Visit 5 compared with Visit 1 ($p = 0.005$) and Visit 3 ($p = 0.048$). Visit also significantly predicted Composite Reaction Time Score [$F(4,75.717) = 9.047$, $p < 0.001$]. Pairwise comparisons showed Composite RT was significantly faster for Visit 4 and 5 than Visit 1 ($ps < 0.001$) and Visit 2 ($p = 0.005$) and Visit 3 ($p = 0.004$) respectively. Visit was a significant predictor of Composite Episodic Memory Score [$F(4,78.34) = 5.471$, $p = 0.001$]. Pairwise comparisons

demonstrated Composite Episodic Memory was significantly improved at Visit 5 compared with Visit 1 ($p < 0.001$). Visit significantly predicted Composite Executive Function [$F(4,83.402) = 5.043$, $p = 0.001$]. For Composite Executive Function, pairwise comparisons revealed performance at Visit 4 was significantly improved when compared with Visit 1 ($p = 0.001$) and Visit 3 ($p = 0.019$). Global Score was significantly predicted by Visit [$F(4,77.658) = 8.810$, $p < 0.001$]. Pairwise comparisons showed performance at Visit 5 was significantly better than at Visit 1 ($p < 0.001$), Visit 2 ($p = 0.016$) and Visit 3 ($p = 0.036$). Performance at Visit 1 was significantly poorer than at Visit 4 ($p = 0.001$).

The complete cognitive data from the ROAB trial can be found in tabulated form in Appendix F.

4.3.4 Mood

Dose was not a significant predictor of mood and there were no Dose by Session interactions.

Session

Session was a significant predictor of PANAS Positive score [$F(2,53.584 = 38.046$, $p < 0.001$]. Pairwise comparisons showed Positive Affect (PA) was significantly higher at 2 hours than at 4 hours and 6 hours ($ps < 0.001$) and PA was significantly higher at 4 hours than at 6 hours ($p = 0.001$), revealing a progressive decline in positive mood throughout the duration of the test day. Session was also a significant predictor of PANAS Negative Affect (NA) [$F(2,48.880 = 4.405$, $p = 0.017$]. Pairwise comparisons revealed significantly higher NA at 4 hours than 6 hours ($p = 0.029$) and approaching significance when compared with 2 hours ($p = 0.057$). These changes in affect occurred alongside progressive cognitive decline throughout the day.

Visit

Visit was a significant predictor of PANAS Positive score [F(4,94.599 = 18.514, p < 0.001]. Pairwise comparisons showed at Visit 2 PA was significantly higher than at Visit 1, 3, 4 and 5 (ps < 0.001). Positive affect at Visit 1 was significantly higher than at Visit 3 (p = 0.018) and Visit 5 (p < 0.001). Visit significantly predicted PANAS Negative score [F(4,76.812 = 8.690, p < 0.001]. Pairwise comparisons showed that negative affect was significantly higher at Visit 3 than Visit 1,2 4 and 5, (ps < 0.001)

4.3.5 Cardiovascular

4.3.5.1 Blood pressure

Dose

Dose was a significant predictor of SBP [F(4,231.331 = 3.069, p = 0.017] and DBP [F(4,210.271 = 3.223, p = 0.014]. Pairwise comparisons showed SBP was significantly lower with WBE 222 than placebo (p = 0.038) and DBP was significantly lower for WBE 222 than placebo (p = 0.027) and WBE 888 (p = 0.025). See figure 4.11 and 4.12 for results.

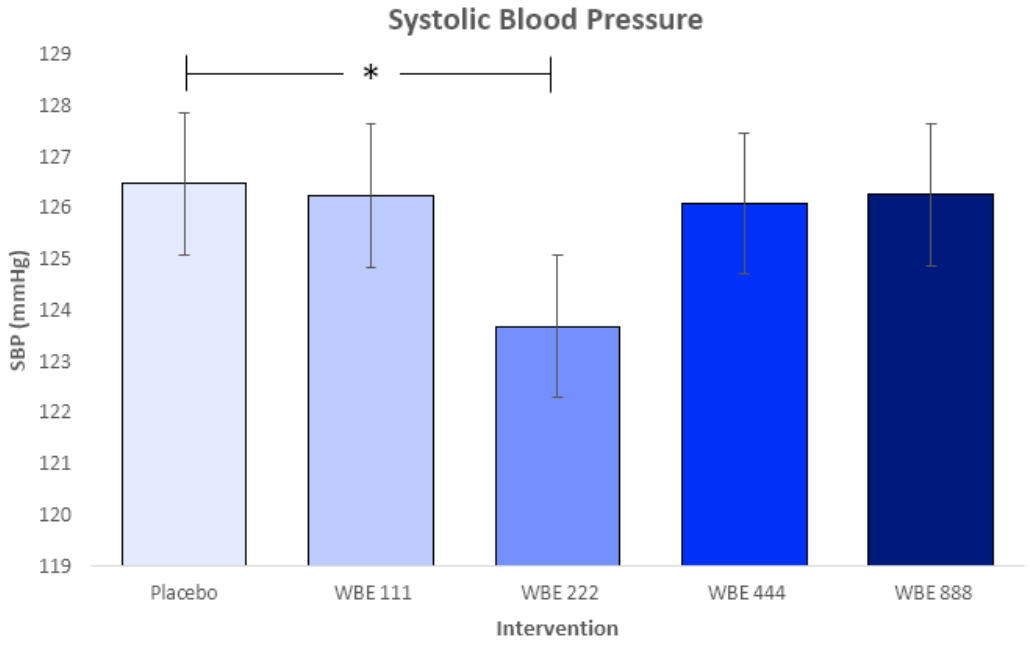


Figure 4. 11: Mean Systolic Blood Pressure as a function of intervention (± SE), showing significantly lower SBP with WBE 222 than placebo. (* p = 0.038)

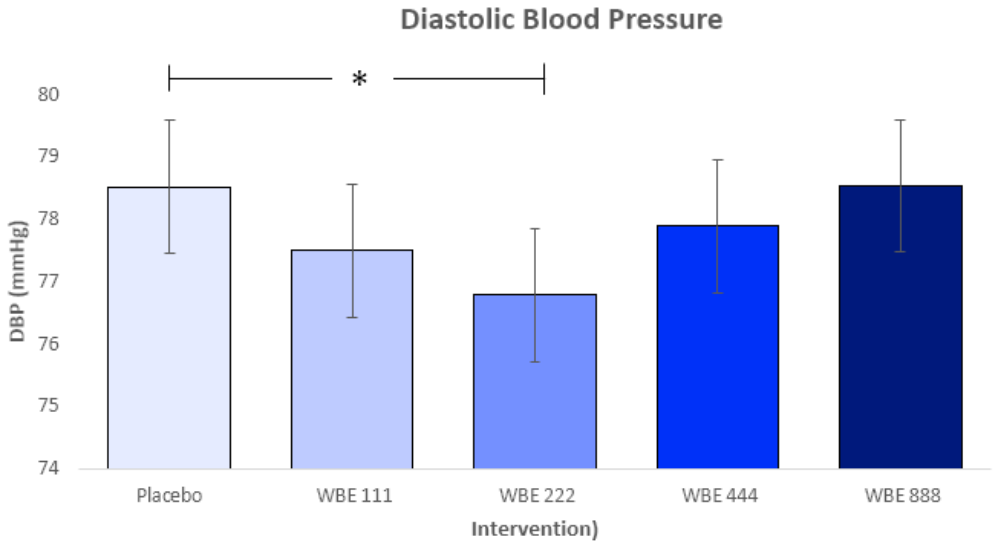


Figure 4. 12: Mean Diastolic Blood Pressure as a function of intervention (± SE) showing significantly lower DBP with WBE 222 than placebo. (* p = 0.027)

Session

Session was a significant predictor of SBP [F(2,51.600 = 54.010, p < 0.001] and DBP [F(2,53.555 = 79.135, p < 0.001]. Pairwise comparisons revealed that Systolic BP was significantly higher at 2 hours than at 4 hours (p = 0.005) and significantly higher at 6 hours than at 2 hours and 4 hours (ps < 0.001). Diastolic BP was significantly higher at 6 hours than at 2 hours (p = 0.005) and 4 hours (p < 0.001) and significantly higher at 2 hours than 4 hours (p < 0.001).

There were no significant Dose by Session interactions for blood pressure and no Visit effects.

4.3.5.2 HR

There were no significant Dose effects for heart rate and no significant Dose by Session interactions

Session

Session was a significant predictor of HR [F(2,52.893 = 40.308, p < 0.001]. Pairwise comparisons revealed HR was significantly higher at 4 hours than at 2 hours and 6 hours (ps < 0.001).

It appears WBE 222 reduces SBP and DBP throughout the course of the day, without impact on heart rate.

Visit

Visit was a significant predictor of HR [F(4,77.462 = 2.942, p = 0.026]. However, pairwise comparisons showed no significant differences between visits.

4.4. Discussion

The aims of this study were to investigate the effects of a range of doses of wild blueberry extract following a single one-off administration, on episodic memory, executive function and cardiovascular measures in healthy older adults. With respect to episodic memory, significant improvements were found for word recognition reaction times following WBE 444 compared with placebo. Faster reaction times were also demonstrated following WBE 111 in the Switching Task, an assessment of executive function. Additionally, the composite executive function score showed a significant dip at 4 hours post-intervention following placebo which was alleviated by all doses of WBE. Strikingly, WBE 222 produced acute reductions in both systolic and diastolic blood pressure compared with placebo.

4.4.1 Episodic Memory

In terms of episodic memory outcomes, WBE 444 significantly improved reaction time for RAVLT – Word Recognition, compared with placebo. It is difficult to comment on whether this replicates previous research as RAVLT - Word Recognition accuracy is more commonly reported in previous flavonoid intervention studies (Dodd et al., 2019, Barfoot et al., 2019; Igwe et al., 2017; Kent et al., 2017; Whyte et al., 2016; Whyte & Williams, 2015). Although positive effects on word recognition accuracy in older adults were found following acute WB powder (Dodd et al., 2019) and chronic WBE 111 (Whyte et al., 2018), these were not detected in the current study. However, no decrements to word recognition accuracy were apparent following WBE, indicating the absence of a speed-accuracy trade-off. Word recognition speed has been shown to decline with age (Laver, 2000; Rojas, Riffo, & Guerra, 2022) and in MCI patients (Bush, Allen, Kaut, & Ogrocki, 2007). Therefore demonstration of improvements following WBE supplementation in this ageing sample is particularly relevant. Further, word recognition accuracy is maintained in older adults compared to young adults (Laver, 2000) and

with increasing age above 60 years (Rojas et al., 2022) despite slowing of word recognition. The benefits to word recognition speed in the absence of changes to word recognition accuracy found by the current study may reflect a greater scope for improvement in this measure. Thus to consider word recognition speed concurrently with accuracy may be more useful than either outcome in isolation.

Acute WBE supplementation elicited no benefits to performance in the Corsi Blocks task with respect to accuracy or reaction time measures, nor composite Episodic Memory Score. Although the Corsi Blocks task is widely used to assess visuospatial memory (Kessels, van Den Berg, Ruis, & Brands, 2008), its use in flavonoid intervention studies in older adults is limited. The chronic study by Whyte et al. (2018) showed WBE 111 was associated with better performance in the Total Number of Correct Sequences. In comparison, the lack of significant findings in this current study could indicate no acute changes to visuospatial memory or low sensitivity of the CORSI Block task to these changes. This suggests chronic WBE treatment of several weeks, is required to produce measurable improvements in the CORSI Blocks task.

4.4.2 Executive Function

With respect to executive function outcomes, significantly faster reaction times were also observed following WBE 111 compared with placebo in the Switching task, notably without deficit to accuracy scores. These findings concur with improved Switching task reaction times following a mixed berry intervention in young healthy adults (Whyte et al., 2019). WBE supplementation did not elicit any benefits over placebo to performance in the Choice Reaction task nor in the Trail Making Task A and B. In previous flavonoid and cognition research, CRT has produced inconsistent results when used in acute studies in young adults, whereby RTs were slower following blackcurrant juice compared with

placebo (Watson et al., 2018). In contrast, CRT contributed to a combined Reaction Time score, which was improved by grape juice (Haskell-Ramsay, Stuart, Okello, & Watson, 2017).

TMT A/B has been used more widely to assess cognitive performance in flavonoid intervention studies in older adult populations, including improved performance following chronic cocoa (Desideri et al., 2012; Mastroiacovo et al., 2014) and berry interventions (Crews Jr et al., 2005; Kent et al., 2017; Lee, Torosyan, & Silverman, 2017; McNamara et al., 2018; Schrager, Hilton, Gould, & Kelly, 2015). In two acute studies in older adults, no effects were seen in TMT A/B performance with plum juice (Igwe et al., 2017) nor in the acute arm of a cocoa intervention study. (Sorond, Lipsitz, Hollenberg, & Fisher, 2008). For improvements in TMT A/B scores to become detectable, chronic berry flavonoid consumption may be required.

Reaction times were improved by WBE in relation to aspects of the different domains of episodic memory and executive function. Importantly, TST and Delayed word recognition both require discrimination of pre-learned information and are therefore arguably more cognitively demanding than either basic reaction time or recall tasks. Positive effects of WBE on TST and delayed word recognition reaction times, but a lack of significant findings in the less demanding CRT and TMT-A and TMT-B, add further credence that berry flavonoids are most effective at improving cognition in more cognitively challenging conditions (Whyte et al., 2016, Miller et al., 2018). Interestingly accuracy scores in neither TST nor Delayed word recognition were improved by WBE. Mean group averages for delayed word recognition accuracy were in keeping with older adult norms (Gale et al. 2017). However, in the absence of available age-adjusted population norms for TST accuracy, the mean group averages which ranged from 97.9-98.1%, imply that that the subjects were performing at ceiling. The task may have lacked sensitivity to WBE intervention. Indeed, young adults showed lower accuracy scores in the same task (Whyte et al., 2019). These results highlight the importance of the task battery employed to detect the effects of flavonoid interventions on cognition. Future

WBE research in older adults should therefore be guided by this and the more challenging cognitive tasks selected.

The executive function composite score, combining accuracy and reaction time measures, showed a post-lunch dip at 4 hours, but only for placebo. Importantly, WBE alleviated this post-lunch decline in executive function. The effect of Session (time post-intervention) can also be seen in poorer performance at 4 hours post-intervention with RAVLT - Delayed Word Recall, RAVLT - Foil Words Rejected, RAVLT - Total Recall, Switching task accuracy, Composite Accuracy score, greater RAVLT - RI and higher PANAS NA. These effects may be attributed to and indicate the study subjects experienced a post-lunch decline in cognitive performance, which has been shown to start at 1 hour after the start of consuming lunch (Blake, 1971). Detrimental effects on mood after lunch have also been observed (A. P. Smith & Miles, 1986). Additionally, the 4-hour post-intervention time point approximated to 13:30h clock time. This is in keeping with studies which have shown a daily dip in alertness and attention most commonly recorded at around 14:00h (Bjerner, Holm, & Swensson, 1955; Hildebrandt, Rohmert, & Rutenfranz, 1975; Prokop & Prokop, 1955) and has been attributed to a combination endogenous circadian rhythms and post-prandial effects.

4.4.3 Blood Pressure and Heart Rate

With respect to cardiovascular outcomes, WBE 222 produced acute reductions in both systolic and diastolic blood pressure compared to placebo. Previous acute anthocyanin-rich berry treatments have shown mixed results in relation to BP. Igwe et al. (2017) demonstrated significant reductions in both SBP and DBP following acute consumption of plum juice. Significant reductions in SBP, but not DBP, were recorded at 1h, 2h and 3h post-supplementation with cherry juice (Keane et al., 2016), conversely only DBP was significantly reduced by a single dose of Haskap berry supplementation (Bell and Williams, 2019). However, acute cranberry (Rodriguez-Mateos et al., 2016) and blueberry

interventions (Rodriguez-Mateos et al., 2013 and Dodd et al., 2019) have not yielded similar blood pressure effects in the past. The disparate findings may be attributed to the differing flavonoid profiles of the interventions employed. Indeed, anthocyanins have been observed to exhibit less robust blood pressure effects than other flavonoid-subclasses (Ciumărnean et al., 2020). The clinical importance of considering DBP and SBP in combination has been discussed, in particular pulse pressure (PP); the difference between SBP and DBP, is an important predictor of CV risk in older adults (Glynn, Chae, Guralnik, Taylor, & Hennekens, 2000). Notably, WBE 222 reduced both SBP and DBP, thus exhibiting drug therapy-like antihypertensive effects. Further, moderate reductions in BP were measured here in a healthy older adult sample, where homeostatic mechanisms may have prevented larger BP reductions. The difference in means between WBE 222 and placebo were -2.80 mmHg for SBP and -1.73 mmHg for DBP. Importantly these reductions are similar in magnitude to those achieved through chronic pharmacological treatment with Ramipril, an angiotensin-converting enzyme inhibitor (Sleight et al., 2001). Despite only modest reductions in SBP (-3.3mgHg), these have been associated with significant impact on clinical endpoints of CV death, MI or stroke, with 22% risk reduction. The cardiovascular benefits of ramipril have been observed in normotensive subjects with a history of coronary disease. This suggests that the blood pressure reductions measured in this current study with WBE 222 have potential clinical significance, especially with longer-term treatment. Importantly, the acute hypotensive effects of WBE demonstrated in the current study, have shown to be maintained following chronic treatment at 3 and 6 months with a lower dose of WBE 111 (Whyte et al.2018). However, WBE 111 did not show acute hypotensive effects in the current study, which suggests that at this particular dose longer term treatment is required for effects on blood pressure to become apparent. As hypothesised, WBE showed cardiovascular benefits after acute consumption, albeit, in absence of a clear dose-dependent response.

Reduced blood pressure has previously been accompanied by improved performance in trail-making speed and verbal fluency with cocoa flavonoid supplementation (Mastroiacovo et al., 2014). In

particular, Whyte et al. (2018) found a reduction in systolic blood pressure alongside improved episodic memory and visuospatial memory, following three months' supplementation with WBE in healthy older adults. The BP effects may be attributed to the vasodilatory actions of blueberry flavonoids (Rodriguez-Mateos et al., 2013) which are mediated through endothelial-dependent mechanisms (reviewed by Ciumarnean et al., 2020). Additionally, cognitive function measured using MMSE was negatively associated with arterial stiffness (Fukuhara et al., 2006) and significant correlations between arterial stiffness and endothelial dysfunction have been shown (Nigam, Mitchell, Lambert, & Tardif, 2003). It is therefore reasonable to expect benefits to endothelial function to subsequently improve cerebral blood flow (CBF) and thus cognitive function. Indeed, Akazawa et al. (2021) recently demonstrated significant correlation between endothelial function and cerebral blood flow (CBF) in a cohort of middle-aged men. Improved BP therefore provides a potential mechanistic explanation of the improvements to the cognitive measures evidenced in the current study.

4.4.4 Summary

As hypothesised, WBE was shown to improve aspects of episodic memory, executive function and cardiovascular health compared to placebo. Specifically faster reaction times for executive function were observed following WBE 111 and for episodic memory following WBE 444. All tested doses of WBE alleviated the post-lunch dip in EF. Importantly, WBE 222 reduced both SBP and DBP, and therefore identified as the optimal tested dose, in that both BP and cognitive effects were observed following ingestion. Results from this study extend previous research by providing evidence that blueberry benefits to cognition are not limited to episodic memory following acute supplementation. In particular, improvements to executive function can be achieved after a single dose of WBE. Specifically, WBE alleviated the post-prandial decline in executive function. The cardiovascular outcomes reinforce previously elicited reductions in blood pressure, particularly in

combination with observed cognitive benefits. However, a clear dose-dependent response was not evidenced. It was expected that between-treatment differences would be greatest at 2h and 6h post-intervention, in keeping with observations of peak blueberry polyphenol metabolite circulation (Rodriguez-Mateos et al., 2013), instead the post-lunch dip (at 4h post-intervention) appears to have influenced performance in the cognitive outcome measures.

Outcomes of the current study corroborate previous findings that situations of increased cognitive demand are particularly sensitive to berry flavonoid treatment (Whyte et al. 2016, Whyte et al., 2017, Whyte et al., 2019, Whyte et al., 2021, Barfoot et al,. 2021 and Miller et al., 2018). We showed reaction times in the more cognitively-taxing Switching task and delayed word recognition were improved by WBE. Plus, cognitive fatigue which developed throughout the course of the day, particularly during the post-lunch dip, was alleviated by WBE. In addition to post-prandial effects after consuming lunch, circadian rhythms are also known to induce reductions in cognitive performance, particularly at 14:00h. The 4h post-intervention timepoint in the current study approximated to 13:30h which did not completely coincide with circadian-related changes to performance.

To further clarify whether treatment with WBE 222 can overcome periods of cognitive decline during the course of the day alongside reductions in blood pressure, this work may be extended to investigate not only the post-prandial dip at one hour after lunch, but also circadian rhythm-induced cognitive decline commonly seen at 14:00h. By aligning post-prandial and circadian effects at 14:00h, the post-lunch decline in cognitive performance is accentuated and serves as the timepoint at which to deliver the cognitive test battery. Peak blueberry phenolic metabolites are produced two hours post-intervention (Rodriguez-Mateos et al., 2013). WBE 222 should therefore be administered at 12:00h in order for maximum metabolite levels to coincide with cognitive testing. Further research will act as a confirmatory investigation into the acute effects of WBE 222 on cognitive

performance during the post-lunch dip. The outcome of this confirmatory study is presented in the following chapter.

Chapter 5: Blueberry Extract Acute Trial – BEAT

5.1 Introduction

In the ROAB trial (Chapter 4), improvements to reaction times were observed in healthy older adults in relation to RAVLT word recognition (a measure of episodic memory) following WBE 444 and for the Switching Task (a measure of executive function) following WBE 111. As regards to changes in cognitive performance during the course of the day, all doses of WBE (WBE 111, WBE 222, WBE 444 and WBE 888) were shown to alleviate the decline in executive function seen following placebo at 4 hours post-intervention. WBE 222 also elicited significantly lower systolic and diastolic blood pressures relative to placebo.

Diurnal peaks and troughs in physiological functions attributed to opposing effects of the arousing circadian system and sleep pressure of homeostatic regulation, are linked with alterations in cognitive processes and performance (Ceglarek et al., 2021; Schmidt, Collette, Cajochen, & Peigneux, 2007; Tassi, Pellerin, Moessinger, Eschenlauer, & Muzet, 2000; Valdez, Ramírez, & García, 2012). Studies which reveal a daily dip in alertness and attention show this has most commonly been recorded at 14:00h (Monk, 2005; Valdez, 2019) and manifests in real-life situations such as increased incidents of traffic accidents (Garbarino, Lino, Beelke, Carli, & Ferrillo, 2001) and errors in interpretation of radiology results (Alshabibi, Suleiman, Tapia, Heard, & Brennan, 2020). One proposed mechanism for this slump in cognitive performance is that during this period, alerting circadian effects are transiently insufficient to counterbalance the sleep pressure of homeostatic systems (Cajochen, Blatter, & Wallach, 2004). Additionally, a post-prandial decline in cognitive performance has been found to commence one hour after consumption of lunch (Müller, Libuda, Terschlüsen, & Kersting, 2013). These effects on performance have come to be collectively termed the ‘post lunch dip’.

In the ROAB trial, the decline in executive function following placebo at 4 hours post intervention was recorded at approximately 13:30h, approaching the post-lunch dip at 14:00h (Monk, 2005; Valdez et al., 2019) and at 1 hour after lunch. These observations suggest that a post-lunch dip occurred during the test day of the ROAB trial. Alleviation of this decline in executive function at 4 hours by WBE indicate that WBE supplementation may overcome the post-lunch dip. Importantly, WBE 222 was identified as the optimal dose tested since benefits to both cardiovascular and executive function measures were demonstrated. Our finding of BP reductions alongside improved cognitive performance suggests a vasodilatory mechanism of action (see Chapter 1 for a discussion of proposed mechanisms of action). Further investigation is required to show whether alleviation of the post-lunch dip following acute WBE 222 treatment can be replicated with cognitive testing to coincide with peak flavonoid metabolite concentrations.

Randomised controlled trials investigating the daily cycle of fluctuations in cognitive performance have typically employed one of two paradigms to overcome exogenous factors known to affect circadian rhythms. In a constant routine (CR) protocol, factors are controlled, such as constant light levels, temperature of the environment and food intake at set intervals providing a specific number of calories (Schmidt et al., 2007). In forced desynchrony (FD) protocols subjects are assessed under significantly reduced or extended periods of wakefulness (Schmidt et al., 2007). These have revealed circadian and homeostatic influences on executive function, memory and attention (Goel, Basner, Rao, & Dinges, 2013; Manly, Lewis, Robertson, Watson, & Datta, 2002; Valdez, 2019). Circadian variations have been demonstrated in relation to different aspects of executive function. García, Ramírez, and Valdez (2016) showed circadian effects on self-monitoring (the ability to adapt to changes in the environment), where cognitive performance was measured by correct responses and latency in a tracking task every 100 minutes during a 30-hour CR protocol. Further, circadian and

homeostatic processes have been found to influence cognitive flexibility (Bratzke, Rolke, Steinborn, & Ulrich, 2009), where switch cost in a switching task was measured 3 hourly in a 40-hour CR paradigm. However, findings related to inhibitory control have been mixed. Circadian modulation of inhibition has been demonstrated in a Go/No-Go task administered 4 hourly under a 4 0-hour CR protocol (Zeeuw et al., 2018). In a time-of-day study, errors of commission for the sustained attention to response test (SART) recorded during the course of a 'normal day' (in the absence of control for exogenous/masking factors) at 1am, 7am, 1pm and 7pm, showed circadian influences (Manly et al., 2002). These findings were not replicated under a FD protocol adopting eighteen 28-hour days using a modified version of SART. Instead greater homeostatic influence over inhibitory control was shown, indicating the impact of time awake (Harrison, Jones, & Waterhouse, 2007). Although time of day effects were not evident from performance in the Stroop task under CR protocols (Bratzke, Steinborn, Rolke, & Ulrich, 2012; Sagaspe et al., 2006), circadian effects have been observed for measures of inhibition as well as flexibility in two Stroop-like tasks under 29-hour CR protocols (García, Ramírez, Martínez, & Valdez, 2012; RamÍrez, GarcÍa, & Valdez, 2012). In-keeping with the findings of circadian and homeostatic effects on executive function, significant effects of session were detected in the ROAB trial, which indicated a post-lunch dip in composite executive function score. A post-lunch dip was also indicated for cognitive flexibility as assessed by TST accuracy (a measure of executive function) at 4-hours post-intervention. Further, aspects of RAVLT involving interference management (foil words rejected and retroactive interference) also showed a post-lunch dip whereby poorer performance at 4 hours compared with 2 hours, were observed.

Evidence is emerging to indicate that working memory may also be modulated by circadian rhythms. Reduced working memory efficiency in the morning compared with the afternoon has been demonstrated (Fabbri et al., 2008; Ceglarek et al., 2021). In particular, circadian and homeostatic variations have been revealed using the Digit Symbol Substitution Test (DSST) in a 28-hour FD study

(Wright Jr, Hull, & Czeisler, 2002) as well as phonological and visuospatial working memory tasks when examined hourly under a 30h CR protocol in female students aged 16-19 years (Ramírez et al., 2006). Time awake effects have been shown in both spatial and verbal 1, 2 and 3-back tasks (Groeger et al., 2008) whilst circadian effects on performance on the n-back task has been inconsistent. Van Eekelen and Kerkopf (2003) found circadian effects on overall n-back performance, as well as the individual 2-back and 3-back trials in young adults. Although circadian variations in the 2-back task were also reported by Zeeuw et al. (2018), the same result was not replicated for the 3-back task in young female adults. The impact of task complexity on circadian patterns of performance in young adults therefore remains unconfirmed. The effects of circadian and homeostatic rhythms on episodic memory is yet to be fully understood due to the limited research in this domain. Circadian variations have been indicated by performance in the probed recall memory (PRM) using both CR (Cajochen et al., 1999; Wright et al., 2002) and FD protocols (Wyatt et al., 1999). Further, circadian fluctuations may explain the effects of session in RAVLT delayed recall and total recall seen in the ROAB trial. Significantly poorer performance was demonstrated at 4 hours post-intervention compared with 2 hours which is suggestive of a post-lunch dip.

According to Posner and Rafal's model of attention, alertness can be grouped into two types; tonic alertness refers to the ability to respond to the general environment and phasic alertness is the ability to respond to a stimulus following a priming signal (Rafal & Posner, 1987). All of the individual components of attention have shown to be influenced by circadian and homeostatic effects. For example, alertness and selective attention measured by reaction times and accuracy scores for aspects of a continuous performance task (CPT)**,** showed time of day fluctuations (Valdez et al., 2005) with a peak in tonic alertness observed between 10:00 and 12:00 (Valdez, Ramírez, García, Talamantes, & Cortez, 2010). Both these studies were conducted under CR protocols of 28h and 30h respectively, where cognitive performance was recorded hourly. Homeostatic and circadian fluctuations in selective attention have also been demonstrated using the Rapid Serial Visual

Presentation (RSVP) (Gallegos et al., 2019), Bowles-Langley Test (BLT) (Schnupp, Heinze, & Golz, 2017) and in dual task performance (Van Eekelen and Kerkopf, 2003, Jasper et al., 2010; Rodgers & Holding, 1991). Similarly, Jasper et al., (2010) found circadian effects with a pronounced post-lunch dip in a dual task which combined a tracking task with a memory task. Rodgers and Holding (1991) also found circadian variations in dual task performance in a study which utilised a time-of-day design. Cognitive testing was completed at 08:00, 11:00, 14:00, 17:00, 20:00 and 23:00. A dip in performance at 14:00 was revealed. It could be argued that this design better reflects the real-life pattern of cognitive performance rhythms to include the effects of exogenous factors (e.g. consumption of lunch) and may explain the notable dip in selective attention at 14:00 in-line with the post-lunch dip. Indeed, A. P. Smith (2021) found the consumption of lunch was associated with reduced selective attention, indicated by slower reaction times in a categoric search task. These findings are in-keeping with earlier work by Monk et al. (1996) that a post-lunch dip attributed to circadian rhythms occurs even in the absence of lunch consumption. In turn consumption of lunch accentuates the decline in cognitive performance (Müller et al., 2013). Circadian and homeostatic fluctuations have shown to influence sustained attention when assessed using the Psychomotor Vigilance Test (PVT), both in studies using FD protocols (Wright et al., 2002 and McHill et al., 2018), and CR designs (Zeeuw et al. 2018). Outcome measures of median reaction times and number of lapses of attention were most commonly used as indicators of sustained attention. Results from the Continuous Performance Task (CPT) have been more equivocal. General sustained attention was not associated with circadian effects when assessed by CPT performance (Valdez et al., 2005). However, a subsequent study showed specific aspects of sustained attention; general stability of efficiency (measured by the variability of SDs of reaction times and accuracy scores) as well as some elements of short-term stability of efficiency (short-hit runs, short-error runs and long-error runs), were influenced by both circadian and homeostatic variations. Time on task stability and long-hit runs did not demonstrate circadian fluctuations but were shown to be associated with homeostatic effects only (Valdez et al., 2010). While specific cognitive domains may be modulated by circadian and

homeostatic fluctuations to varying degrees, overall cognitive performance reaches a trough between 04:00 and 07:00 (Valdez et al., 2019). Improvements seen towards midday are followed by a post-lunch dip, most often observed between 14:00 and 16:00 (Monk et al., 2005), after which cognitive performance continues to increase throughout the evening before the most substantial decline reported after 22:00 (Valdez et al., 2019).

An important limitation of cognitive chronobiological research is that the majority of studies have been conducted in young adults. Few RCTs have investigated the specific pattern of changes to cognitive performance during the course of the day in older adults. Two such studies used 40-hour CR protocols to assess the cognitive performance of young and older adults (Blatter et al., 2006; Sagaspe et al. 2012). Blatter et al. (2006) showed circadian fluctuations in sustained attention are less pronounced in older adults compared with young adults, as measured by the PVT. Similarly, reduced peaks and troughs in inhibition were found using a Go/No-go task (Sagaspe et al., 2012). Additionally, in older adults circadian and homeostatic effects have shown to modulate performance according to task difficulty. Blatter et al. (2005) demonstrated that planning performance only showed circadian rhythms in more difficult versions of a maze task in a 40-hour CR protocol. The reduced amplitude of circadian changes in attention and circadian effects which may be limited to the more difficult aspects of a task in older adults, were reflected by the absence of session effects (indicative of time-of-day effects) in the ROAB trial for the simpler cognitive tasks (Corsi and TMT) and where attention is a key component of performance (CRT).

A limited number of studies have investigated alleviation of the post-lunch dip in cognitive performance by nutritional interventions. Dhillon et al. (2017) reported a significant dip in memory performance post-lunch following a high carbohydrate lunch. In comparison, this decline was alleviated by 57.7% by an almond-enriched lunch. Memory performance was indicated by measures of immediate recall, delayed recall and verbal list recognition, recorded immediately after lunch and

30 to 35 minutes later. In terms of flavonoids and the post-lunch dip, ginkgo biloba (GB) has been examined for its ability to overcome the drop in cognition after lunch (Mattes & Pawlik, 2004). A post-lunch dip in alertness was demonstrated using a self-reported measure. However, objective assessment of vigilance with the letter-cancellation task during subjects' habitual lunchtime and 30 minutes later did not show a significant decline. In turn, it was not possible to examine the effects of GB on the post-lunch dip. In contrast, findings from the ROAB trial suggest that WBE treatment is able to overcome the post-lunch decline in cognition.

The primary objective of the current study was to replicate alleviation of the cognitive decline during the post-lunch dip at 14:00 by WBE 222 supplementation. A randomised, double-blinded, placebo-controlled study design was employed to investigate the effects of WBE 222 on episodic memory and executive function in a population of older adults aged 68-75 years old during the post-lunch dip. In order to maximise opportunities to detect treatment-related changes, cognitive tasks which showed sensitivity to WBE treatment in the ROAB trial were selected; RAVLT (verbal episodic memory) and Switching task (executive function), and Corsi Blocks task to assess spatial episodic memory. The secondary objective of this study was to replicate the cardiovascular effects following WBE 222 in healthy older adults demonstrated in the ROAB trial, where reductions in systolic and diastolic blood pressure were recorded. Importantly, blueberry phenolic metabolites are known to reach a peak in plasma concentrations two hours post-intervention (Rodriguez-Mateos et al., 2013). WBE 222 was therefore administered at 12:00h in order for maximum metabolite levels to coincide with cognitive testing. The cognitive test battery was delivered at two time points; baseline (08:30) and during the post-lunch dip (14:00h), following lunch at 13:00h.

In accordance with findings from the ROAB trial, it was predicted that improved or maintained performance will be detected in measures of episodic memory and executive function during the

post-lunch dip, after WBE 222 consumption compared with placebo. Systolic blood pressure and diastolic blood pressure were expected to be reduced following WBE 222 compared with placebo.

5.2 Methods

5.2.1 Participants

This study was reviewed and given a favourable ethical opinion for conduct by the University of Reading Research Ethics Committee (UREC 19/19).

5.2.1.1 Determination of Sample Size

Based on the combined reaction time scores in the ROAB trial, an a priori repeated measures, within factors power analysis using G*Power 3.1 was performed to determine the required sample size for cognitive effects. This analysis showed that, using an alpha level of 0.05, an effect size (F) of 0.21 and a power of 0.8, 86 participants would have been required to detect cognitive changes.

5.2.1.2 Recruitment

As per Study 1, healthy older adults aged 68 – 75 years from Reading, UK and the local area were recruited via the Psychology Department's Older Adult Panel, local community noticeboards, the University of the Third Age and other older adult activity groups. In March 2020, government restrictions to prevent the spread of COVID-19 meant that further recruitment to the study could not continue. At this point, all forty-five volunteers who had attended a familiarisation visit had also progressed to randomisation and completed all stages of testing. Participants received an expenses payment of £50 on completion of testing. See figure 5.1 for consort diagram of the recruitment stages.

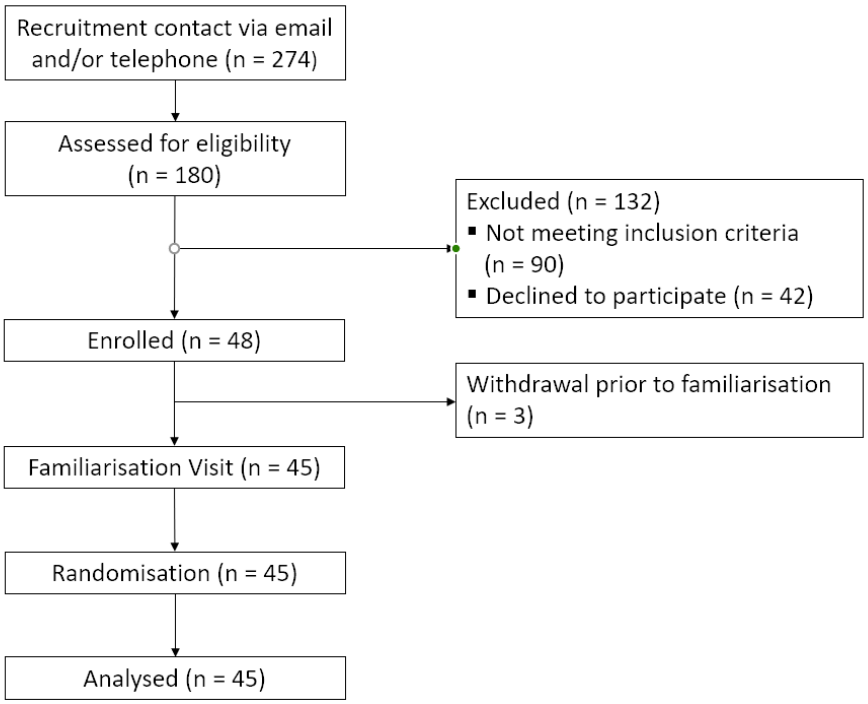


Figure 5. 1: Consort diagram showing subject numbers at each stage of recruitment and participation in the study

5.2.1.3 Subject Information

5.2.1.3.1 Screening at recruitment:

Screening for suitability of participants was completed prior to enrolment to the experiment according to the inclusion and exclusion criteria outlined in Table 4.1 see Chapter 4 Section 4.2.1.3.1.

5.2.1.3.2 Demographics

Demographic data for all 45 participants are summarised in Table 5.1

Table 5. 1: Subject demographic information

Baseline Characteristics	Mean	SD	Range
Age at enrolment (y)	71.02	2.03	68-75
Gender	18M/27F	N/A	N/A
BMI (kg/m^2)	25.05	2.95	20.04 – 29.97
MMSE	28.53	1.38	25-30
NART (number of errors)	8.04	4.44	1.0 – 21.0
FoF 1	5.27	0.98	3.1-6.7
FoF2	5.36	1.19	2.6-6.6
Raven's Progressive Matrices	48.98	7.03	30 – 60
YALE physical activity survey – energy expenditure (kcal/week)	6050	2384.61	2205-11655
Energy consumption (kcal/day)	1904	778.82	893-6038
Fruit consumption (g/day)	339	241.25	100-977
Vegetable consumption (g/day)	339	136.41	117-662

5.2.1.4 Informed Consent

All prospective participants were provided with the study information sheet via e-mail or post, prior to screening. This gave detailed information about the procedures involved in the experiment (see

Appendix C). Individuals who then opted to take part in the experiment were required to give signed consent at the start of their first familiarisation visit. See Appendix D for a copy of the consent form.

5.2.1.5 Low Polyphenol Diet

As per the ROAB trial, during the 24 hours prior to each test visit, participants were required to adhere to a low polyphenol diet and to abstain from consuming caffeinated beverages and alcohol. The guidance on a low polyphenol diet provided to participants can be found in Appendix D. On test days, participants arrived at the laboratory fasted for 12 hours. On arrival at 08.00h subjects received a standardised low polyphenol breakfast as detailed in Chapter 4 section 4.2.1.5, with water to drink.

5.2.2 Study Design

5.2.2.1 Overall Study Design and Plan

The study was designed as a randomised, double-blinded, placebo-controlled, cross-over trial. Subjects were required to attend three visits each separated by a 7-day washout period. The first of these was a screening/familiarisation visit, using the same questionnaires detailed in Chapter 4 Section 4.2.2.1.

5.2.2.2 Treatments

All treatments were administered as a single opaque white capsule. WBE 222 mg and placebo were formulated as per Study 1 see Table 5.2. All capsules were provided by Naturex and stored under dry conditions at room temperature.

Table 5. 2: **Intervention Arms Included in the Study**

Intervention	Formulation and Dosage Regimen
Placebo	Formulation containing inert artificially coloured maltodextrin, once daily, in one single hard capsule
WBE 222 mg	Formulation containing 200 mg wild blueberry extract + 22 mg L-Cysteine + 2 mg L-Glutathione, once daily, in one single hard capsule

5.2.2.3 Method of Assigning Subjects to Treatment Order

On completion of the practice visit, participants were randomised to receive either placebo on test day 1 followed by wild blueberry extract 222 mg on test day 2 or vice versa, according to participant number. All eligible participants took the assigned capsule on site, when prompted by the investigator. The randomisation was carried out using a computerised random number generator (www.random.org) by NATUREX-DBS. The investigators and the participants remained unaware of the relationship between participant number and the order of the treatments. The study was therefore double-blinded.

5.2.3 Cognitive Tests

The cognitive tests selected showed sensitivity to WBE supplementation in the ROAB trial. The Corsi Blocks task was also chosen to assess spatial episodic memory. Alternate versions of each of the cognitive tasks were created and Latin square counterbalancing was used to generate the order of

presentation across test sessions to limit learning. Detailed descriptions of RAVLT, Corsi Blocks task and Switching task can be found in Chapter 4 Section 4.2.3. Additionally, for RAVLT new word lists were generated which were matched for familiarity and concreteness. In the current study, the number of cycles presented in the Switching task was increased to 96 cycles.

5.2.3.1 Composite Scores

Each of Composite Accuracy, Composite Reaction Time, Composite Episodic Memory, Composite Executive Function and Global Score were calculated using the cognitive endpoints above.
Composite Accuracy comprised of measurements of Corsi longest sequence remembered, Corsi total correct number of sequences remembered, RAVLT word recognition accuracy, RAVLT delayed word recall, RAVLT total recall, RAVLT PI, RAVLT RI and Switching Task accuracy.
The Composite Reaction Time score was made up of Switching Task reaction time and RAVLT word recognition reaction time. Composite Episodic Memory combined scores for Corsi longest sequence remembered, Corsi total number of correct sequences remembered, RAVLT word recognition accuracy, RAVLT delayed word recall accuracy, RAVLT total recall, RAVLT PI, RAVLT RI and RAVLT word recognition reaction time, Switching Task accuracy, Switching Task reaction time were used to calculate the Composite Executive Function score. The Global score was calculated based on a combination of all the above measures.
See Chapter 4 Section 4.2.3.2.4 for further details on the method of composite calculation.

5.2.4. Cardiovascular Measures

5.2.4.1 Tertiary Endpoints: Blood Pressure

Measures of blood pressure were conducted as per the ROAB trial, see Chapter 4 Section 4.2.4.1

5.2.5. Procedure

The double-blind, crossover study design is illustrated in figure 5.2. Subjects attended the laboratory on three separate occasions each separated by one week. For 24 hours prior to each visit, participants followed a low polyphenol diet. They attended each visit 12 hours fasted. On arrival at 08.00h subjects received a standardized low polyphenol breakfast of two croissants (total energy 338kcal, fat 18.2g, carbohydrate 38g, protein 5.2g), with water to drink. At 08.30h subjects then completed a test battery consisting of cognition and blood pressure measures. Testing took place in the following order: RAVLT (immediate recall), Corsi Blocks task and Switching Task, blood pressure and heart rate. The intervention was administered in the form of one single oral capsule at 12.00h (2 hours prior to completing the test battery). A standardised low polyphenol lunch consisting of a sliced white bread cheese sandwich and a packet of ready salted crisps (total energy 400.5kcal, fat 20.8g, carbohydrate 48.7g, protein 23.7g) was consumed at 13.00h, one hour prior to completing the test battery at 14.00h. During breaks between testing and lunch, participants were invited to sit in the waiting room where they were permitted to engage in activities such as reading books and magazines or use their own electronic devices such as mobile phones, personal computers and tablets. A television was not provided. Participants were also permitted to leave the laboratory and were asked to drink water only, refrain from eating and avoid taking part in any rigorous exercise.

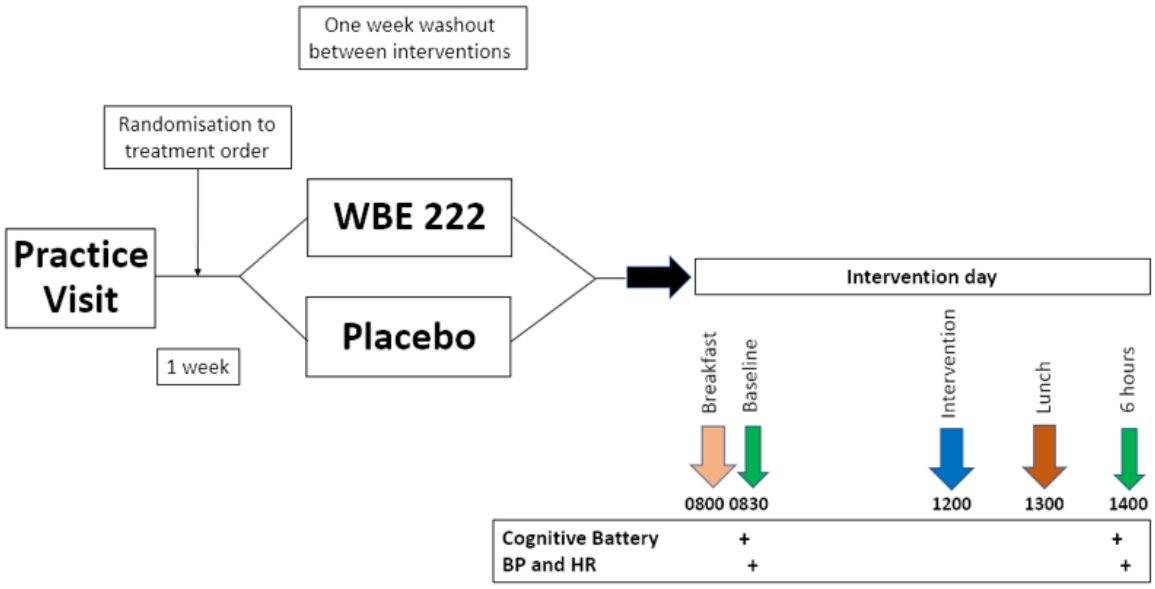


Figure 5. 2: Study design and timeline of procedure on intervention days

5.2.6 Statistical and Analytical Plans

All data were analysed using IBM SPSS statistics version 25. Z score analysis was used to identify outliers; datapoints with z score>3.29 were removed prior to statistical analysis (Tabaschnick & Fidell, 2013). A linear mixed model (LMM), using an unstructured covariance matrix, was used to analyse data for all measures. Baseline performance was included as a repeated covariate. Visit (Visit 1 and Visit 2 irrespective of treatment) and Treatment (WBE 222 and placebo) were included as fixed factors in the model. Visit was included in order to model any residual practice effects (Bell et al., 2018). The aim of the analysis plan was to determine whether Treatment (with Wild Blueberry extract) was a significant predictor of cognitive performance or blood pressure. Post hoc pairwise comparisons were used to investigate any significant effects of Treatment. A Sidak correction was applied to all multiple comparisons. Significant comparisons are reported ($p<0.05$). For the Switching task only, the additional factor of switch sequence and its treatment interaction were added to the LMM analysis.

5.3 Results

Episodic Memory

Means and standard errors for all Episodic Memory outcomes recorded in the BEAT trial are shown in Table 5.3.

RAVLT

RAVLT performance was similar following placebo and WBE 222 such that Treatment was found not to be a significant predictor for RAVLT. Mean word recognition reaction times of 1235ms and 1206ms were recorded for placebo and WBE 222 respectively, and for word recognition accuracy and delayed recall accuracy mean performances were 80.4% and 6.5 words following both placebo and WBE 222 (see Table 5.3 for detailed RAVLT results). Visit was also not a significant predictor for RAVLT. The current results do not provide evidence of benefits to verbal episodic memory following WBE 222 treatment at 14:00

Table 5. 3: Mean (SE) of Episodic Memory Outcomes following WBE 222 and Placebo.

Measure	Intervention	Timepoint	
		Baseline (included as a covariate in the analysis)	**14:00h**
RAVLT:			
Word Recognition – accuracy	Placebo	0.845 (0.017)	0.804 (0.019)
	WBE222	0.879 (0.017)	0.804 (0.019)
p-value			0.987
Word Recognition – reaction time (ms)	Placebo	1214.73 (50.596)	1234.61 (38.729)
	WBE222	1200.74 (50.911)	1205.58 (39.072)
p-value			0.523
Delayed Word Recall – accuracy	Placebo	8.791 (0.545)	6.511 (0.398)
	WBE222	9.216 (0.543)	6.549 (0.398)
p-value			0.940
Total Recall	Placebo	49.829 (1.443)	46.312 (0.989)
	WBE222	50.634 (1.445)	45.421 (0.989)
p-value			0.527
Proactive Interference	Placebo	0.936 (0.050)	1.031 (0.052)
	WBE222	0.949 (0.050)	1.012 (0.052)
p-value			0.742
Immediate Recall	Placebo	6.843 (0.247)	5.803 (0.211)
	WBE222	6.654 (0.246)	5.958(0.212)
p-value			0.485
CORSI Blocks Task:			
Longest Sequence	Placebo	7.350 (0.221)	7.392 (0.190)
	WBE222	6.980 (0.224)	7.353 (0.192)
p-value			0.852
Total Correct Sequences	Placebo	0.524 (0.019)	0.537 (0.015)
	WBE222	0.528 (0.019)	0.507 (0.015)
p-value			**0.034***
Latency (ms)	Placebo	1017.217 (29.946)	1013.178 (20.983)
	WBE222	1014.043 (30.175)	1023.39 (21.191)
p-value			0.716

Statistical differences from placebo are indicated *p < 0.05.

Corsi

Treatment was a significant predictor of Total Correct Sequences [F(1,29.6) = 4.9, p = 0.034]. Performance with placebo was found to be improved over WBE 222. Overall, in the current study measures on the Corsi Blocks task do not provide evidence of benefits of WBE 222 to visuospatial episodic memory at 14:00. See figure 5.3 for representation of results. Although not specifically analysed, the detailed results for the Corsi Blocks task (found in Table 5.3), show that the performance for Longest correct sequence following WBE 222 improved from baseline to 14:00h compared to no change following placebo and that latency time increased from baseline to 14.00h.

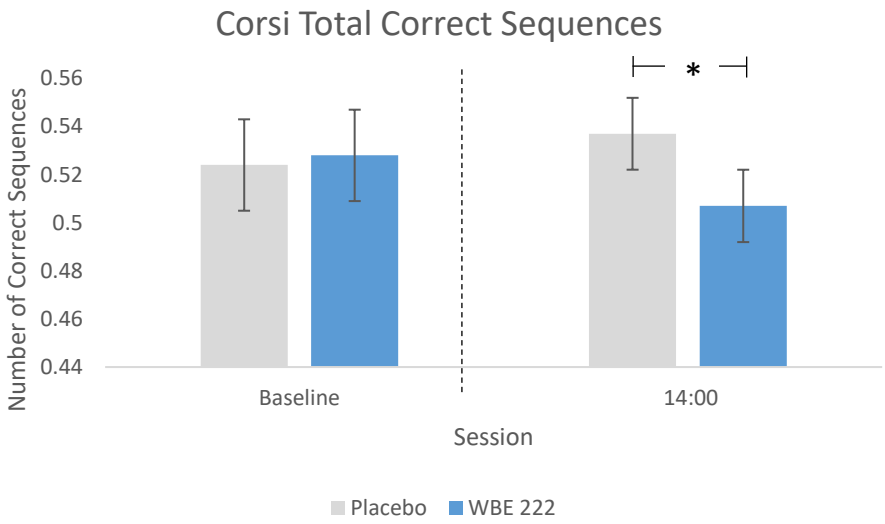


Figure 5. 3: Mean Corsi Total Correct Sequences (± SE) as a function of Treatment, showing significantly higher performance with placebo than WBE 222 (*p = 0.034). Baseline performance, included as a covariate in the analysis, is shown separated by the dotted line.

Executive Function

Means and standard errors for all Executive Function outcomes recorded in the BEAT trial are shown in Table 5.4.

Table 5. 4: Mean (SE) of Executive Function Outcomes following WBE 222 and Placebo

Measure	Intervention	Timepoint	
		Baseline (included as a covariate in the analysis)	**14:00h**
Switching Task:			
Accuracy	Placebo	0.973 (0.006)	0.976 (0.003)
	WBE222	0.970 (0.006)	0.974 (0.003)
p-value			0.162
Reaction Time (ms)	Placebo	897.233 (30.802)	886.235 (9.984)
	WBE222	923.003 (30.815)	870.961 (10.035)
p-value			**0.012***

Statistical differences from placebo are indicated *p < 0.05

Switching Task

Treatment was a significant predictor of Reaction Time [F(1,303.5) = 6.4, p = 0.012]. Reaction times were significantly faster with WBE 222 than placebo. See Table 5.4 for complete Switching Task results. This finding indicates WBE 222 supplementation is able to improve executive function reaction times at 14:00. Visual inspection of the graphical depiction of Switching Task Reaction Time results (See Figure 5.4) suggest that reaction times were reduced following WBE 222 but remained unchanged following placebo.

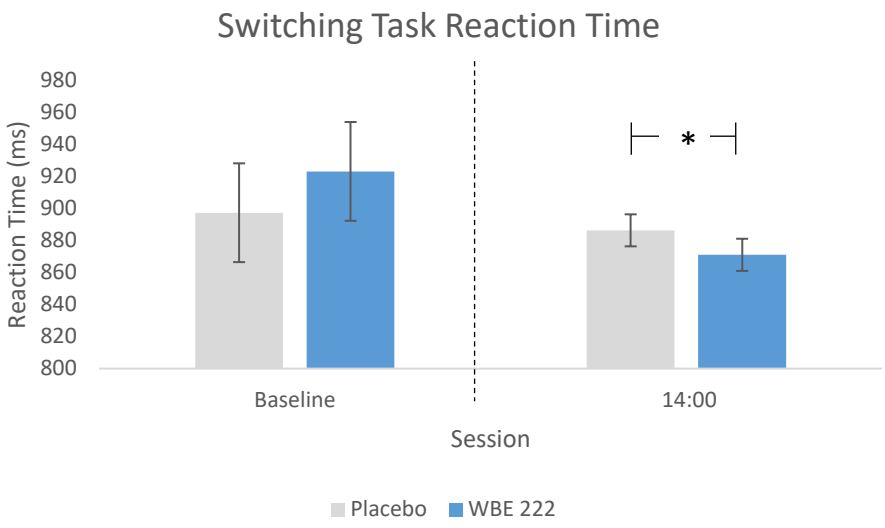


Figure 5. 4: Mean Switching Task Reaction Time (± SE) as a function of Treatment, showing significantly faster reaction times with WBE 222 than placebo. (*p = 0.012). Baseline performance, included as a covariate in the analysis, is shown separated by the dotted line.

Visit was a significant predictor of Switching Task Reaction Time [F(1,304.9 = 6.4, p = 0.011]. Reaction times were found to be significantly faster during Visit 2 than Visit 1. This may be attributable to practice effects despite all participants completing a practice visit prior to commencing the study.

Composite Scores

Means and standard errors for all Composite Cognition Scores recorded in the BEAT trial are shown in Table 5.5. Treatment and Visit were not significant predictors of the Composite scores.

Table 5. 5: Mean (SE) of Composite Cognition Scores following WBE 222 and Placebo

Measure	Intervention	Timepoint	
		Baseline (included as a covariate in the analysis)	**14:00h**
Composite Accuracy	Placebo	-0.28 (0.084)	0.012 (0.053)
	WBE222	0.15 (0.084)	-0.011 (0.053)
p-value			0.647
Composite Reaction Time	Placebo	0.006 (0.103)	-0.006 (0.059)
	WBE 222	-0.011 (0.103)	0.019 (0.059)
p-value			0.700
Composite Episodic Memory	Placebo	-0.31 (0.080)	0.011 (0.032)
	WBE222	0.18 (0.080)	-0.009 (0.032)
p-value			0.525
Composite Executive Function	Placebo	0.30 (0.087)	-0.010 (0.043)
	WBE222	-0.46 (0.087)	0.003 (0.043)
p-value			0.516
Global	Placebo	0.003 (0.073)	0.008 (0.031)
	WBE222	-0.006 (0.073)	-0.009 (0.031)
p-value			0.541

Cardiovascular

Means and standard errors for all Blood Pressure measurements recorded in the BEAT trial are shown in Table 5.6.

No cardiovascular effects were detected with WBE 222 treatment.

Table 5. 6: Mean (SE) of Blood Pressure measurements following WBE 222 and Placebo

Measure	Intervention	Timepoint	
		Baseline (included as a covariate in the analysis)	**14:00h**
Systolic BP (mm/Hg)	Placebo	122.996 (2.397)	124.542 (1.337)
	WBE222	123.467 (2.395)	124.176 (1.348)
p-value			0.808
Diastolic BP (mm/Hg)	Placebo	79.406 (4.429)	75.487 (0.952)
	WBE 222	77.298 (4.420)	75.467 (0.952)
p-value			0.984

Blood Pressure

Treatment and Visit were not significant predictors of systolic and diastolic blood pressure. Here no reductions to either SBP or DBP were detected. Results of the current study do not provide evidence of benefits to blood pressure following treatment with WBE 222. See figures 5.5 and 5.6 for results.

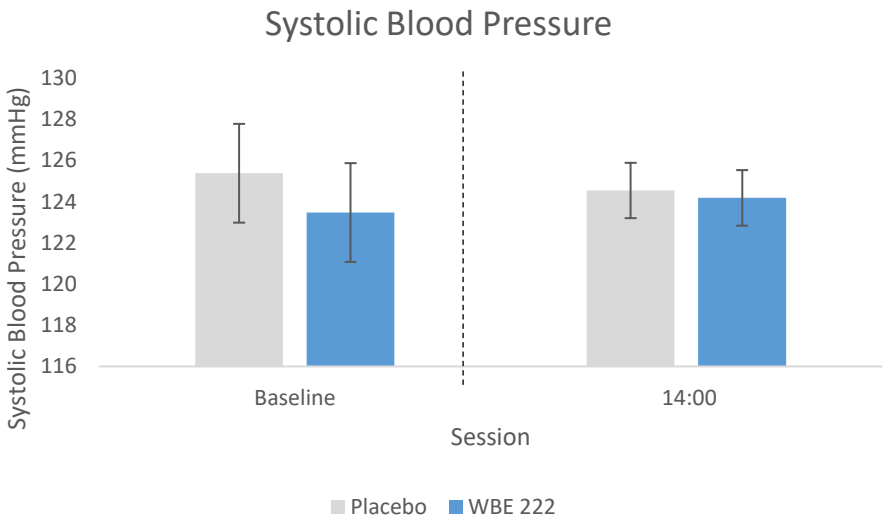


Figure 5. 5: Mean SBP (± SE) as a function of Treatment, showing SBP was not significantly different following WBE 222 than placebo (p = 0.808). Baseline SBP, included as a covariate in the analysis, is shown separated by the dotted line.

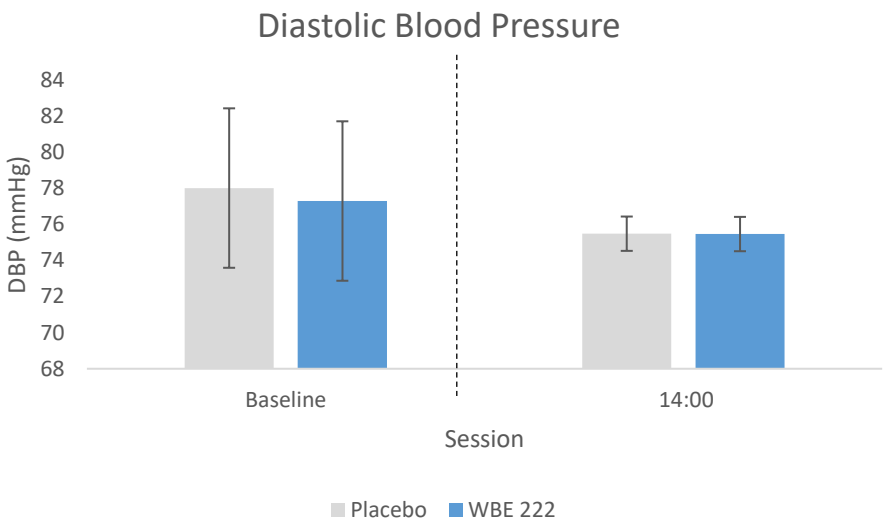


Figure 5. 6: Mean DBP (± SE) as a function of Treatment, showing DBP was not significantly different following WBE 222 than placebo (p = 0.808). Baseline DBP, included as a covariate in the analysis, is shown separated by the dotted line.

5.4 Discussion

The aims of this study were to investigate the effects of a single treatment of WBE 222 on episodic memory, executive function and cardiovascular measures in healthy older adults during the post-lunch dip. Circadian and homeostatic effects have been shown to culminate in a decline in cognitive performance during the day, most commonly observed at 14:00. Additionally, a post-prandial reduction in cognitive ability has been demonstrated 1 hour after lunch consumption. Collectively known as the post-lunch dip, evidence indicates that it affects the domains of executive function, memory and attention. This study was designed to examine the effects of WBE 222 at 14:00 when a post-lunch dip was anticipated and further enhanced by consumption of lunch 1 hour prior to cognitive testing. WBE 222 supplementation was scheduled such that known peaks in plasma blueberry flavonoid metabolites at 2 hours would coincide with a predicted drop in post-lunch performance. However, a post-lunch dip was not observed, and the largely null findings of this study

do not provide evidence for acute cognitive or cardiovascular benefits of WBE 222 in healthy older adults.

Episodic Memory

In the current study supplementation with WBE 222 was unable to produce benefits to any RAVLT outcome measures recorded at 14:00. Considering the baseline scores for RAVLT variables, a pattern of poorer performance at 14:00 following both interventions can be seen. This is in-keeping with cognitive chronobiology research which indicates older adults show synchrony effects, such that they generally perform at peak in the morning compared to when tested in the afternoon (May & Hasher, 1998). However, in general, the changes from baseline were not of notable size, which suggests that a clear post-lunch dip did not occur in relation to RAVLT performance. Surprisingly, poorer accuracy in the Corsi Block task was elicited following WBE 222 compared with placebo. Whilst a decline from baseline in total correct sequences was observed, a slowing of reaction times but improvement in longest correct sequence was found following WBE 222. This could indicate following WBE 222 participants focused their effort on reproducing the longer block sequences to the detriment of speed of response and overall number of correct sequences recalled. However, reasons for disparate levels of motivation following the different interventions remain unknown. The lack of positive findings here suggests there are no acute changes to visuospatial and verbal episodic memory following a single dose of WBE 222 that are detectable using the Corsi Block task and RAVLT. Importantly, examination of baseline scores for RAVLT and Corsi Blocks task variables suggest a post-lunch dip was not induced in relation to EM in this study. Whilst this might be expected since circadian variations in measures of memory performance are less pronounced than for executive function in older adults (May et al., 1993; Intons-Petersen et al., 1998; May and Hasher, 1998; Hasher et al., 2002; West et al., 2002), an absence of a prominent decline in EM performance in turn means that there was insufficient scope for alleviation by WBE 222.

Executive Function

Significantly faster reaction times (approximately 15ms), without detriment to accuracy scores, were observed following WBE 222 compared with placebo in the Switching task. This is in-keeping with the ROAB trial, where benefits to Switching task reaction times, a main effect of treatment, following WBE 111 were observed (approximately 11ms faster than placebo). Findings from the current study provide further evidence to support conclusions that WBE improves reaction times in healthy older adults in relation to executive function. This was detected in the early afternoon when a post-lunch dip was predicted to occur. However, baseline measurements indicate that reaction times did not change following placebo at 14:00. Therefore, a post-lunch dip was not evident, rather WBE 222 improved EF reaction times from baseline.

In older adults, time-of-day studies show that interference and inhibition control are particularly susceptible to circadian fluctuations (Hasher et al., 2002; West et al., 2002). Further, circadian fluctuations were revealed to be dependent on task difficulty where time-of-day variations were only evident in more challenging versions of a maze task (Blatter et al., 2005). The Switching task arguably places greater cognitive demand on subjects than simple recall tasks in that it entails mental flexibility to adapt to a pre-learned rule according to presented information, such that a post-lunch dip in performance might be expected. However, the amplitude of the peaks and troughs in inhibition are reduced in older adults compared to those recorded in young adults (Sagaspe et al., 2012) and may explain the absence of a post-lunch dip in EF found in the current study.

Cardiovascular

In the current study supplementation with WBE 222 did not demonstrate effects on SBP or DBP. The absence of blood pressure outcomes alongside limited improvements to cognitive measures is

consistent with the proposed mechanism of vasodilation common to both cognition and cardiovascular benefits of flavonoid supplementation. Indeed, in the ROAB trial main effects of treatment were shown for both systolic and diastolic blood pressure whereby significant reductions in blood pressure were recorded following WBE 222 compared with placebo. Treatment with WBE also elicited faster reaction times in the Switching task and delayed word recognition as well as alleviation of the post-lunch decline in executive function score. As to understanding possible reasons for such disparate BP findings between the ROAB and BEAT trials, this would be an important next step given the potential association between blood pressure and cerebral blood flow, and the mechanistic link this provides between blood pressure and cognitive performance. For example, blood pressure response to blueberry supplementation has been shown to depend on baseline BP where significant reductions in DBP were only evident in pre-hypertensive subjects (McAnulty et al., 2014). Comparison of mean baseline BPs could indicate if this is a determinant of BP effects shown in the ROAB trial but not in the current study.

The BEAT trial was designed to align the commonly observed post-lunch dip with cognitive testing and known plasma peaks in blueberry flavonoid metabolites. It was anticipated that such testing would maximise benefits of WBE treatment in light of positive findings demonstrated in the ROAB trial (Chapter 4). Switching Task reaction times were found to be faster following WBE supplementation consistently in both the ROAB trial (WBE 444 compared to placebo) and the current study (WBE 222 compared to placebo). However, the cognitive benefits demonstrated following WBE 111 to word recognition reaction times in the ROAB trial were not replicated by treatment with WBE 222 in the BEAT trial. The somewhat disparate results of the ROAB and BEAT trials could be explained by possible differences in cognitive and physical fatigue of subjects between the studies at comparable timepoints. For the ROAB trial, the 4 h post-intervention test battery was the third test battery subjects completed (clock-time approximately 13.30h). This equates to three hours of testing by the end of the third session. Subjects had one 2-hour and one 1-

hour break between starting the test day and this third test battery. However, for the current study the test day only involved two test sessions, a total of two hours testing with one 2.5h break, followed by two 1-hour breaks. The increased rest period and decreased cognitive burden placed on subjects for the current study may mean that they were less cognitively and physically fatigued relative to their counterparts in the ROAB trial. Under such conditions, a post-lunch dip was not apparent in the current study to explain why the benefits of WBE observed in the ROAB trial were not replicated. In this regard, it was not possible to measure the true effects of WBE 222 on the post-lunch dip.

Further, the positive effects on EF found by the ROAB trial at 4 h post-intervention may be associated with increases in blueberry flavonoid metabolites approaching the peak found at 6 hours rather than the earlier peak between 1 and 2 hours (Rodriguez-Mateos et al., 2013). In such a case, testing at 2 h post-intervention only in the BEAT trial may therefore have been too early to coincide with EF benefits. The extent to which the cognitive effects of blueberry flavonoids can be detected outside of known peaks in plasma metabolites is still unclear since testing of whole blueberry powder at 5 hours post-intervention by Dodd et al. (2019) did not yield significant benefits to cognitive performance.

Summary

It was not possible to measure the effects of WBE 222 on the post-lunch dip in the absence of a clear decline in cognitive performance at the expected timepoint. As hypothesized, WBE 222 was shown to improve aspects of executive function. Specifically, WBE 222 improved Switching task reaction times compared to placebo at 14:00 (2 hours post-intervention). In this regard, results from this study reinforce findings from the ROAB trial showing improvements to executive function can be achieved after a single dose of WBE. However, improvements to episodic memory and

cardiovascular measures were not evidenced. Results of this study failed to demonstrate faster reaction times in relation to episodic memory, unlike those observed following a higher dose of WBE (WBE 444) in the ROAB trial. Importantly, significant reductions in SBP and DBP recorded following WBE 222 in the ROAB trial were not replicated in the current study.

The limited cognitive benefits observed in this study coincided with an absence of cardiovascular effects. In contrast, both cognitive and cardiovascular improvements were demonstrated in the ROAB trial. This is in keeping with the proposed mechanism of vasodilation underpinning the mechanisms through which blueberry flavonoids exert their effects on cognitive performance and reduce blood pressure.

To better understand the effects of WBE supplementation on the post-lunch dip, future research may consider the provision of a high carbohydrate and high fat lunch to increase the likelihood of a post-lunch dip occurring. These have shown negative effects on cognition post-prandially, including reduced attention (Wells, Read, Uvnas-Moberg, & Alster, 1997) and increased reaction times (A. P. Smith, 1993). In the current study, the standardized lunch was not altered from the ROAB trial to maintain palatability and consistency across the two studies. It was also outside the scope of the current study to control for the homeostatic effects on the post-lunch dip. Future studies may look to extend participant 'time awake' periods to increase homeostatic pressure. This is an area worthy of exploration as improvements to cognition at this key time of day are likely to have safety and general wellbeing implications for individuals.

In conclusion, acute WBE 222 supplementation was observed to improve reaction times of healthy older adults in an executive function task. The inconsistent findings in relation to episodic memory and cardiovascular effects following WBE treatment observed across the ROAB and BEAT trials reflect that of the existing flavonoid-cognition literature.

Chapter 6: Final Discussion

6.1 Summary of Findings

The socio-economic burden of increased incidence of neurodegenerative diseases including dementia, associated with progressively ageing populations has led to substantial focus on diet and lifestyle factors as preventative and treatment measures. Flavonoids are one such dietary component to have received significant attention in relation to a protective role against pathological cognitive decline and improvements to cognitive function in healthy populations. This thesis examined the effects of flavonoid consumption on cognitive function by meta-analysis of human intervention studies and assessed moderating factors of this effect. Key findings from this showed flavonoid consumption has a significant positive effect on cognitive function. Further, significant effects of flavonoids were demonstrated in middle-aged and older adults, following consumption of berry, cocoa or Ginkgo biloba products.

In light of significant flavonoid effects in older adults and following berry supplementation, the subsequent aims of this thesis were to determine the acute cognitive and cardiovascular effects of a wild blueberry extract in an older adult population, to extend the acute effects of blueberry supplementation previously observed in children across 1 -6h timeframe (Whyte et al., 2015; 2016;2017, Barfoot et al., 2019) and chronic results following wild blueberry extract (Whyte et al., 2018). Cognitive and cardiovascular parameters were investigated across two experiments.

The aims of the ROAB trial were to determine wild blueberry extract effects on cognitive function and whether these are dose and time-dependent. The cognitive and cardiovascular effects of a range of WBE doses were investigated in a within-subject design with measurements taken at 2 hours, 4 hours and 6 hours post-intervention to reflect observed peaks in FMD and metabolites following acute blueberry treatment (Rodriguez-Mateos et al., 2013). This showed benefits to EM

and EF speed, and alleviation of a post-lunch decline in EF. This suggests that blueberry flavonoids are able to overcome the post-lunch dip. In keeping with proposed vasodilatory mechanisms of cognitive and cardiovascular benefits, SBP and DBP were significantly reduced following WBE 222. Although a clear dose response was not found, WBE 222 was identified as the optimum tested dose as it was associated with both BP effects and alleviation of a post-lunch dip in EF.

Subsequently, WBE 222 was examined in the BEAT trial which aimed to replicate alleviation of cognitive decline during a predicted post-lunch dip whilst also improving cardiovascular outcomes. The post-lunch dip is associated with increased risk of accidents and negatively impacts on the performance of day-to day activities (Garbarino et al., 2001). Overcoming this therefore has positive implications on the safety and wellbeing of individuals. A within-subject design was adopted with cognitive and cardiovascular measurements taken 1 hour after lunch at 14:00 to coincide with the post-lunch dip and post-prandial decline in cognitive performance. This showed improved EF speed consistent with ROAB. However, EM speed and alleviation of a post-lunch dip (in EF) was not replicated and cardiovascular effects were not evident.

Experimental details and outcomes are discussed further below.

6.1.1 Dietary Flavonoids and Human Cognition: Meta-analyses.

Meta-analysis of the existing human flavonoid-cognitive literature was performed. Several published systematic reviews have given a narrative commentary of the current research into the effects of flavonoids on cognitive function in favour of flavonoid intake. The mixed outcomes of individual studies indicate that completion of the meta-analyses was timely. Further, previous meta-analyses have focussed on specific populations and/or flavonoid-rich sources such that broad conclusions over flavonoid efficacy could not be made.

It was hypothesised that flavonoids would show a significant positive effect on cognitive function overall. Additionally, the mixed findings between individual studies suggest that cognitive benefits of flavonoids might therefore be dependent on several experimental characteristics of the flavonoid intervention, including dose and source, duration of intervention and population examined, such as age and cognitive health status, and the cognitive ability assessed. These were therefore expected to be significant determinants of the cognitive effects of flavonoids. Comparative subgroup analyses according to the moderators flavonoid source, duration of supplementation, age of participant, and cognitive health status were conducted. However, comparative analysis was not possible where studies reported data for multiple subgroups within a category; therefore separate meta-analyses were completed for each of dose, acute/chronic studies and cognitive ability.

As predicted flavonoid intake was found to have a significant positive effect on cognitive performance, albeit small in magnitude. Of the four comparative subgroup analyses completed, only subject cognitive health status was found to be a significant moderator of cognitive effects. Although significant benefits were shown in both cognitively healthy and unhealthy populations, flavonoid effects were found to be most prominent in the cognitively unhealthy in which there is likely greater scope for behavioural change. The subgroup analyses also revealed significant flavonoid effects in middle-aged and older adults but not other age groups, which may reflect the onset of age-related cognitive decline, meaning detection of cognitive changes are more likely. Positive effects of flavonoids were found following berry, cocoa and Ginkgo biloba supplementation but not citrus, soya, pine bark or tea, indicative of mechanistic differences between the flavonoid subclasses and reflects the limited data relating to citrus, soya, pine bark or tea. Significant flavonoid effects were found in chronic studies. Specifically, the duration of intervention analysis showed these effects were most likely observed following supplementation of 6 weeks and longer, which suggests that the cognitive effects of flavonoids are cumulative. Analysis of the individual cognitive abilities showed significant flavonoid effects on long term memory, processing speed and mood. However, as

comparative analysis could not be completed, it is not possible to conclude whether any cognitive abilities are particularly responsive to flavonoid intervention.

In light of findings that older adults might be more susceptible to the effects of flavonoids, and that the effects of berries are particularly pronounced, an acute trial was designed to investigate the effects of a wild blueberry extract in older adults.

6.1.2 The ROAB trial

Following on from Dietary Flavonoids and Human Cognition: Meta-analyses, an investigation into the effects of wild blueberry extract on cognitive performance and cardiovascular outcomes was performed. In the ROAB trial, a double-blind, within-subjects design compared the effects of four separate wild blueberry extract doses (111mg, 222mg 444mg and 888mg) with a placebo (maltodextrin). Measures of cognition, mood, blood pressure and heart rate were recorded at baseline, 2 hours, 4 hours and 6 hours post-intervention to encompass timeframes of observed FMD and metabolite peaks following acute blueberry supplementation (Rodriguez-Mateos et al., 2013). It was hypothesised that positive effects of blueberry flavonoids on EF, EM and blood pressure would be dose- and time-dependent. It was predicted that EF and EM performance would be most improved according to increase in dose and these would be most prominent at 2 hours and 6 hours coinciding with FMD and metabolite peaks (Rodriguez-Mateos et al., 2013).

Individual test outcomes and composite scores for EM, EF, reaction times, accuracy and a global composite score were derived from a test battery comprised of RAVLT, Corsi Blocks task, Switching task, Trail Making tasks A and B and Choice Reaction Time. Results showed improved RAVLT word recognition reaction times following WBE 444 compared with placebo and faster reaction times following WBE 111 in the Switching Task, indicating benefits of WBE to psychomotor function.

Additionally, a significant dip at 4 hours post-intervention in the composite executive function score found following placebo was attenuated by WBE. The drop in executive function overlapped with the post-lunch dip, indicating that WBE may alleviate the post-lunch decline in cognitive abilities. Acute reductions in both systolic and diastolic blood pressure were demonstrated following WBE 222 consumption compared with placebo. The observed cognitive effects coincided with reductions in BP support purported vasodilatory mechanisms common to both outcome measures. Although a predicted dose-dependent response for cognitive and cardiovascular measures was not evident, WBE 222 was identified as the optimum tested dose, in that intake of WBE 222 elicited both cognitive and cardiovascular benefits. The BEAT trial was designed and carried out to specifically investigate the effects of WBE 222 on the post-lunch dip.

6.1.3 The BEAT trial

Subsequent to the cognitive and cardiovascular observations from the ROAB trial, a further experiment to replicate the effects of wild blueberry extract 222mg on the maintenance of cognitive performance during an expected post-lunch dip was completed. The BEAT trial was a double-blind, within-subjects study in which cognitive assessment using tests where WBE effects were found was conducted at 14:00h, when a post-lunch dip was anticipated. Testing was scheduled 1 hour after consumption of lunch, when post-prandial declines in cognitive function are also reported. The interventions were administered 2 hours prior to cognitive testing such that known peak plasma concentrations of blueberry polyphenol metabolites (Rodriguez-Mateos et al., 2013) would coincide with measurement of cognitive ability.

It was predicted that in accordance with findings from the ROAB trial, cognitive performance measured by RAVLT, Corsi Blocks task, Switching task and composite scores for EM, EF, reaction times, accuracy and a global composite score, would be maintained and blood pressure reduced

during the post-lunch dip after WBE 222 consumption relative to placebo. The positive cognitive effects were limited to faster Switching task reaction times following WBE 222 relative to placebo. Importantly, the results showed that a clear post-lunch dip in cognitive function did not occur, such that the largely null cognitive findings are likely explained by insufficient scope for WBE 222 effects to be detected. Further, no effects of WBE 222 on BP were observed. Overall, this study did not provide evidence for alleviation of the post-lunch dip by WBE 222 consumption.

6.2 General Discussion

Taken together the experimental chapters of this thesis demonstrate that although in general dietary flavonoids have a positive effect on cognitive function found by meta-analysis, overall acute supplementation with a flavonoid-rich wild blueberry extract did not elicit significant improvements to cognitive outcomes.

6.2.1 Cognitive Effects

Positive effects of acute WB powder (Dodd et al., 2019) and chronic WBE 111 (Whyte et al., 2018) on RAVLT -Word Recognition accuracy have previously been reported in older adults, demonstrating benefits of blueberry flavonoids on long term memory. This is in keeping with outcomes of Dietary Flavonoids and Human Cognition: Meta-analyses, which found significant effects of flavonoid intake on performance in this cognitive domain. In contrast, acute WBE administration did not elicit benefits to word recognition accuracy, rather reaction times for word recognition were significantly improved following WBE 444 relative to placebo in the ROAB trial. The faster reaction times more likely demonstrate positive effects of WBE 444 on psychomotor function as opposed to long term verbal memory. Further, no effects of WBE 222 were found for any aspect of RAVLT performance in the BEAT trial, again indicating that WBE does not improve verbal memory.

Across the ROAB and BEAT trials acute WBE supplementation did not elicit any benefits to performance in the Corsi Blocks task. In fact poorer accuracy was found following WBE 222 consumption in the BEAT trial. This is in contrast to results of the chronic study by Whyte et al. (2018) which showed WBE 111 was associated with a trend towards better performance in the Total Number of Correct Sequences. The absence of positive findings in the chapters of this thesis indicate acute administration of WBE doses between 111mg and 888mg do not confer advantages to visuospatial memory. Seemingly, chronic WBE treatment of several weeks, is required to produce measurable improvements in the Corsi Blocks task. This is in agreement with the findings of Dietary Flavonoids and Human Cognition: Meta-analyses, which showed cognitive effects of flavonoids were most apparent following at least 6 weeks of supplementation.

Faster Switching task reaction times were found in relation to placebo following WBE 111 in the ROAB trial and WBE 222 in the BEAT trial. The consistent outcomes indicate that this particular effect is relatively robust. These findings concur with improved Switching task reaction times following an acute mixed berry intervention in young healthy adults (Whyte et al., 2019). However, accuracy scores in the Switching task were not altered by WBE intake, which indicates that the benefits to Switching task reaction times likely represent changes in improved psychomotor function rather than executive functioning. Together with the null findings in the ROAB trial for Choice Reaction task and Trail Making Task A and B, the chapters of this thesis indicate that acute WBE doses between 111mg and 888mg do not improve executive function.

The improvements to reaction times following WBE 444, 222 and 111 are likely representative of benefits to psychomotor function. These findings are in agreement with the results of Chapter 3: Dietary Flavonoids and Human Cognition: Meta-analyses, which found significant positive effects to psychomotor speed following flavonoid treatment, and more specifically most commonly observed

following acute administration of flavonoids (Bell et al. 2015). Interestingly, the Switching task and Delayed word recognition both require discrimination of pre-learned information or task rules and are therefore more cognitively demanding than simple reaction time or recall tasks. Positive effects observed in these two tasks support proposals that berry flavonoids are most effective at improving cognition in more cognitively challenging conditions (Whyte et al., 2016, Miller et al., 2018). In further support of this notion, the greater cognitive and physical demand of the testing schedule placed on participants of the ROAB trial compared to the BEAT trial, may in part explain the disparate cognitive outcomes. The benefits of WBE, although limited, were more apparent in the ROAB trial where a post-lunch dip was evident. In particular, a post-lunch dip in executive function composite score was alleviated by WBE in the ROAB trial. In contrast, a post-lunch dip was not evident for any cognitive outcomes measured by the BEAT trial. These findings indicate that to increase the likelihood of detecting the cognitive effects of berry flavonoids, a sufficiently demanding test schedule comprised of appropriately challenging tasks should be incorporated into the design of a study.

The cognitive effects of acute flavonoid intake have shown to be dose-dependent (Kennedy et al., 2000; Scholey et al,. 2010). However, a dose-response was not apparent in the ROAB trial despite previous findings of more pronounced cognitive responses following higher doses of berry flavonoids (Whyte et al., 2016, Bell & Williams. 2018). Although a minimum dose for the cognitive benefits of berry flavonoids has not been defined, these have most commonly been reported following doses of between 127mg anthocyanins (Whyte et al., 2016) and 254mg anthocyanins (Whyte et al., 2019). Indeed, no cognitive effects were found following a single 73.5mg dose of tart cherry anthocyanins (Keane et al., 2016). This suggests that the flavonoid content of the tested WBE interventions; 7mg, 14mg, 38mg and 76mg respectively were insufficient to elicit robust cognitive changes and therefore explain an absence of a dose-response.

6.2.2 Mood

Flavonoids were found to have significant positive effects on mood by Dietary Flavonoids and Human Cognition: Meta-analyses. However, acute effects of WBE on mood were not apparent in the ROAB trial, which is also contrary to observed improvements to Positive Affect following acute blueberry supplementation in children and young adults (Khalid et al., 2017) and chronic supplementation in adolescents (Fisk et al,. 2020) each containing 253mg anthocyanins. Whyte et al. (2018) also assessed the effects of WBE 111 (7mg anthocyanins) on mood and found no alterations to PANAS-NOW scores after 3 months. Taken together these findings suggest that the absence of positive effects on mood following chronic WBE consumption shown by Whyte et al. (2018) and following acute intake found in the chapters of this thesis, are likely explained by too low a dose of blueberry flavonoids to elicit changes in mood.

6.2.3 Cardiovascular effects

Previously acute berry flavonoid treatments have shown mixed effects on BP. Reductions in BP were found following supplementation with plum juice (Igwe et al. 2017), cherry juice (Keane et al., 2016) and haskap berry (Bell and Williams, 2019). However, acute cranberry (Rodriguez-Mateos et al., 2016) and blueberry interventions (Rodriguez-Mateos et al., 2013 and Dodd et al., 2019) did not elicit similar blood pressure effects. Additionally, anthocyanins have shown less robust blood pressure effects than other flavonoid-subclasses (Ciumarnean et al., 2020) which the findings of the chapters of this thesis are in agreement with, in that the BEAT trial failed to replicate the BP reductions following WBE 222 observed in the ROAB trial. Factors such as baseline BP, which was shown to determine BP response to blueberry supplementation (McAnulty et al., 2014), could further explain the disparate BP outcomes in this thesis. It may be useful to compare mean baseline

BPs from the ROAB trial and BEAT trial to examine the influence of baseline measures on the findings here.

Whyte et al (2018) found a reduction in systolic blood pressure alongside improved episodic memory and visuospatial memory, following three months' supplementation with WBE in healthy older adults. This supports the proposal that the BP and cognitive effects of blueberry flavonoids may be attributed to endothelial-dependent vasodilation. Improved endothelial function has been found to correlate with cerebral blood flow (Akazawa et al., 2021), following which improvements to cognitive function might be expected. The BP findings from the chapters of this thesis are broadly consistent with this mechanism of action, in that in the ROAB trial acute SBP and DBP reductions coincided with faster reaction times in the Switching task and delayed word recognition, as well as alleviation of the post-lunch decline in executive function score. Accordingly, an absence of blood pressure outcomes were found alongside limited improvements to cognitive measures in the BEAT trial. However, it should be noted that direct measures of vasodilation and CBF were not taken here and require further investigation.

6.3 Limitations

6.3.1 Recruitment

Initial retention of recruited volunteers to the ROAB trial was difficult. Thirty per cent of volunteers (12 out of 40) who attended a familiarisation visit, withdrew because of the demands of the task battery and length of the test day. In comparison all 45 volunteers who completed a familiarisation visit went on to complete the BEAT trial, further demonstrating the difference in cognitive and physical fatigue experienced by participants between the two studies. Interestingly, across both

studies, once randomised to an intervention schedule all participants continued to complete all subsequent test sessions.

Older adults were mainly recruited via the Psychology Department's Older Adult Panel and the University of the Third Age, such that the demographics of participants may have been biased towards those with an interest in health, nutrition and cognition. Indeed, the majority of participants were educated to at least secondary level and of white ethnicity. Further, the start of the study days at 8am, meant that older adults were unable to use free public transport (off-peak hours only) which deterred a number of potential participants and favoured individuals who were able to drive to the University. In retrospect, it would be useful to highlight that travel expenses are recompensed. The potential sample bias in these studies means that participants may have performed better than average on the cognitive tasks and possibly at ceiling, such that the observed magnitude of WBE effects may have been understated.

In March 2020, UK government restrictions to prevent the spread of COVID-19 meant that recruitment to the BEAT trial could not continue. At this point, 45 participants had completed all test sessions. There was a shortfall of 41 participants compared to the target sample size of 86 and therefore a potential risk of type II error. The target sample size was computed according to the effect size of WBE treatment on reaction times measured in the ROAB trial. Indeed, benefits to EF reaction times were successfully demonstrated following WBE 222 by the BEAT trial despite the recruitment limitations. This suggests that the number of participants completing the BEAT trial was sufficient to detect cognitive effects. It might therefore be reasonable to expect evidence of emerging effects for other cognitive measures where an effect exists. However, there were no patterns or trends towards significance in any of the other reported outcomes. The BEAT trial power calculation provided an estimate of the number of participants required to detect a difference between the effects of WBE 222 and placebo on cognitive performance during the post-lunch dip.

Importantly, a post-lunch dip did not occur during the BEAT trial which is unlikely to have been affected by continued recruitment. Consequently, this suggests that additional significant cognitive outcomes were unlikely to become apparent with a larger sample size and that the conclusions drawn from this thesis are unlikely to have been altered by further recruitment to the BEAT trial.

6.3.2 Habitual Diet

To minimise the effects of habitual diet on outcome measures, participants were required to adhere to a low polyphenol diet for 24 hours and fast for 12 hours before testing. Participants were provided with a list of low polyphenol foods as guidance for what items could be consumed prior to test days. Compliance was confirmed verbally at each test day. Although this was not more formally documented, participant enquiries regarding the polyphenol content of foods they were planning to consume indicate a high level of commitment to the restricted diet and suggest that claims of compliance were accurate. Future studies could use 24 hour food diaries to document adherence to the low flavonoid diet. To further reduce dietary confounds the same standardised breakfast was eaten at the start of each visit. Restrictions of budget meant that provisions of a standardised dinner the night before testing was not possible. Additionally, there remains debate over the real world relevance of testing under tightly controlled diet conditions.

6.3.3 Flavonoid Stability

The antioxidants, L-cysteine and L-glutathione, were included in the formulation of WBE to enhance the stability of the blueberry anthocyanins and therefore increase anthocyanin bioavailability (Eisenberger, 2014). The extent to which anthocyanin bioavailability was enhanced was not measured here. In light of more recent findings which suggest that glutathione do not protect flavonoids from oxidation (Gambuti, Picariello, Rolle, & Moio, 2017) but may increase oxidative

degradation during storage (Z. Zhang, Zhang, Fan, & Kilmartin, 2022), it would be prudent to measure concentrations of plasma anthocyanins in future research. Specifically, levels of bioactive metabolites which correlate with cognitive performance, such as hippuric acid (Barfoot et al., 2021) would be most relevant.

6.4 Future Research

This thesis examined the effects of WBE on cognitive and cardiovascular function in healthy older adults. The data collected here cannot therefore be applied to other populations. Flavonoids were shown to be effective at modulating the cognitive performance of middle-aged adults by Dietary Flavonoids and Human Cognition: Meta-analyses. Extending the current work to investigate the dose- and time-dependent effects of WBE in middle-aged adults would be a reasonable next step and in light of findings that age-related cognitive decline commences in this age group, the cognitive benefits of a dietary supplement are therefore of particular interest. The findings of the current thesis suggest that doses of WBE of between 111mg and 888mg were too low, instead should encompass anthocyanin concentrations between 127mg and 254 mg at which cognitive effects have previously been detected (Whyte et al., 2016, Whyte et al., 2019). Testing of much higher doses would support elucidation of the shape of the dose-response curve, which is likely to be non-linear in light of significant flavonoid effects of low and medium tested doses, but not high doses (see Chapter 3: by Dietary Flavonoids and Human Cognition: Meta-analyses). This plateau of increase in cognitive benefits with increased flavonoid dose could reflect saturation of metabolic enzymes, such as LPH.

Observations from this thesis indicate that under conditions of increased cognitive fatigue when a post-lunch dip is induced, blueberry-flavonoid effects may become apparent. To investigate this further, future research should look to enhance the likelihood of a post-lunch dip, through provision

of macronutrients known to negatively impact post-prandial cognition, such as found in a high carbohydrate and high fat lunch (Wells et al., 1994; Smith et al., 1993). Homeostatic effects could also be increased by standardising the 'time awake' window prior to cognitive testing, such that it is extended to generate cognitive and physical fatigue. Further the chronotype of participants could be assessed, such as by the Munich Chronotype Questionnaire, to enable identification of any subgroups who may be more susceptible to a post-lunch dip and therefore respond to blueberry supplementation. Importantly, a suitably demanding task battery comprised of tasks for which acute blueberry effects have been demonstrated should be selected. Under these conditions the cognitive effects of blueberry consumption are more likely to be elicited and importantly likely reflect cognitive and physical demands experienced in real-life situations.

This thesis did not assess the purported stabilisation of anthocyanins in the WBE interventions by the addition of L-cysteine and L-glutathione to the formulation. This could be done through monitoring of plasma concentrations during the acute post-intervention phase. However, there is increasing evidence which shows that bioactive flavonoid metabolites rather than their native forms are responsible for physiological changes, including modulation of cognition. In light of this, it would be reasonable to employ dosage design techniques which either promote in vivo metabolism of flavonoids or protect administered doses of monomeric or aglycone forms, such as micro- or nano-encapsulation (Tran & Tran, 2021). The cognitive effects of this type of formulation have not been assessed compared to standard extraction and encapsulation or whole fruit products and should be included in any well-designed placebo-controlled study. To understand, not only how flavonoid bioavailability may be altered by administration of monomeric or aglycone blueberry flavonoids, markers of mechanisms of action should also be measured. This could include endothelial function (FMD) and CBF (fMRI).

6.5 Final Conclusions

The research within this thesis found significant positive effects of dietary flavonoids on human cognitive function. In particular, these effects are determined by subject age, cognitive health status, flavonoid dose, duration and source. In the first investigation of the acute cognitive and cardiovascular effects of WBE supplementation in older adults, evidence for benefits are weak in this research and most likely limited by the low flavonoid content of the intervention. Improvements to psychomotor function in relation to executive function were found in both studies. However, reaction times for episodic memory and blood pressure reductions were only apparent during a schedule which increased cognitive and physical fatigue. Under such conditions, WBE treatment was found to alleviate a post-lunch dip in executive function. It is unclear whether stabilisation of anthocyanins in the WBE interventions by the addition of L-cysteine and L-glutathione was successful. Future research should consider the enhanced delivery of monomeric and aglycone blueberry flavonoids to improve bioavailability of bioactive metabolites and modulation of cognitive function.

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Appendices

Appendix A: Summary of Key Cognitive Tests used in Flavonoid-Cognition Research

Name of Test	Cognitive ability assessed	Advantages/Disadvantages
Animal Naming Test (Campagna et al., 2017)	Word fluency	Simple to administer. Ceiling effects. (Labenz et al., 2019)
Appointment Test (Kaschel, 1994)	Long term memory encoding and retrieval	High ecological validity (Kaschel et al. 2011)
Attention Network Test (Fan, McCandliss, Sommer, Raz, & Posner, 2002)	Reaction/Decision speed	Computerised. Task difficulty modifiable Low reliability of orienting and alerting scores. (MacLeod et al., 2010)
Benton Visual Retention Test (Benton Sivan, 1992)	Long term memory encoding and retrieval	Alternate forms available for repeat testing. Poor test-retest reliability. (de Jager et al., 2014)
Berg Card Sorting Test (Berg, 1948)	Fluid reasoning	Computerised. Inconsistent methods of scoring (Fox, Mueller, Gray, Raber, & Piper, 2013)
Boston Naming Test (Kaplan, Goodglass, & Weintraub, 2001)	Acquired knowledge	Good test-retest reliability. Performance depends on attention. Most reliable for individuals with low-average naming ability. (Harry & Crowe, 2014)
Buschke Selective Reminding Test (Buschke, 1973)	Long term memory encoding and retrieval	Normative data. Sensitivity to impairment. Requires ender correction for scores (Leitner, Miller, & Libben, 2019)
California Verbal Learning Test (Delis, Kramer, Kaplan, & Ober, 2000)	Long term memory encoding and retrieval	Good test-retest reliability. Construct validity. Significant role of attention in performance. Practice effects. (Leitner et al. 2019)
CERAD (Consortium to Establish a Registry for Alzheimer's Disease) (Morris, Mohs, & Rogers, 1989)	Long term memory encoding and retrieval	Test-retest reliability. Standardised. Clinical assessment for AD. (Fillenbaum et al. 2008)

Choice Reaction Time (Posner & Rothbart. 1980)	Reaction/Decision speed	Correlation with other tests of information processing. Limited by motor function (de Jager et al. 2014)
Complex Figure Test (Osterrieth. 1944)	Processing speed	Correlates strongly with other visual memory tests. (de Jager et al. 2014)
Complex visual vigilance (Lieberman, Coffey, & Kobrick, 1998)	Processing speed	Influenced by motor function
Contextual memory (Pipingas et al., 2008)	Long term memory encoding and retrieval	Computerised
Continuous Performance Task (Klee & Garfinkel, 1983)	Processing speed	Validated across age groups and ADHD patients. Influenced by motivation. (van den Bosch, Rombouts, & van Asma, 1996)
Corsi Block Tapping Test (Corsi, 1972)	Working memory	Computerised versions available. Versions not standardised (Arce & McMullen, 2021)
Delayed Matching to Sample Test (Holdstock, Shaw, & Aggleton, 1995)	Working memory	Computerised. Alternate forms available for repeat testing (Hartman, Dumas, & Nielsen, 2001)
Digit Symbol Substitution Test (McLeod, Griffiths, Bigelow, & Yingling, 1982)	Processing speed	Minimal effect of mood on performance Practice effects. (Zihl, Fink, Pargent, Ziegler, & Bühner, 2014)
Digit Vigilance Test (Lewis & Rennick, 1979)	Processing speed	Good test-restest and alternate form reliability (Kelland & Lewis, 1996)
Doors and People test (Baddeley, Emslie, & Nino-Smith, 1994)	Long term memory encoding and retrieval	Simple to administer. Normative data for ages 16+ years only. (Davis, Bradshaw, & Szabadi, 1999)
Driving recognition score (Kaschel et al., 2011)	Long term memory encoding and retrieval	High ecological validity
East Boston Memory test - delayed recall (Scherr et al., 1988)	Long term memory encoding and retrieval	Brief (5 minutes) and sensitive measure of verbal memory (Gfeller & Horn, 1996)
Finger Tapping Tasks	Processing speed	Simple. Variations in versions – interpretation across studies difficult (Witt, Laird, & Meyerand, 2008)
Flanker Task (Eriksen & Eriksen, 1974)	Reaction/Decision speed	Reliability and validity (Sanders, Hortobágyi, Balasingham, Van der Zee, & van Heuvelen, 2019)

Go NoGo Task (Gordon & Caramazza, 1982)	Processing speed	Child and adult versions. Valid and reliable (Thomas et al. 2016).
Grooved Pegboard (Matthews & Kløve, 1964)	Processing speed	Normative data. Influenced my handedness. (Tolle, Rahman-Filipiak, Hale, Kitchen Andren, & Spencer, 2020)
Groton Maze Learning Test (Snyder et al. 2005)	Visuospatial ability	Construction validity in children and adults. (Pietrzak et al., 2008)
Hopkins Verbal Learning Test (Brandt, 1991)	Long term memory encoding and retrieval	Good validity compared to other verbal memory tests. Ceiling effects reported in unimpaired adults. (de Jager et al. 2014)
Intradimensional-Extradimensional (IDED) attention-shifting task (Sahakian & Owen, 1992)	Fluid reasoning	Normative data. Computerised. Modest construct validity. (Smith et al. 2012)
International Shopping List (Lim et al., 2009)	Long term memory encoding and retrieval	Cross-cultural application Proactive interference of performance with short retest intervals (Rahimi-Golkhandan, Maruff, Darby, & Wilson, 2012)
N-back (Kirchner, 1958)	Working memory	Manipulate difficulty via load level and different stimuli. Moderate reliability as measure of working memory. Ceiling effects at lower levels in particular. (Jaeggi, Buschkuehl, Perrig, & Meier, 2010)
Paced Auditory Serial Addition test (Gronwall & Sampson, 1974)	Working memory	Practice effects – especially short-term repeated testing (Tombaugh, 2006)
Pattern Recognition Memory (Sahakian and Owen. 1992)	Working memory	Semi-computerised. Modest construct sensitivity. (Lenehan, Summers, Saunders, Summers, & Vickers, 2016)
Picture Matching Task (Bisanz, Danner, & Resnick, 1979)	Fluid reasoning	Not limited by language – use with children
Picture Recall Task (Sahakian and Owen. 1992)	Long term memory encoding and retrieval	Semi-computerised. Modify stimulus and interstimulus duration. Modest construct sensitivity. (Lenehan et al. 2016)
Rapid Visual Information Processing (Sahakian and Owen. 1992)	Processing speed	Semi-computerised. Modest construct sensitivity. (Lenehan et al. 2016)
Ravens Progressive Matrices (Raven & Court, 1998)	Fluid reasoning	Non-verbal assessment. Good convergent validity. Moderate discriminant validity. (Schweizer, Goldhammer, Rauch, & Moosbrugger, 2007)
RBANS Attention (Randolph, 1998)	Processing speed	Normative data. Brief instrument. Validity. (Shura et al., 2018)
RBANS Delayed Memory (Randolph. 1998)	Long term memory encoding and retrieval	Normative data. Brief instrument. Validity. (Shura et al. 2018)

RBANS Immediate Memory (Randolph. 1998)	Long term memory encoding and retrieval	Normative data. Brief instrument. Validity. (Shura et al. 2018)
RBANS Language (Randolph. 1998)	Word fluency	Normative data. Brief instrument. Validity. (Shura et al. 2018)
RBANS Visuospatial/Constructional (Randolph. 1998)	Visuospatial ability	Normative data. Brief instrument. Validity. (Shura et al. 2018)
Rey Auditory Verbal Learning Test (Rey, 1958)	Long term memory encoding and retrieval	Easy to administer. High test-retest reliability. Practice effects (de Sousa Magalhães, Fernandes Malloy-Diniz, & Cavalheiro Hamdan, 2012)(de Sousa et al. 2012)
Rey Osterrieth Complex Figure Test (Osterrieth. 1944)	Visuospatial ability	Normative data in children and adults. Varied scoring criteria. Moderate reliability (Loring, Martin, Meador, & Lee, 1990)
Selective Reminding Test (Grober & Buschke, 1987)	Long term memory encoding and retrieval	Good correlation with other verbal memory tests. Standardised. Practice effects. (de Jager et al. 2014)
Serial 3s Subtraction Task (Hayman, 1941)	Processing speed	Good construct validity. Normative data. Strategy employment for computerised versions. (Bristow, Jih, Slabich, & Gunn, 2016)
Serial 7s Subtraction Task (Hayman. 1941)	Processing speed	Good construct validity. Normative data. Strategy employment for computerised versions. (Bristow et al. 2016)
Simple Reaction Time (Simpson, Surmon, Wesnes, & Wilcock, 1991)	Reaction/Decision speed	Correlation with tests of information processing Limited by motor function (de Jager et al. 2014)
Stockings of Cambridge (Sahakian and Owen. 1992)	Fluid reasoning	Semi-computerised. Modest construct sensitivity. (Lenehan et al. 2016)
Spatial Span (Sahakian and Owen. 1992)	Working memory	Semi-computerised. Modest construct sensitivity. (Lenehan et al. 2016)
Spatial Working Memory (Sahakian and Owen. 1992)	Working memory	Semi-computerised. Modest construct sensitivity. (Lenehan et al. 2016)
Stroop (Golden, Freshwater, & Golden, 1978)	Processing speed	Children and adults. Computerised versions – internal consistency Different versions – not comparable across studies (Basu, 2023)
Sustained Attention to Response Task (Robertson et al., 1997)	Processing speed	Validated across age groups. Potential speed-accuracy trade-off. Influenced by motivation (Dang, Figueroa, & Helton, 2018)

Symbol Digit Modalities Test (Smith. 1973)	Processing speed	Test-retest reliability. Equivalent alternate forms. (Benedict et al., 2017)
Task Switching Task (Miller et al. 2018)	Reaction/Decision speed	Computerised. Lengthy task, performance dependent on attention.
Tower of London (Shallice, 1982)	Fluid reasoning	Abstract nature – low ecological validity Practice effects (Humes, Welsh, Retzlaff, & Cookson, 1997)
Tower of Hanoi (Simon, 1975)	Fluid reasoning	Abstract nature – low ecological validity Practice effects. (Humes et al. 1997)
Trail Making Task (Reitan, 1958)	Processing speed	Varied methods of administration. Not comparable across studies. Practice effects (de Jager et al. 2014)
Verbal Recognition Memory (Sahakian and Owen. 1992)	Long term memory encoding and retrieval	Semi-computerised. Modest construct sensitivity. (Lenehan et al. 2016)
Virtual Morris Water Maze	Visuospatial ability	Inconsistencies of procedures used. (Thornberry, Cimadevilla, & Commins, 2021)
Visual Object Learning Test	Long term memory encoding and retrieval	Good validity. Difficult to eliminate verbal associations (de Jaeger et al. 2014)
Visual Scanning Test (Weintraub, Haines, & Randle, 1985)	Processing speed	Simple to administer. Physical presentation. Performance depend on motor function. (de Jaeger et al. 2014)
Visual Spatial Learning Test (Malec et al., 1992)	Working memory	Non-verbal, minimal language and motor skill requirements. Normative date for adults. (Malec et al. 1992)
WAIS-IV (Wechsler. 2008)		Normative data 16-90 years. Low internal consistency reliability. (Gignac & Watkins, 2013)
WAIS-IV block design (Wechsler. 2008)	Acquired knowledge	Normative data 16-90 years. Low internal consistency reliability. (Gignac & Watkins, 2013)
WAIS-IV digit span (Wechsler. 2008)	Working memory	Normative data 16-90 years. Low internal consistency reliability. (Gignac & Watkins, 2013)
WAIS-IV symbol search (Wechsler. 2008)	Processing speed	Normative data 16-90 years. Low internal consistency reliability. (Gignac & Watkins, 2013)
WAIS-IV: Similarity (Wechsler. 2008)	Acquired knowledge	Normative data 16-90 years. Low internal consistency reliability. (Gignac & Watkins, 2013)

WAIS-IV Arithmetic (Wechsler. 2008)	Working memory	Normative data 16-90 years. Low internal consistency reliability. (Gignac & Watkins, 2013)
WAIS-IV Comprehension (Wechsler. 2008)	Acquired knowledge	Normative data 16-90 years. Low internal consistency reliability. (Gignac & Watkins, 2013)
WAIS-IV Information (Wechsler. 2008)	Acquired knowledge	Normative data 16-90 years. Low internal consistency reliability. (Gignac et al. 2013))
WAIS-IV vocabulary (Wechsler. 2008)	Acquired knowledge	Normative data 16-90 years. Low internal consistency reliability. (Gignac & Watkins, 2013)
Wisconsin Card Sorting Test (Berg, 1948)	Fluid reasoning	Computerised versions available. Test-retest reliability. Inconsistent methods of scoring. (Kopp, Lange, & Steinke, 2021)
WMS-IV Faces (Wechsler. 2009)	Long term memory encoding and retrieval	Normative data 16-90 years. Moderate reliability (Kent. 2013)
WMS-IV Verbal Paired Associates (Wechsler. 2009)	Long term memory encoding and retrieval	Normative data. Good validity compared with other verbal memory tests. Practice and ceilling effects (Uttl, Graf, & Richter, 2002)
WMS-IV Logical memory (Wechsler. 2009)	Long term memory encoding and retrieval	Good construct validity. Practice effects. (Kent. 2013)
WMS-IV Visual Reproduction (Wechsler. 2009)	Long term memory encoding and retrieval	Correlates with visuospatial processing (executive function). Influence of motor skills. (Kent. 2013)

RBANS – Repeatable Battery for the Assessment of Neuropsychological Status

WAIS-IV - Wechsler Adult Intelligence Scale—Fourth edition

WMS-IV - Wechsler Memory Scale–Fourth Edition

Appendix B: Health screening questionnaire

*** Please answer the following questions as accurately as possible. All information provided will remain confidential ***

Demographic Information:

Age _____ Height _____

Gender _____ Weight _____

Questions about health and lifestyle: (Circle the relevant answer)

Do you smoke? Yes / No

If 'Yes', please state: Number of cigarettes per day _____

Number of cigars per day _____

Amount of tobacco per day _____

Do you drink alcohol? Yes/No

If 'Yes', approximately how many units per week? _____

Do you drink tea? Yes / No

If 'Yes', approximately how many cups of tea per day? _____

Do you drink coffee? Yes / No

If 'Yes', approximately how many cups of coffee per day? _____

Are you vegetarian or vegan? Yes / No

Are you currently on a weight-reducing or other special diet? Yes/No

If 'Yes', please give details:

Do you exercise regularly? Yes/No

If 'Yes', please give an estimate of the number of hours you spend exercising per week.

_____

Do you have allergies to any foods? In particular, any fruits or cocoa, nut, milk or gluten based products? Yes/No

If 'Yes', please list foods you have an allergy to below:

Please indicate if you had or have any if the following conditions: (place a tick next to any relevant conditions)

[ ] Diabetes – if yes, please circle: Type I / Type II

[ ] Heart disease – if yes, have you had open heart/bypass surgery? Yes / No

[ ] High blood pressure – if yes, are you currently taking medication to control your blood pressure? Yes / No

Did you start taking medication immediately after diagnosis? Yes / No

How long have you been taking medication for?_____

[ ] Nervous system disease / Degenerative disorder (e.g. Multiple Sclerosis)

[ ] Stroke or Transient Ischaemic Attack

[ ] Chronic kidney disease/impaired kidney function

[ ] Chronic liver disease/impaired liver function

[ ] Chronic thyroid disease/impaired thyroid function

[ ] Mental or emotional problems (e.g. depression) – if yes, please give details including any current medication

_____

[ ] Renal or Gastrointestinal disorders – if yes, please give details

_____

[ ] Any other serious illnesses – if yes, please give details

_____

Are you currently, or have you been within the last month, on any of the following medication?

[ ] Warfarin - if yes, please give date you commenced taking the medication and daily dose. _____

[ ] Heparin - if yes, please give date you commenced taking the medication and daily dose._____

[ ] Rivaroxaban - if yes, please give date you commenced taking the medication and daily dose. _____

[ ] Dabigatran - if yes, please give date you commenced taking the medication and daily dose._____

[ ] Apixaban - if yes, please give date you commenced taking the medication and daily dose. _____

[ ] Aspirin - if yes, please give date you commenced taking the medication and daily dose. _____

[ ] Plavix (Clopidogrel) - if yes, please give date you commenced taking the medication and daily dose. _____

[ ] Persantin (Dipyridamole) - if yes, please give date you commenced taking the medication and daily dose. _____

[ ] Other anticoagulant medication- if yes, please give details including the date you commenced taking the medication and daily dose. _____
_____

[ ] Any other medication – if yes, please give details including the date you commenced taking the medication and daily dose. _____
_____
_____

Appendix C.1: Information Sheets for ROAB trial

Blueberry study information

We hope to provide all the information you will need about the study in order for you to make an informed decision about whether you would like 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 Adrian Whyte either by telephone: 0118 3788523 or email:

Background to the study

We are interested in finding out about the effects on attention and memory when an individual is given different doses of blueberry extract. Blueberries naturally contain high amounts of flavonoids which are found in a number of foodstuffs including vegetables, fruits and fruit juices. Recent research from our lab with older adults has shown that sustained consumption of wild blueberry extract can lead to improvements in memory performance and we are presently interested in whether there are short term effects following administration of a single dose. There will be about 30 participants in this study.

Why have I been asked to take part?

As a natural part of growing older, our cognitive abilities, such as memory and ability to concentrate, become less sharp. With research indicating that our blueberry extract might contribute to delaying this decline in performance, we are keen to recruit healthy participants aged 68 to 75 years to help us investigate the effect of different doses in relation to this.

Who is running the study?

The study is run by research staff in the School of Psychology & Clinical Language Sciences at the University of Reading and is funded by Naturex - DBS, a group which specialises in plant extraction and the development of natural ingredients. It has been reviewed by the University Research Ethics Committee and has been given a favourable ethical opinion for conduct, which means that an independent group did not raise any objections to the study on ethical grounds and have permitted the study to proceed.

What does the study involve?

The study will involve 7 visits to the University each separated by 1 week. The visits will consist of an initial familiarisation visit and 6 test visits where, throughout the day, you will perform a number of cognitive games. On each visit you will consume a different dose of the wild blueberry extract or a placebo contained in 3 capsules. You will not know which dose you are taking.

Familiarisation Visit: The familiarisation visit will begin at 0800 where a provided breakfast will be consumed followed by your height, weight and BMI being measured. Practices of all four cognitive game sessions which will comprise the main test visit will also take place at 0900, 1130, 1330 and 1630, with lunch being provided at 1200. Immediately following each session, your blood pressure and heart rate will be measured. Also during this first visit additional health and cognitive questionnaires will be administered and you will also be given a sample of the wild blueberry capsules to try.

Test Visits: As with the familiarisation visit, each test visit will begin with breakfast at 0800 followed by your height, weight, and BMI being measured. Cognitive game sessions will take place at 0900, 1130, 1330, and 1630, with the study capsules being consumed at 0930 and lunch being provided at 1200. Throughout each test day, your blood pressure and heart rate will be measured at regular intervals.

At the beginning and end of the study, we will also ask you to complete two, 3 day, food diaries.
Participation is voluntary, and, having started the study, you are free to withdraw at any point without the requirement to give a reason for doing so. The principal investigator may also ask you to stop the study for several reasons, including incompliance or safety.

What happens to the data?

All information collected will remain fully confidential and be assigned an anonymous number so that no name will appear on any of the documents. All data will be kept safely locked at the University of Reading and only the named researchers 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.
It is planned that the results of this study will be published in an academic journal. Full anonymity will be respected in this publication. Only the overall group results will be reported and no direct reference will be made to any contributing individual.
Additionally, if you so request, the results of the study will be forwarded to you upon its completion either by post or email.

Will I be paid to participate?

Yes, your time and contribution is highly valued by the University and you will be paid £70 for your involvement over the seven weeks.

I'd like to participate, what happens next?

Please reply directly to via email using the following address: . The email should include your name, contact telephone number and time of day at which it is most convenient for you to be contacted.

Appendix C.2: Information Sheets for BEAT Trial

Study: Investigation of the acute cognitive effects seen after administration of 222mg of a proprietary wild blueberry intervention in healthy older adults
Researchers: Prof. Claire Williams, Dr. Daniel Lamport, Nancy Cheng
Tel: 0118 3788523
Email: **claire.williams@reading.ac.uk**
daniel.lamport@reading.ac.uk
nancy.cheng@pgr.reading.ac.uk

Blueberry Study Information

We hope to provide all the information you will need about the study in order for you to make an informed decision about whether you would like 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 Nancy Cheng by email: nancy.cheng@pgr.reading.ac.uk.

Background to the study

We are interested in finding out about the effects on attention and memory when an individual is given a dose of 222mg wild blueberry extract (WBE). Blueberries naturally contain high amounts of flavonoids which are found in a number of foodstuffs including vegetables, fruits and fruit juices. Recent research from our lab with older adults has shown that sustained consumption of wild blueberry extract can lead to improvements in memory performance and we are presently interested in whether there are short term effects following administration of a single dose. There will be about 50 participants in this study.

Why have I been asked to take part?

As a natural part of growing older, our cognitive abilities, such as memory and ability to concentrate, become less sharp. With research indicating that our blueberry extract might contribute to delaying this decline in performance, we are keen to recruit healthy participants aged 68 to 75 years to help us investigate the effect of different doses in relation to this.

Who is running the study?

The study is run by research staff in the School of Psychology & Clinical Language Sciences at the University of Reading and is funded by Naturex, a group which specialises in plant extraction and the development of natural ingredients. It has been reviewed by the University Research Ethics Committee and has been given a favourable ethical opinion for conduct, which means that an independent group did not raise any objections to the study on ethical grounds and have permitted the study to proceed.

What does the study involve?

The study will involve 3 visits to the University each separated by 1 week. The visits will consist of an initial familiarisation visit and 2 test visits where you will perform a number of cognitive games once in the morning and once during the afternoon. On each visit you will consume either 222mg wild blueberry extract or a placebo contained in a single capsule. You will not know which dose you are taking.

Familiarisation Visit: The familiarisation visit will begin at 0800 where a provided breakfast will be consumed followed by your height, weight and BMI being measured. Practices of all both cognitive game sessions which will comprise the main test visit will take place at 0830 and 1400, with lunch being provided at 1300. Immediately following each session, your

blood pressure and heart rate will be measured. During this first visit additional health and cognitive questionnaires will be administered and you will also be given a sample of the wild blueberry capsules to try.

Test Visits: As with the familiarisation visit, each test visit will begin with breakfast at 0800 followed by your height, weight, and BMI being measured. Cognitive game sessions will take place at 0830 and 1400, with the study capsules being consumed at 1200 and lunch being provided at 1300. During each test day, your blood pressure and heart rate will be measured on completion of the cognitive games sessions.

At the beginning of the study, we will ask you to complete a 3-day food diary.

Participation is voluntary, and having started the study, you are free to withdraw at any point without the requirement to give a reason for doing so. The principal investigator may also ask you to stop the study for several reasons, including incompliance or safety.

What happens to the data?

All information collected will remain fully confidential and be assigned an anonymous number so that no name will appear on any of the documents. All data will be kept safely locked at the University of Reading and only the named researchers at the top of this sheet will have access. The data will be used only for research purposes and will be destroyed 5 years after the completion of the study.

It is planned that the results of this study will be published in an academic journal. Full anonymity will be respected in this publication. Only the overall group results will be reported and no direct reference will be made to any contributing individual.

Additionally, if you so request, the results of the study will be forwarded to you upon its completion either by post or email.

Will I be paid to participate?

Yes, your time and contribution is highly valued by the University and you will be paid £50 for your involvement over the three weeks.

I'd like to participate, what happens next?

Please reply directly to Nancy Cheng via email using the following address: nancy.cheng@pgr.reading.ac.uk. The email should include your name, contact telephone number and time of day at which it is most convenient for you to be contacted.

Appendix D.1: ROAB Trial Consent Form

Title of study: An investigation of the cognitive benefits seen after administration of a proprietary wild blueberry in healthy older adults.

When you have read each statement below, please put your initials in the box next to it to show that you understand and agree with the statement.

□ I …… agree to participate in the study 'An investigation of the cognitive benefits seen after administration of a proprietary wild blueberry in healthy older adults' at the School of Psychology & Clinical Language Sciences, University of Reading.

□ 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 agree to the arrangements described in the Information Sheet in so far as they relate to my participation.

□ I am aware that this application has been reviewed by the School of Psychology and Clinical Language Sciences Research Ethics Committee and has been given a favourable ethical opinion for conduct

□ I understand that all personal information will remain confidential to the investigators and arrangements for the storage and eventual disposal of any identifiable material have been made clear to me.

□ The contents of the capsules have been explained to me and I am happy to consume them during each study visit.

□ I understand that participation in this study is voluntary and that I can withdraw at any time without having to give an explanation.

□ I am happy to proceed with participation.

Signature ……..

Name (in capitals) ……..

Date ……

Appendix D.2: BEAT Trial Consent Form

Title of study: **Investigation of the acute cognitive effects seen after administration of 222mg of a proprietary wild blueberry intervention in healthy older adults**
When you have read each statement below, please put your initials in the box next to it to show that you understand and agree with the statement.

- ☐ I …… agree to participate in the study 'Investigation of the acute cognitive effects seen after administration of 222mg of a proprietary wild blueberry intervention in healthy older adults' at the School of Psychology & Clinical Language Sciences, University of Reading.
- ☐ 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 agree to the arrangements described in the Information Sheet in so far as they relate to my participation.
- ☐ I am aware that this application has been reviewed by the School of Psychology and Clinical Language Sciences Research Ethics Committee and has been given a favourable ethical opinion for conduct
- ☐ I understand that all personal information will remain confidential to the investigators and arrangements for the storage and eventual disposal of any identifiable material have been made clear to me.
- ☐ The contents of the capsules have been explained to me and I am happy to consume them during each study visit.
- ☐ I understand that participation in this study is voluntary and that I can withdraw at any time without having to give an explanation.
- ☐ If any adverse information regarding my health is revealed during the study, I am happy for the relevant details to be provided to my General Practitioner (GP)
- ☐ I am happy to proceed with participation.

Signature …….

Name (in capitals) …….

Date ……

Researcher Name:
Signature:
Date:

Appendix E: Low Polyphenol Diet

Please **avoid** eating foods shown below for **24** hours before **each** visit to the Nutritional Psychology Unit and for the duration of each study day. Please avoid caffeinated beverages for 24 hours.

- All berries
- Fruit and vegetables (except potatoes)
- Fruit juices
- Jams and preserves
- Red wine
- Fruit teas
- Soy products
- Chocolate/cocoa
- Tea (black, green, earl grey etc)
- Coffee
- All high energy and/or caffeinated drinks, eg: Coca-Cola, Red Bull, Lucozade.
- All dietary supplements
- Alcohol or other recreational drugs

Suggested foods you may eat include those shown below.

- Potatoes
- Rice
- Sweetcorn
- Mushrooms
- Carrots
- Bananas
- Pasta
- Meat/fish
- Dairy products including milk, cheese & non-fruit yoghurt
- Non-fortified cereals or porridge
- White bread

Please fast (no food or beverages except water) for 12 hours overnight prior to attending each study day

Appendix F: ROAB Trial Cognitive Data

Mean (SE) of Episodic Memory Outcomes following varying doses of Wild Blueberry extract

	Intervention				
Measure	**Placebo**	**WBE 111**	**WBE 222**	**WBE 444**	**WBE 888**
RAVLT:					
Word Recognition – accuracy	0.792 (0.025)	0.758 (0.025)	0.772 (0.025)	0.797 (0.025)	0.778 (0.025)
p-value		0.281	0.919	1.000	0.987
Word Recognition – reaction time (ms)	1189.789 (43.791)	1183.151 (43.723)	1142.587 (43.750)	1067.966 (43.763)	1174.933 (43.718)
p-value		1.000	0.700	**0.001***	1.000
Delayed Word Recall – accuracy	6.181 (0.468)	6.061 (0.471)	6.495 (0.467)	5.791 (0.470)	6.689 (0.468)
p-value		1.000	0.988	0.956	0.752
Learning	5.624 (0.335)	5.760 (0.337)	5.621 (0.334)	5.407 (0.335)	5.572 (0.335)
p-value		1.000	1.000	0.997	1.000
Total Recall	45.174 (1.532)	45.371 (1.538)	44.776 (1.530)	43.999 (1.531)	44.973 (1.530)
p-value		1.000	1.000	0.717	1.000
Proactive Interference	0.300 (0.237)	0.295 (0.235)	0.296 (0.229)	0.514 (0.233)	0.503 (0.230)
p-value		1.000	1.000	0.999	0.999
Retroactive Interference	2.833 (0.269)	3.384 (0.269)	2.872 (0.268)	2.617 (0.269)	2.750 (0.268)
p-value		0.168	1.000	0.990	1.000
Immediate Recall	5.515 (0.257)	5.738 (0.259)	5.535 (0.256)	5.720 (0.257)	5.719 (0.257)
p-value		0.956	1.000	0.973	0.972
Foil Words Rejected	0.923 (0.011)	0.935 (0.011)	0.932 (0.011)	0.929 (0.011)	0.920 (0.011)
p-value		0.426	0.825	0.991	1.000
CORSI Blocks task:					
Longest Sequence	7.735 (0.182)	7.601 (0.182)	7.795 (0.182)	7.789 (0.182)	7.623 (0.182)
p-value		0.986	1.000	1.000	0.996
Total Correct Sequences	0.561 (0.019)	0.561 (0.019)	0.569 (0.019)	0.569 (0.019)	0.558 (0.019)
p-value		1.000	0.988	0.995	1.000
Latency (ms)	871.190 (22.467)	871.809 (22.501)	871.109 (22.404)	870.829 (22.464)	870.03 (22.456)
p-value		1.000	1.000	1.000	1.000

Reported values are estimated marginal means with baseline as covariate. Statistical differences from placebo are indicated *p < 0.05.

Mean (SE) Executive Function Cognition scores following varying doses of Wild Blueberry extract.

	Intervention				
Measure	**Placebo**	**WBE 111**	**WBE 222**	**WBE 444**	**WBE 888**
Switching Task:					
Accuracy	0.981 (0.003)	0.979 (0.003)	0.979 (0.003)	0.980 (0.003)	0.981 (0.003)
p-value		0.283	0.091	0.686	1.000
Reaction Time (ms)	772.808 (12.984)	761.524 (12.983)	779.585 (12.981)	780.500 (12.979)	772.340 (12.988)
p-value		**0.018***	0.480	0.298	1.000
Trail Making Task:					
TMT A (ms)	33,293.05 (889.994)	32,969.86 (891.747)	33,223.20 (889.085)	33,420.96 (891.000)	32,726.43 (891.870)
p-value		1.000	1.000	1.000	0.984
TMT B (ms)	48,841.62 (2,412.497)	48,567.72 (2,413.472)	49,337.73 (2,418.415)	49,431.30 (2,409.345)	45,958.55 (2,421.806)
p-value		1.000	1.000	1.000	0.304
TMT B – A (ms)	14,861.37 (1,927.546)	15,363.11 (1,932.702)	16,366.08 (1,934.010)	15,871.36 (1,926.766)	14,131.87 (1,936.781)
p-value		1.000	0.954	0.997	1.000
Choice Reaction Time:					
Reaction Time (ms)	396.783 (6.642)	397.998 (6.649)	399.615 (6.637)	402.373 (6.641)	395.862 (6.640)
p-value		1.000	0.885	0.105	1.000
Accuracy	0.992 (0.002)	0.992 (0.002)	0.993 (0.002)	0.992 (0.002)	0.993 (0.002)
p-value		1.000	1.000	1.000	1.000

Reported values are estimated marginal means with baseline as covariate. Statistical differences from placebo are indicated *p < 0.05.

Mean (SE) Composite Cognition scores following varying doses of Wild Blueberry extract.

	Intervention				
Measure	**Placebo**	**WBE 111**	**WBE 222**	**WBE 444**	**WBE 888**
Composite Accuracy	-0.024	-0.01	-0.022	-0.041	-0.04
	(0.062)	(0.062)	(0.062)	(0.062)	(0.062)
p-value		1.000	1.000	1.000	1.000
Composite Reaction Time	0.011	-0.006	-0.02	-0.017	-0.045
	(0.077)	(0.077)	(0.077)	(0.077)	(0.077)
		1.000	0.990	0.996	0.644
p-value					
Composite Episodic Memory	-0.038	-0.005	-0.006	-0.015	-0.042
	(0.055)	(0.055)	(0.055)	(0.055)	(0.055)
		0.984	0.992	0.999	1.000
p-value					
Composite Executive Function	-0.002	0.022	-0.021	-0.008	0.036
	(0.068)	(0.068)	(0.068)	(0.068)	(0.068)
		0.999	1.000	1.000	0.965
p-value					
Global	-0.031	-0.002	-0.001	-0.007	-0.016
	(0.058)	(0.058)	(0.059)	(0.058)	(0.058)
p-value		0.943	0.955	0.992	1.000

Reported values are estimated marginal means with baseline as covariate. Statistical differences from placebo are indicated *p < 0.05.

Mean (SE) Mood and Cardiovascular outcomes following varying doses of Wild Blueberry extract.

	Intervention				
Measure	**Placebo**	**WBE 111**	**WBE 222**	**WBE 444**	**WBE 888**
PANAS Positive	5.657	5.691	5.745	5.604	5.739
	(0.137)	(0.137)	(0.138)	(0.138)	(0.138)
p-value		1.000	0.837	0.995	0.911
PANAS Negative	2.118	2.108	2.098	2.086	2.080
	(0.016)	(0.016)	(0.016)	(0.016)	(0.016)
p-value		1.000	0.975	0.641	0.359
Systolic Blood Pressure (mm/Hg)	126.481	126.248	123.686	126.091	126.268
	(1.394)	(1.388)	(1.383)	(1.393)	(1.398)
p-value		1.000	**0.038***	1.000	1.000
Diastolic Blood Pressure (mm/Hg)	78.514	77.498	76.782	77.888	78.539
	(1.062)	(1061)	(1.060)	(1.064)	(1.063)
p-value		0.529	**0.027***	0.967	1.000
Heart Rate (bpm)	63.618	62.901	63.670	62.832	62.903
	(0.741)	(0.741)	(0.741)	(0.746)	(0.750)
p-value		0.827	1.000	0.804	0.838