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# The clinical effects of flavonoid-rich nutritional interventions on neurocognitive function

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## DECLARATION

The candidate confirms that the work submitted is her own and that appropriate credit has been given within the thesis where reference has been made to the work of others.

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Marina Gougoulidou

August 2021

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## ABSTRACT

It has been widely accepted that lifestyle and dietary habits strongly influence health and the occurrence of disease. Previous research has shown that dietary polyphenols can enhance brain function, which has been seen as improvements in both cognitive performance and mood, suggesting that they can be used as intervention strategies for the prevention, delay or treatment of neurodegenerative diseases and mental illness. The mechanisms whereby polyphenols induce those health promoting effects have been emerging but are still unclear. There is evidence suggesting that certain polyphenolic compounds can cross the blood brain barrier and positively modulate specific neurotransmitter receptors. Further, there is evidence that they can improve arterial function leading to improvements in cerebral blood flow, and that they can positively modulate glucoregulatory mechanisms.

In this thesis, we investigate the effects of dietary polyphenols that naturally occur in cocoa and grapeseed on cognitive function and mood in healthy older adults and their potential underpinning mechanisms. We further explore the effects of cocoa polyphenols on executive function by systematically reviewing published clinical trials, as an attempt to implement a domain specific approach, whilst highlighting the importance of executive (dys)function in brain health. Finally, we discuss the effects of hop flavonoids on mood and neurochemical parameters. We consider their potential as mood enhancing treatments and introduce a clinical trial protocol for the investigation of those effects.

We showed that 900mg of cocoa flavanol intake for 12 weeks showed a tendency towards improved vascular function, FMD response ($F(2,59.26)=2.59$; $p=0.084$) and a significantly reduced mean heart rate in the cocoa arm (mean diff=-2.39; $p=0.019$) and that an acute dose of 600mg total grapeseed polyphenols improved blood glucose peak time ($p=0.002$) and mood by increasing difference in sedation ($F(1,30)=7.045$; $p=0.012$) . We speculate that overall improvements in cognitive function observed after chronic supplementation of a cocoa polyphenol-rich treatment might be attributed to the methylxanthines found in both active treatment and control, however that is not in line with our findings regarding vascular function, therefore further investigation is required to establish whether the cocoa methylxanthines present in our treatments can have any biological or cognitive effects. In addition, we argue that grapeseed polyphenols may have

protective effects against cognitive decline due to fatigue on a postprandial level. We believe that the observed changes in mood following both intervention strategies are encouraging but require further investigation on the associated biochemical mechanisms of action. We finally set the foundations for clinical research on hop phenolics, a promising area that is presently underresearched, and we discuss unanswered questions and future directions in the field.

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## ABBREVIATIONS

**5-HIAA** 5-Hydroxyindoleacetic Acid

**5-HT** 5-Hydroxytryptamine or Serotonin

**5-HT$_5$** Type of Serotonin Receptor

**ABV** Alcohol By Volume

**Akt** (or PKB) Protein Kinase B

**ALP** Alkaline Phosphatase

**ALT** Alanine Aminotransferase

**BBB** Blood-Brain Barrier

**BDNF** Brain Derived Neurotrophic Factor

**B-L VAS** Bond-Lader Visual Analogue Scales

**BSI** Brief Symptom Inventory

**CBD** Cannabidiol

**CBF** Cerebral Blood Flow

**CINN** Centre for Integrative Neuroscience and Neurodynamics

**CNS** Central Nervous System

**CPT** Continuous Performance Task

**CREB** CyclicAMP-Response Element-Binding protein

**CRP** C-Reactive Protein

**CRPUS** Ultra-Sensitive C-Reactive Protein

**CVD** Cardiovascular Disease

**DASS-21** Depression Anxiety and Stress Scale

**EDTA** Ethylenediaminetetraacetic Acid

**EGCG** Epigallocatechin Gallate

**ELISA** Enzyme-Linked Immunosorbent Assay

**ERK** Extracellular Receptorkinase

**FMD** Flow Mediated Dilation

**fMRI** Functional Magnetic Resonance Imaging

**GABA** Gamma Amino Butyric Acid

**gamma GT** Gamma-Glutamyl Transferase

**GCP/ICH** Good Clinical Practice/International Conference of Harmonisation

**HDL** High Density Lipoprotein (Cholesterol)

**HOMA-IR** Homeostatic Model Assessment for Insulin Resistance

**HPLC** High-Performance Liquid Chromatography

**HVA** Homovanillic Acid

**iAUC** Incremental Area Under the Curve

**IQ** Intelligence Quotient

**LDL** Low Density Lipoprotein (Cholesterol)

**LLM** Linear Mixed Model

**LPH** lactase-phlorizin hydrolase

**MAO** Monoamine Oxidase

**MCI** Mild Cognitive Impairment

**$ML_1$** Type of Melatonin Receptor

**MMSE** Mini Mental State Examination

**NART** National Adult Reading Test

**NEFA** Non-Esterified Fatty Acids

**NEFAHR** Non Esterified Fatty Acids (HR enzymatic colorimetric assay)

**NO** Nitric Oxide

**PASE** Physical Activity Scale for the Elderly

**RAVLT** Rey Auditory Verbal Learning Test

**RSVP** Rapid Serial Visual Presentation

**RVE** Robust Variance Estimation

**RVIP** Rapid Visual Information Processing

**S100b** S100 calcium-binding protein Beta

**SOP** Standard Operating Procedure

**SSAI** Spielberger State Anxiety Inventory

**SSRI** Selective Serotonin Reuptake Inhibitor

**STAI** Spielberger Trait Anxiety Inventory

**T1D** Type 1 Diabetes

**TRIG** Triglycerides

**WHR** Waist to Hip Ratio

INTRODUCTION

# Polyphenols and the Brain: An introduction to the Thesis and the Broader Context

## Overview

Plants have been historically known to have therapeutic properties. Traditional herbal medicine was for centuries aiming to treat anything from common medical conditions to rare diseases that were poorly, if at all, understood. With the surge of modern medicine, science and technology, traditional herbal medicine has not necessarily become redundant or obsolete, but, rather, has set foundations for scientific research in various therapeutic areas, where plant-based extracts, or isolated compounds have been shown to have beneficial biological properties. Although we know a lot about plant extracts as nutraceuticals, there are still knowledge gaps and uncertainties that current scientific research is trying to address. Seeing as there is a global rise in cases of chronic disease, including life-threatening conditions, some of which preventable, nutritional scientific research has had the mission to establish ways of preventing them [1]. Changes in lifestyle and diet can, in many cases, prevent, or even reverse certain modifiable factors associated with chronic diseases, such as cardiovascular disease [2]. The role of nutritional supplements in this mission has been, and is still being extensively investigated, as it could open new horizons in medicine and pharmacy. Nutraceutical products are widely available, safe, promising and they have been gaining increasing popularity in the last decades. Whilst modern pharmacy is mainly focused on treating the disease, clinical nutrition is playing important part in the prevention of disease. In that sense, nutraceutical and pharmaceutical research are both important and influential in the improvement of the healthcare system.

Focusing on the field of nutritional neuropsychology, two of the broad areas of interest are cognitive (dys)function and mental health. Various nutritional supplements and plant extracts have been investigated as potential treatments for depression and other mood related disorders. Recent research has been exploring the possibilities of certain

naturally occurring compounds interacting with neurotransmitter receptors, as potential mechanisms whereby those compounds exert beneficial effects on mood and mood disorders. The clinical strategy behind those avenues is simply to create easy access to non-prescription treatments for common mental illness such as depression and anxiety, in a day and age where mental health is heavily challenged. With regard to cognitive performance, there is an ever-increasing interest in intervention strategies that could have a positive impact on the aging population. As the older population has progressively grown over the years due to an increase in the average life expectancy [3], conditions like dementia and other neurodegenerative diseases have been brought into attention. Given that we cannot stop aging and that there is no treatment for reversing neurodegeneration (rather, there are treatments for alleviating the symptoms of neurodegenerative diseases like dementia), it is crucial that we find ways of improving and slowing down the aging process and delaying the onset of those diseases. This is extremely challenging as the aetiology of cognitive decline and impairment is uncertain and multifaceted. Given that malnutrition has been associated with cognitive decline [4] and that cardiovascular risk factor control has been suggested as a strategy for delaying or avoiding the occurrence of cognitive decline[5] it is plausible that we can address the growing issue of cognitive decline and patient dependency through nutritional interventions that target the biochemical mechanisms underlying the cognitive decline, both in the CNS and the vascular system. Current research is trying to decipher the biological mechanisms underpinning the progression of such diseases of the CNS and possible ways of manipulating them. Both mental health and age-related cognitive dysfunction are areas of utmost complexity and, although we understand the central nervous system more than ever before, there are still a lot of unanswered questions. Nutrition research is again playing a vital role in understanding certain biological processes related to brain function and various nutraceutical products have been researched for their brain health promoting properties.

Naturally occurring plant constituents of various sources that allegedly have the ability to cross the blood brain barrier and therein wield psychoactive effects are of important scientific interest. In this thesis, I focus on polyphenolic compounds from various food sources that are common in the western diet. Polyphenols are a family of phytochemicals that derive from secondary plant metabolism, naturally occurring in a wide range of plant-based foods and drinks. They share a chemical structure that is

distinguishable by a three carbon ring configuration: two aromatic rings (A and B) bound by three carbon atoms thus forming a third oxygenated heterocycle (ring C) [6]. Due to the variations in the saturation of the carbon rings, their alkylation, glycosylation and the hydroxylation pattern of the molecules, thousands of polyphenolic compounds have been identified and classified into seven subclasses: flavonols, flavones, flavanones, flavanonols, flavanols, anthocyanidins, and isoflavones, all referred to as flavonoids [7]. Table 0.1. summarises the main flavonoid subgroups, their chemical structures, main food sources, and it further outlines the metabolism these compounds undergo after ingestion. Figure 0.1. illustrates the human flavonoid metabolism (Schematic adapted from Thilakarathna et al., 2013 [8]).

Table 0. 1. Flavonoid Subgroups, sources, and Summary of Metabolism

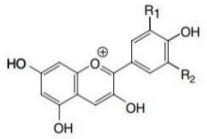
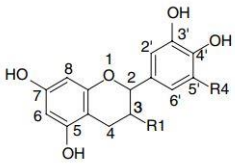
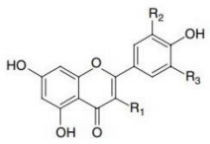
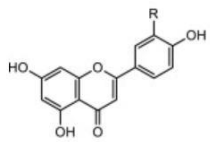
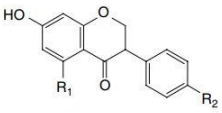
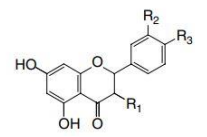
| Group | Structural Formula | Examples | Sources | METABOLISM | | |
|---|---|---|---|---|---|---|
| Anthocya-nidins |  | Pelargonidin, Cyanidin, Delphinidin, Paeonidin, Petunidin, Malvidin | Berries, Red Wine | **Phase I/II Metabolism** (Small Intestine and Liver)<br>Sulphates<br>Glucuronides<br>O-methylated Conjugates<br>O-methylated Aglycone | **Phase III metabolism** (Further intracellular metabolism)<br>Blood Brain Barrier (depending on lipophilicity)<br>oxidative metabolism, P4s0-related metabolism and conjugation with thiols | **Bacterial Metabolism** (colon)<br>Protocatechuic Acid, Benzoic Acid, Phenylacetic acid, Phenylpropionic Acid |
| Flavanols |  | Catechin, Epicatechin, Epigallocatechin Epigallocatechin gallate | Green tea, Cocoa | | | |
| Flavonols |  | Kaempferol, Quercetin, Myricetin, Isorhamnetin | Onion, Broccoli | | | |
| Flavones |  | Luteolin, Apigenin | Parsley, Celery | | | |
| Iso-flavones |  | Genistein, Daidzein | Soy | **Flavonoid Metabolites:**<br>• Vascular benefits<br>• Synaptic Plasticity<br>• Neurogenesis<br>• Anti-inflammatory | | |
| Flava-nones |  | Naringenin, Hesperetin | Citrus fruits, tomato | | | |

*Table adapted from Spencer et al., 2008 [9] and Vauzour 2014 [10].*

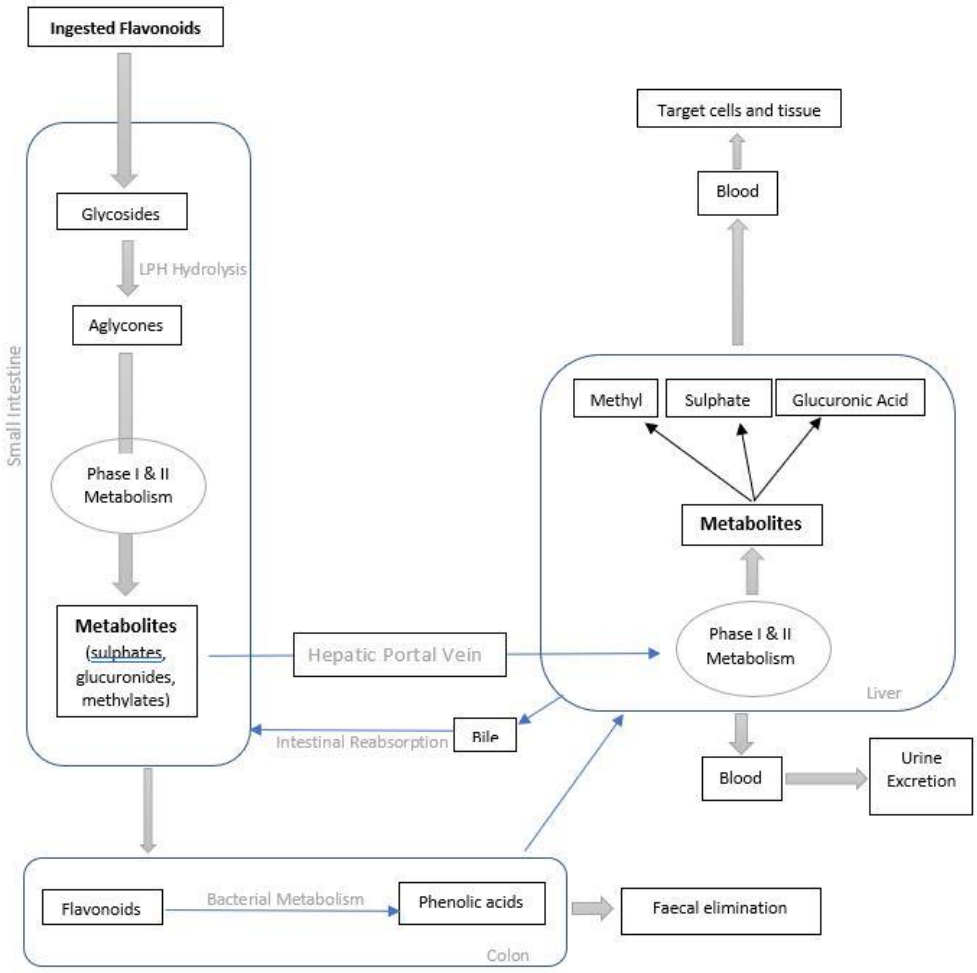



Figure 0. 1. Schematic of human flavonoid metabolism.

Ingested flavonoids undergo extensive intestinal metabolism. Metabolites are then transported to the liver where they undergo further metabolism. The liver metabolites can be transported to targeted cells and tissues for further intracellular metabolism, including penetrating the blood brain barrier, depending on their lipophilicity. They are then excreted to bile or eliminated via urine and/or faeces. The aglycones or flavonoid metabolites that reach the colon can undergo microbial degradation and reabsorption (LPH: lactase-phlorizin hydrolase) [8].

Furthermore, Table 0.2. summarises the main metabolic conversions flavonoids undergo in the small intestine and their metabolites that are reported to have biological activity [8]. The flavonoid metabolites can then exert various effects as explained below (Figure 0.2) and further in the following chapters.

Table 0. 2. Summary of Bioavailability and metabolic conversion of flavonoid sub-groups.

| **Flavonoid** | **Bioavailability and Main Metabolic conversion** |
|---|---|
| Flavan-3-ols (monomeric) and Proanthocyanidins | Major bioactive forms: conjugates of epicatechin; catechin: methyl, sulfate and glucuronic acid conjugates; epicatechin: mainly to sulfate conjugates, no glucuronidation;<br>oligomeric procyanidins can be absorbed in small intestine |
| Flavonols | Potentially active metabolites: glucuronides |
| Anthocyanidins | Sometimes found with sugars intact in circulation.<br>Major intestinal metabolites: glucuronide and sulfate conjugates of protocatechuic acid and phloroglucinaldehyde; anthocyanin derivatives metabolically more resistant than parent compounds |
| Isoflavones | Aglycone more bioavailable; possible deglycosylation prior hepatic metabolism |
| Flavanones | Rapid absorption, low bioavailability |

Although flavonoids are considered 'non-nutrients', as they are not essential for survival and growth, they have been shown to have health promoting properties, demonstrated in both animal and human studies. It is therefore believed to be important by some that polyphenol-rich foods be part of a healthy diet. Both habitual intake through plant-based foods, as well as in the form of nutritional supplements have been investigated extensively. However, given the dramatic variability in polyphenol content between different foods, as well as the differences in bioavailability and metabolism they undergo, specific polyphenolic sources are, rather commonly in clinical research, isolated and investigated independently in order to better understand the observed health benefits and underlying mechanisms involved and to an extent understand their medicinal potential.

In the context of this thesis, I have attempted to understand the effects of certain polyphenolic compounds from different food sources that, reportedly, upon ingestion and metabolism, are likely to be able to penetrate the blood brain barrier due to their high lipophilicity, thus exerting beneficial effects on neurocognitive function and mood. More specifically, flavonoids found in grapeseed, cocoa and hops, from the flavonoid subclasses of anthocyanidins, flavanols, and flavanones (in their prenylated form) respectively and

their metabolites. Based on previous research, these compounds have been shown to share the ability to directly or indirectly affect with the central nervous system, in ways that are still unclear. The observed beneficial outcomes following the intake of these flavonoids, allegedly include enhancement of cognitive performance, improvements in mood and promoting vascular health, as explained in more detail in the subsequent chapters. Several mechanisms underlying the above-mentioned effects have been suggested. Directly by crossing the Blood Brain Barrier and interacting with neuronal receptors, signalling kinases, and neurotrophins, thus leading to changes in synaptic function, which results in improvements in cognitive functioning, or indirectly by increasing peripheral and, as a result, cerebral blood flow via interacting with NO pathways in the peripheral and cerebrovascular endothelium [11]. Figure 0.2 illustrates the proposed beneficial effects of flavonoids and their metabolites on brain function and summarises the suggested underlying mechanisms of action.

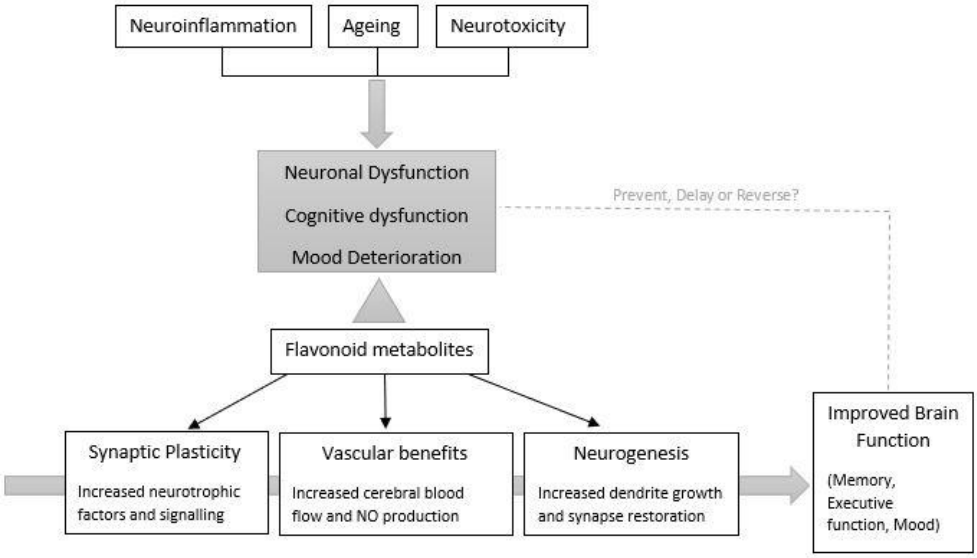



Figure 0. 2. Proposed beneficial effects of flavonoids on brain function and summary of suggested mechanisms of action *(figure adapted from Vauzour, 2014)*

Due to the complex nature of those effects and their proposed mechanisms of action, a multidisciplinary approach was implemented. My team and I looked at, not only cognitive behavioural and other brain related outcomes, but also vascular and metabolic parameters that may be of relevant importance in the broader area of polyphenols and health, as an attempt to better understand the biological activity of these compounds. I further attempted to comprehend the meaning of self-rated mood changes and establish

quantifiable and objective ways of measuring mood, one of the biggest challenges and unaddressed gaps in nutrition research. I conducted extensive literature research in order to, not only understand the current knowledge and answer questions, but to formulate new research questions as well. As such, the primary objective of this thesis is to investigate whether flavonoid-rich nutritional interventions can lead to improvements in mood and cognitive function, based on the hypothesis that both acute and chronic flavonoid supplementation can indeed improve mood and cognitive performance, directly and indirectly.

The primary and secondary objectives of each chapter are explained below, in chronological order, to better illustrate the conception and rationale behind them.

## Objective one

Building on previous research within our group on the effects of berry polyphenols on brain function, we aimed to investigate the postprandial effects of a polyphenol-rich grapeseed extract on cognitive performance, mood, salivary cortisol and glucose metabolism by conducting a randomised controlled trial in a sample of healthy and cognitively intact older adults (see Chapter 3). This study aimed to test claims from previous research that one of the mechanisms underlying the effects of berry flavonoids on brain function is through modulating glucoregulatory mechanisms. As such, we hypothesised that a single dose of a flavonoid rich grapeseed based drink can improve cognitive performance in healthy older adults and that this improvement may be mediated by improvements in glucose metabolism, cortisol response, and changes in mood. Although mood was originally a secondary outcome tested in this clinical trial, it, later on, had an important part in formulating further research questions and possible answers. In addition, during this trial we tested and optimised a complex and multifaceted cognitive test battery that was designed to cover a range of cognitive domains, such as episodic memory, sustained attention, working memory and more, which was then used in subsequent research. The polyphenolic compounds of interest in this study were predominantly phenolic acids and proanthocyanidins.

## Objective two

Similarly, to add to previous research, this time on cocoa flavanols, we conducted a randomised controlled trial to investigate the chronic effects of a cocoa flavanol-rich capsule on cognitive function, mood and vascular function, and to explore potential biochemical mechanisms underlying those effects, in a sample of healthy and cognitively intact older adults (see Chapter 2). Although flavanol-rich cocoa drinks have been previously used to a great extent, both in our department and by other research groups, this time we used a cocoa extract capsule instead. This meant that we could achieve more effective blinding as well as higher doses and fast absorption more easily. We hypothesised that 24 weeks of daily supplementation of our flavanol-rich cocoa treatment would result in enhanced cognitive performance and mood and that it would improve markers of endothelial function, glucoregulation as well as serum brain derived neurotrophic factor, which have been previously suggested as potential mechanisms though which cocoa flavanols enhance brain function. We further hypothesised that said effects would sustain 12 weeks after the cessation of the treatment. The main polyphenolic compounds of interest here were flavan-3-ols, however naturally occurring methylxanthines such as theobromine and caffeine, also came into the discussion as potential bioactive constituents of our treatment.

## Objective three

Based on the cocoa flavanol study mentioned above, I identified some literature discrepancies and issues in the assessment of cognitive performance. Cognitive function is multifaceted and diverse; therefore, the methods used across different studies in the literature are very inconsistent making it difficult to draw meaningful conclusions and understand the bigger picture. As such, I aimed to take a domain-specific approach and conduct a systematic review of placebo controlled trials on the effects of cocoa flavonoid based interventions on executive functioning. Since impairments in executive functioning are associated with higher healthcare costs, compared to other cognitive functions, such as memory (as discussed in Chapter 1), the systematic review aimed to rationalise the use of flavanol-rich cocoa treatments for the enhancement of executive function. As such, the primary hypothesis was that cocoa flavanol treatments can, both acutely and chronically,

improve executive function, based on a collection of published randomised, placebo controlled trials that met our eligibility criteria.

## Objective four

Based on results and trends from the clinical trials mentioned above on mood related outcomes, I aimed to conduct a third clinical trial to further investigate those effects. I hypothesised that a hop-rich beer can, both chronically and acutely, improve mood in young adults that are likely to suffer from a common mood disorder. The rationale was (a) to use a more potent polyphenolic phytochemical that could potentially cause more pronounced mood effects than the once observed in the previous Chapters and (b) to try to establish different, more objective ways of measuring mood, other than self-rated mood questionnaires. Based on previous research in our lab on the polyphenolic constituents of hops found in beer, this clinical trial aimed to investigate the acute and chronic effects of those phytochemicals on mood and biochemical parameters, such as neurotransmitters and their metabolites, which may be related to mood, and to attempt to establish biochemical markers of mood. The population sample this time is young adults with signs of common mood disorders, such as depression and anxiety, where mood changes may be more noticeable. Some of the main polyphenolic compounds of interest here were xanthohumol and prenylated flavonoids, such as prenylnaringenin.

## Summary

The purpose of this thesis was to explore how different polyphenol-rich nutritional interventions can affect the brain, from a clinical perspective. Although the focus is not mechanistic, we touch on some of the suggested mechanisms through which polyphenolics exert biological activity in the brain and the periphery. In this way, the readers can grasp the complicated concept of using nutritional products with psychoactive properties as a potential treatments for age related cognitive dysfunction as well as mental health disorders, a research area that has still a lot to bring to light. This piece of research also aimed to create scope for future investigation on the effects of dietary polyphenolics, especially on mood and mood disorders, which has so far been investigated to a lesser extent, compared with neurocognitive function, but is still of great clinical importance. We identified and attempted to address issues and limitations that clinical research in this field

has been facing, which is reflected in our study designs. As an endeavour to piece together the parts of the bigger picture in the field, we discuss how our findings contribute (or, in some cases, do not contribute) to the general knowledge and we talk about conflicting findings and opinions. Finally, this thesis aims to create insightful questions and premises for discussion; ultimately, '*The scientist is not a person who gives the right answers; it is the one who asks the right questions*'-Claude Levi-Strauss.

CHAPTER 1

# The effects of flavanol-rich cocoa interventions on executive function: A systematic review of placebo-controlled trials

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## Abstract

Cocoa flavanols have been shown to have beneficial effects on various aspects of cognition. A systematic review was conducted to assess whether cocoa flavanol rich interventions have beneficial effects on executive function. To our knowledge, this is the first systematic review of placebo-controlled trials investigating the effects of cocoa flavanols on domain specific aspects of cognition. Web of Science, PubMed, Scopus and PsychINFO were searched from inception to May 2021. Inclusion criteria were placebo-controlled trials that used cocoa flavanol rich interventions and reported at least one cognitive outcome related to executive function. The search generated 8,332 results. Nineteen studies met our eligibility criteria, 12 acute and 7 chronic trials. Eight out of 19 reported statistically significant beneficial effects of cocoa flavanols on executive function. Three articles reported significant physiological effects (improvements in neurochemical and vascular parameters) but not on executive function. Our results suggest that cocoa flavanols may have more obvious acute effects than chronic and that higher doses (≥600 mg total flavanol) may be more effective. Future research is required to provide sufficient data for a quantitative analysis.

## Introduction

Diet represents one of the most important lifestyle factors that can influence brain function, from mental health [12] to cognitive decline and the incidence of neurodegenerative diseases [13]. A lot of attention has been drawn to flavonoids, a group of polyphenolic compounds naturally occurring in many plant-based foods and beverages, like berries, grapes, tea, cocoa and chocolate products and red wine. Flavonoids share a common structure that consists of two aromatic rings and a third oxygenated heterocycle ring and they are divided into seven subclasses: flavonols, flavones, flavanones, flavanonols, flavanols, anthocyanidins, and isoflavones [6]. Much attention has been drawn to flavan-3-ol (also referred to as flavanols), as it is the most abundant flavonoid in our diets [14, 15]. Specifically, cocoa flavan-3-ols have received much attention as cocoa is naturally high in flavanols. Flavanol-rich cocoa is shown to have health promoting properties, such as improving vascular function by increasing peripheral blood flow and flow-mediated dilation of the brachial artery [16] as well as improving cerebral blood flow [17], which may be linked to the beneficial effects of these bioactives on cognition [17]. Indeed, cocoa flavan-3-ols have been shown to have beneficial effects on various aspects of cognitive function [18-27] and potentially the ability to delay age related cognitive decline [28].

Looking at the individual cognitive tasks and domains, it is evident that there is a lot of interest in executive function and many reported effects of cocoa flavan-3-ols are on executive function related tasks [18-20, 22-25, 29-32]. Executive function (also known as cognitive control or executive control) is an umbrella term for a set of premeditated higher cognitive processes used for concentration, sustained attention, fluid reasoning and problem solving and they include three core functions: inhibition, interference control and cognitive flexibility [33]. According to Rabinovici and colleagues, there are four main components in executive function: information updating and monitoring (often referred to as working memory), response inhibition, mental set shifting and fluency [34]. This array of brain processes will be referred to as ‘executive function’ in this review. Although age related cognitive decline, dementia and other neurodegenerative diseases are often associated with memory loss or memory dysfunction, evidence shows that executive function impairment is linked to higher healthcare costs, and rather disproportionately so, in comparison with memory or other cognitive impairments [35]. It is, therefore, important

that we address executive dysfunction separate to other cognitive dysfunctions, as not only does it compromise a person's quality of life but it also poses a healthcare expenditure issue.

Hence, and considering the importance of assessing the effects of nutritional interventions on independent cognitive abilities, instead of a random collection of cognitive tests, which can lead to unintentional interpretation bias, the aim of this review is to systematically assess published placebo controlled trials investigating the link between cocoa flavanol intake and executive function as an attempt to understand the bigger picture of cocoa flavanol rich treatments on executive function and create scope for future research.

## Methods

This review was conducted according to established guidelines and is reported in accordance with the PRISMA guidelines [36]. The search was performed from inception to May 2021 using the following strategy.

### *Search Strategy*

A search of the literature was conducted using the following terms: 'cocoa flavanol'; 'flav* and cognit*', 'human trial AND (cocoa or flav*) AND cognit*', 'cocoa AND cognit*', 'chocolate AND cognit*'**.**

A search of the following databases was performed: Web of Science, PubMed, Scopus, PsychINFO. Web of Science and Scopus searches were filtered for: *Articles*. PubMed searches were filtered for: '*Clinical Trial*', '*Humans*' and '*Randomised controlled trial*'. The articles generated from the search were screened according to inclusion/exclusion criteria explained below.

### *Inclusion/Exclusion Criteria*

Only placebo-controlled trials with participants of any age and gender were included. Only trials that used a high flavanol chocolate or cocoa based intervention, orally administered to human subjects, using appropriate matched controls, were included. Only blinded (single or double blinded) studies were included. Eligible studies had to include at

least one behavioural cognitive outcome pertaining to executive function. Epidemiological/longitudinal/cohort studies were excluded. Studies examining other flavonoid sources, such as tea, berries etc., were excluded. Since most of the included studies report data for a variety of cognitive domains, and in order to effectively group the cognitive tests used, the Cattell –Horn–Carroll framework [37] was implemented to ensure only executive function related outcomes, rather than a mixture of tasks, were included in this systematic review.

### *Study Selection*

The results of the search were assessed and screened for eligibility by two independent researchers (M.G., C.M.). The results of the searches were first screened manually and based on title and abstracts. Irrelevant articles were deleted. The selected articles were then further screened for eligibility. Each article was either discarded, if it did not meet one or more of the eligibility criteria, or it was moved to the next stage. The set of included articles was finalised when both researchers were in agreement. In the event of disagreement, a third person (J.S.) was consulted. The cognitive tests used in the selection of included papers were screened by two independent researchers (M.G, G.D.) to assess which ones tested executive functions. If any of the papers did not include at least one cognitive outcome related to executive function, they were excluded when the two researchers (M.G., G.D.) were in agreement.

### *Quality Assessment*

Risk of bias was assessed using the Jadad scale, which includes 3 assessment points: blinding, randomisation and account of all study participants. For the assessment of blinding studies can have a maximum score of 2 (1 point if blinding is mentioned and one additional point if the method of blinding is appropriate). Similarly, for the assessment of randomisation, studies can have a maximum score of 2 points (1 if randomisation is mentioned and one additional point if the method of randomisation is appropriate). One extra point is given to studies that provide a full account of the fate of all study participants, including drop-outs, non-adherence to protocol or other reasons for exclusion from the

statistical analysis. Therefore, each study can have a maximum score of 5, which suggests low bias and a minimum of zero, which suggests high risk of bias.

## Results

### *Main Search*

The search returned 8,332 articles in total. Of those, 4,208 were duplicate citations. 41 articles were selected for further screening. Of those 41 articles, 21 were excluded: three meeting abstracts, four review articles, one in-vitro study, six papers that did not report any cognitive outcomes, three with unsuitable treatments, one that did not report any executive function outcomes, one that focuses on cocoa methylxanthines, rather than flavanols and two that were not blinded. Figure 1 shows the search and screening process and the reasons for exclusion. Table 1 summarises the characteristics of the included studies.

The trials included in this systematic review were conducted between 2006 and 2021 and included a total of 999 participants, with 10-211 participants per study. Out of the 20 studies included in the systematic review, seven were chronic and 13 were acute trials. Nine out of 20 report statistically significant beneficial effects of cocoa flavanol treatments on executive function outcomes, one study, even though it used a selection of executive function tasks, it only reported significant effects on global cognition, and it does not report changes in executive function separately. Ten studies did not report any changes in executive function after the administration of the cocoa flavanol treatment. Table 2 shows a summary of the results on executive function in cocoa flavanol intervention trials and the flavanol dose used. Six out of 13 acute trials, and three out of seven chronic trials showed significant improvements in executive function. The duration of the chronic trials ranged from 28 days to 12 weeks. In the acute trials the treatments were administered between 1.58h-3.5h prior to cognitive testing.

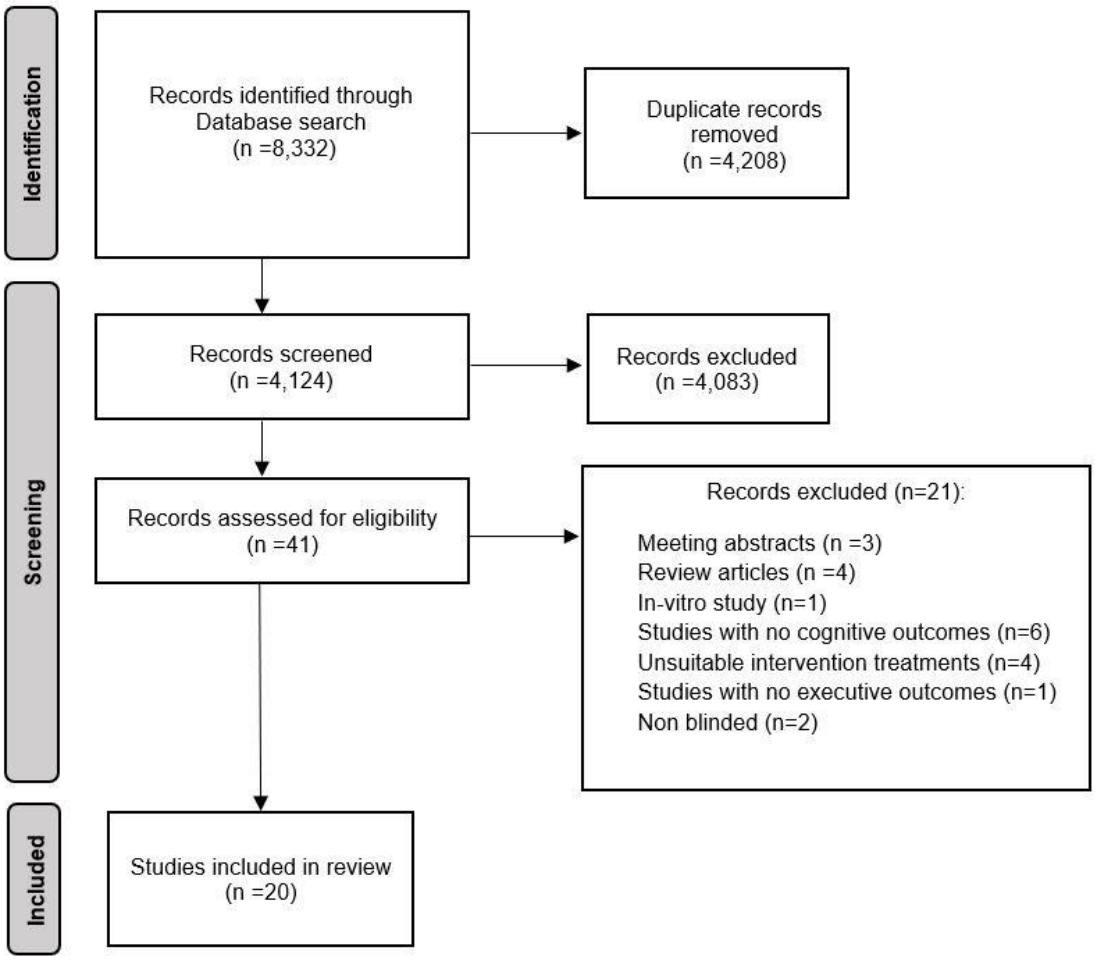


Figure 1. 1 Flow diagram of the search and screening process and reasons for exclusion.

### *Study Participants*

The majority of the studies recruited healthy, cognitively intact, non-smoking adults of both sexes. Eleven studies recruited healthy, young adults, aged between 18 and 45. Six studies recruited healthy older adults, aged over 60 years and one study recruited healthy, middle-aged participants (40-65 years old). One study recruited older participants with mild cognitive decline and one study participants with type 1 diabetes (Table 1). One study investigated the effects of cocoa flavanol combined with exercise on cognitive function and used only young, healthy, well-trained men.

Table 1. 1 Summary of the studies included in the systematic review

| Author | Participants | Intervention | Duration | Design | Executive Function Outcomes | Key Findings |
|---|---|---|---|---|---|---|
| **Crews W. D., et al., 2008 [38]** | Healthy men (n = 41) and women (n = 60) ≥60 years old (38 men and 52 women completed the trial protocol) | One dark chocolate bar (containing 60% cacao, 397.30 mg total proanthocyanins/g) and one 8-ounce (237 mL) cup of cocoa beverage (357.41 mg total proanthocyanins/g) or similarly matched placebo products (Total proanthocyanin concentrations for each placebo bar and beverage: 0.20 mg/g and 40.87 mg/g, respectively) | Once a day for 6 weeks | Randomised, double-blind, fixed-dose, placebo-controlled, parallel-groups [dark chocolate and cocoa (n = 51) or placebo (n = 50)] | Trail Making Test (Total time), Stroop Color-Word Test (raw scores) | No significant group-by-trial interactions were found for any of the variables |

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| **Decroix L., et al., 2016 [39]** | Healthy, well-trained male cyclists, 30 ± 3 years old (N=12) | High cocoa flavanol chocolate milk (903.75 mg flavanol) or Placebo (15 mg flavanol), matched in taste, colour and calories, together with a standardized high carb lunch to increase flavanol absorption. | Acute (95 minutes prior to testing). 7 days wash-out between testing days. | Randomized, double-blind, crossover design. | Stroop test (reaction times) | No effects of cocoa flavanol intake on reaction times. Effects overruled by the strong exercise-induced increases in cerebral perfusion and oxygenation |
| **Decroix L., et al., 2018 [40]** | 20 healthy subjects age 23.2 ± 4.3years | Cocoa Flavanols (530 mg Cocoa Flavanol, 100 mg epicatechin) or matched placebo | Acute (2 h prior to testing) | Randomised, double-blind, crossover design, 4 trials: 2h after acute Cocoa Flavanol or placebo intake, and 30 min after initial exposure to hypoxia or normoxia. | Stroop test accuracy and reaction times; N-BACK (score/1000) | Acute Cocoa Flavanol intake enhanced the hemodynamic response in the Prefrontal Cortex during cognitive tasks activating the Prefrontal Cortex, but did not alter neuronal activity and did not result in improvements in cognitive function |

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| **Desideri G., et al., 2012 [19]** | Subjects with mild cognitive impairment, n=90 | Drink containing 990 mg (high flavanol), 520 mg (intermediate flavanol), or 45 mg (low flavanol). | Once a day for 8 weeks. | Randomised, double-blind, parallel arm design, 3 arms (30 subjects per group). Measurements at baseline and after 8 weeks. | Trail Making A and B (total time in seconds)<br><br>Verbal fluency (words per minute) | Significant improvements in Trail making A and B scores in the High and Intermediate Flavanol groups but not in the Low Flavanol group. Verbal fluency improvements significantly greater in the High Flavanol group compared to Low Flavanol. |
| **Field D. T., et al. 2011 [20]** | 8 males and 22 Females, 18–25 years old (healthy) N=28 (completed protocol and included in analysis) | Commercially available dark chocolate (CHOIX+, 773 mg flavanol) White chocolate control (Waitrose own brand) with a flavanol trace. Dietary restrictions: low flavonoid diet. | Acute (2h prior to testing). 1 week washout between treatments. | Crossover, experimenter blind, randomised, controlled. | Visual Spatial Working Memory Task (% accuracy) | Improvements in cognitive performance (mean % correct was significantly higher in the high Cocoa Flavanol condition, compared to low). |

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| **Francis S. T. , et al., 2006 [21]** | 16 healthy female subjects between the ages of 18-30 years | High flavanol cocoa drink (172 mg flavanols per drink) and low flavanol control (13 mg flavanols per drink). | 1 drink per day for 5 days, 14 day washout between treatments | Randomised, crossover design, final drink consumed 1.5 h prior to fMRI scan | Reaction times, switch cost and error rates for cognitive tasks ('switch' and 'nonswithch' conditions) | Flavanol-rich cocoa intake had no significant effect on the cognitive outcomes, but it modifed the BOLD response to task switching. |
| **Grassi D., et al., 2016 [41]** | 32 healthy adults (25.31±3.60 years old) | 520 vs 88.5 mg of cocoa flavanol in the form of chocolate bars. | Acute (2h prior to testing) | Randomised, crossover design. Two baseline sessions after one night of undisturbed sleep and two experimental sessions after one night of total sleep deprivation (4 experimental session per participant) | 2-back letter task (discrimination index for accuracy) | Cocoa flavanol intake had no effects on any of the cognitive behavioural outcomes.<br><br>Improvements in vascular function outcomes. |

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| **Karabay A., et al., 2018 [29]** | 48 (24 female) healthy subjects, mean age = 22.15 years. | Beverage with High-flavanol Acticoa ™ cocoa powder, containing 8.3 g flavanols/100 g, or alkalized cocoa powder that contained no flavanols (control), water and sugar. 4 conditions: Baseline, Placebo, Low dose (374 mg Cocoa Flavanol ) & High dose(747 mg Cocoa Flavanol). | Acute (2 h prior to testing). 1 week washout between treatments. | Randomized, double-blind, placebo- and baseline-controlled crossover design, 1week washout between 4 conditions. | Rapid Serial Visual Presentation task (RSVP-Target accuracy); Visual Search task (accuracy and reaction times) | No effects of Cocoa Flavanol intake on temporal attention<br><br>Cocoa Flavanol consumption can enhance the efficiency of spatial attention (decreased reaction times in visual search). |
| **Massee L. A., et al., 2015 [22]** | 40 young, healthy participants aged 18–40 years | Cocoa tablet (3058mg T.cacao seed extract standardized to contain 250mg catechin and 5.56mg caffeine) or | Acute (2–3.5 h prior to testing) And Sub-chronic (once a day | Randomized, placebo-controlled, double-blind, parallel arm. | Congruent and Incongruent Stroop test; Spatial Working Memory; Serial threes and sevens; Rapid Visual Information Processing | At the acute time point, consumption of cocoa significantly improved self-reported mental fatigue and performance on the Serial Sevens task in cycle one of the |

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| | | matched placebo tablet. | for 4 weeks). | | (Average reaction time and % accuracy). | Cognitive Demand Battery. No other significant effects were found. |
| **Mastroiacovo D., et al., 2014 [42]** | 90 cognitively intact elderly subjects | 3 treatment drinks: High Flavanol=993mg, Intermediate Flavanol= 520mg, Low Flavanol=48mg total flavanols. | Once a day for 8 weeks. | Randomised, double-blind, controlled, parallel-arm design. | Trail Making A and B (total time in s) Verbal Fluency Test (words per 60s) | Total time for Trail Making A and Trail Making B tasks significantly decreased in the high and intermediate dose conditions. Verbal fluency scores significantly increased in high dose condition. |
| **Neshatdoust S., et al., 2016 [23]** | Healthy older males and females aged 62–75 y, n=40. | High-flavanol cocoa drink (494 mg total flavanols) or a low-flavanol cocoa drink (23 mg total flavanols). | Once a day for 28 days (4 week washout between treatments) . | Randomised, controlled, double-masked, cross over design. | Go-NoGo, Stroop, plus-minus, trail making, letter memory, serial sevens, digital symbol substitution, Rapid Visual Information Processing Task. | Significantly improved global cognition and serum BDNF levels. |

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| **Pase M. P., et al., 2013 [43]** | Healthy middle-aged (40–65 years) n=72 | Dark chocolate drink mix standardized to contain 500 mg, 250 mg or 0 mg of polyphenols (placebo) once daily for 30 days. | Acute (1h prior to testing; tested at baseline, 1, 2.5 and 4h post ingestion) and Sub-chronic (once a day for 30 days). | Randomised, double-blind, placebo controlled, parallel-groups design. | Quality of Working Memory (combined %accuracy from Spatial Working Memory and Numeric Working Memory tasks)<br><br>Continuity of Attention (combined %accuracy from Choice Reaction Time and Digit Vigilance tasks)<br><br>Power of Attention (sum of speed of response in ms, from Simple Reaction Time, Choice Reaction Time and Digit Vigilance tasks). | After 30 days, the high dose of treatment significantly increased self-rated calmness and contentedness relative to placebo.<br><br>No effects on cognitive performance. |

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| **Saunders C. J., et al., 2011 [24]** | 63 healthy older adults (aged 62–75 years) | High flavanol cocoa drink (600 mg) and a macro and micronutrient matched, low flavanol cocoa drink (23 mg). | Acute (2 h prior to testing) 5 week washout between treatments. | Randomised, double blind, cross over design. | GoNogo, Stroop, Letter memory (reaction times) | Significant improvement in executive function (Improved updating tested using the letter memory task, and inhibiting: reduction in response times on GoNogo and Stroop tasks) at 2 h following consumption of the high flavanol drink compared with low flavanol. Reduction in diastolic blood pressure and an attenuation in the rise of stress related systolic blood pressure. |
| **Scholey A. B., et al., 2010 [25]** | 30 healthy men and women (18-35 years old). | Drinks containing 520 mg, 994 mg Cocoa Flavanol and a matched control. | Acute (90 mins prior to testing). 3 day washout between treatments. | Randomised, controlled, double-blind, balanced, three period crossover trial. | Serial 3's and Serial 7's (numbers of correct and errors) Rapid Visual Information Processing (RVIP-%accuracy and reaction times) | The 994 mg Cocoa Flavanol beverage significantly speeded RVIP responses and resulted in more errors during Serial Sevens. Increases in self-reported 'mental fatigue' were significantly attenuated by the |

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| | | | | | | consumption of the 520 mg Cocoa Flavanol beverage only. |
| **Sorond F. A., et al., 2013 [26]** | 60 older people (aged 72.96 ±5.4 years) | Cocoa powder in packets to be mixed with water (flavanol-rich cocoa 609 mg, and flavanol-poor cocoa 13 mg flavanols/serving). | Twice a day for 30 days. | Randomised, controlled, parallel-arm, double-blind. Tested at baseline, 24 h and after 30 days of treatment. | Trail making A and B tasks (total time in seconds) | Improved Trail B scores in people with impaired neurovascular coupling after 30 days of cocoa consumption.<br><br>Cocoa consumption enhanced neurovascular coupling. |
| **Tsukamoto H., et al., 2018 [31]** | 10 healthy young men 22.6 ± 0.3 y.o. | High Cocoa Flavanol (563 mg of Cocoa Flavanol) or energy-matched low Cocoa Flavanol (38 mg of Cocoa Flavanol) beverage | Acute (30 mins prior to testing) | Single-blind, counterbalanced, intervention trial (combined effects of High Cocoa Flavanol intake and moderate exercise on Executive Function and Memory at baseline, 30 and 60 mins post-drink, | Stroop test (accuracy and reaction time) | Executive Function improved immediately after exercise in both conditions. Executive Function was higher after Hight Cocoa Flavanol consumption compared with the Low Cocoa Flavanol during all time periods. High Cocoa Flavanol consumption and moderate-intensity exercise did not have any effect on Memory Function. |

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| | | | | right after 30 min workout, 30 mins post workout and 60 min post workout) | | |
| **Decroix L., et al., 2019 [18]** | 11 patients with Type 1 Diabetes and their healthy matched controls. | High Cocoa Flavanol condition (900 mg Cocoa Flavanol) and Placebo condition (15 mg Cocoa Flavanol). | Acute (2 h prior to testing). 7-day washout between treatments. | Randomised double-blinded, placebo controlled, counterbalanced cross-over design. | Flanker test (executive and inhibitory control). Accuracy, Response time and flanker interference effect outcomes. | Acute cocoa flavanol intake improved reaction time on the flanker test and increased the Blood oxygenation level dependent response in the activated brain areas in patients with Type 1 Diabetes and their matched controls. |
| **Sumiyoshi E., et al., 2019 [32]** | 18 healthy young subjects (both sexes; 20–31 years old). | Dark Chocolate (540mg total polyphenol) (n=10) and cacao-free (0mg polyphenol) white chocolate (n = 8). | Sub-chronic (once a day for 30 days). | Randomised, placebo control, single-blind, counterbalanced parallel arm design. Measurements at baseline, at 30 days | Modified Stroop Colour Word Test as number of correct answers (measures attention, inhibition) and Digital Cancellation Test as total performance and omission ratio (measures | Dark chocolate intake significantly increased the number of correct answers post-treatment compared with white chocolate where there was no effect on the number of correct answers in the Stroop test. |

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| | | | | and follow up after 3 weeks. | information processing speed, focused attention, sustained attention, omission ratio, primarily reflects sustained and selective attention) | Dark chocolate intake significantly increased total performance in trial 3 of the D-CAT task compared to white chocolate.<br><br>Increased Nerve Growth Factor in plasma. |
| **Gratton G., et al., 2020 [44]** | 18 healthy male adults (18-45 years old) | High (681.4mg) or low (4.1mg) cocoa flavanols drinks. | Acute (2h prior to cognitive testing) | Randomised, controlled, crossover design, with minimum 2 weeks wash-out. | Modified Stroop task (4 conditions with progressively increasing task demands: word, colour, Stroop, double Stroop) measured as Inverse efficiency score (time/accuracy in seconds) | The cognitive benefit of high-flavanol cocoa in the Double Stroop over and above the other cognitive tasks was significantly higher compared to low-flavanol.<br>Benefits were only observed on the most demanding condition (double Stroop). |

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| **Sloan R., P., et al., 2021 [45]** | 211 healthy adults (50–75 years old) | 260, 510 and 770 mg/day of cocoa flavanols or matched placebo. | 12 weeks | Randomized, controlled, parallel-arm design. At baseline, 12 weeks post intervention and after an 8-week washout. | List-Sorting task (NIH Toolbox Cognition Battery, reflects working memory) | There was no effect of 12 weeks of flavanol consumption on the list-sorting task.<br><br>Hippocampal-dependent list-learning performance improved after flavanol intake. |

Table 1. 2 Summary of statistically significant effects and flavanol doses

| Paper | Polyphenol Dose/day | Duration | Significant Improvements |
|---|---|---|---|
| **Acute Studies** | | | |
| **Decroix L. et al., 2016** | 903.75 mg flavanol | Acute (95 minutes prior to testing) | No (No intervention-time interaction data reported) |
| **Decroix L. et al., 2018** | 530 mg of Cocoa Flavanol, 100 mg epicatechin | Acute (2 h prior to testing) | No (No cocoa flavanol effects or scores reported) |
| **Field D. T., et al. 2011** | 773 mg cocoa flavanol | Acute (2 h prior to testing) | Yes (Cocoa Flavanol better than low Cocoa Flavanol condition: 87.1 versus 83.5% correct, $F(1,28)=3.41$, $p<0.05$). |
| **Grassi D., et al., 2016** | 520 mg cocoa flavanol | Acute (2h prior to testing) | No (no significant effects of treatment on 2-back accuracy: $F(1,30)=2.51$; $p=0.12$). |
| **Karabay A., et al., 2018** | 374 mg Cocoa Flavanol (low dose) & 747 mg Cocoa Flavanol (high dose) | Acute (2 h prior to testing) | No (no effect of cocoa flavanol on RSVP or Visual search task $F(2, 90) = 1.04$; $p = 0.36$ |

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|---|---|---|---|
| | | | and F(3, 127) = 2.09, p= 0.11, respectively) |
| **Massee L. A., et al., 2015** | 250mg catechin (cocoa tablet) | Acute (2–3.5 h prior to testing) | No (no effects of cocoa flavanol on Stroop accuracy F=0.41, p=0.53) |
| **Pase M. P., et al., 2013** | 500 mg total polyphenol (high dose) 250 mg (lower dose) | Acute (1h prior to testing) (and Sub-chronic) | No (no significant effect of cocoa flavanol on any of the tasks. No p and F values reported) |
| **Saunders C. J., et al., 2011** | 600 mg total flavanol | Acute (2 h prior to testing) | Yes (Significant improvements in letter memory task (p=0.05), Stroop (p=0.03) and GoNogo (p=0.01) following high flavanol intake compared to placebo). |
| **Scholey A. B., et al., 2010** | 994 mg (high dose), 520 mg (lower dose) Cocoa Flavanols | Acute (90 mins prior to testing) | Yes (correct serial three responses significantly increased compared to placebo, F=18.5, p<.001) |

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| **Tsukamoto H., et al., 2018** | 563 mg of Cocoa Flavanol | Acute (30 mins prior to testing) | No (no significant time × condition interaction for Stroop: F(4,36)=0.71, p>0.05) |
| **Decroix L., et al., 2019** | 900 mg Cocoa Flavanol | Acute (2 h prior to testing) | Yes (Significant task and group interaction for Flanker test reaction times F(2)=9.60; p <0.001) |
| **Gratton G., et al., 2020** | 681.4mg of cocoa flavanol | Acute (~2h prior to cognitive testing) | Yes (Significantly improved Inverse efficiency scores for Double Stroop F(3,48)=3.267, p=0.029) |
| | **Chronic and Sub-chronic Studies** | | |
| **Desideri G., et al., 2012** | 990 mg (high flavanol), or 520 mg (intermediate flavanol) | 8 weeks | Yes (Significant time and treatment interactions: F=27.62; P<0.0001 and F=78.19; P<0.0001 for trail making A and B respectively and F=22.79; P<0.0001 for verbal fluency) |

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|---|---|---|---|
| **Crews W. D., et al., 2008** | 456.97 mg total proanthocyanins | 6 weeks | No (no effects of cocoa flavanol on Stroop, 3.07±6.68 for cocoa vs 2.20±4.8 for placebo, p=0.481) |
| **Francis S. T., et al., 2006** | 172 mg cocoa flavanols | 5 days | No (no significant difference in the 'switch cost' between the two drinks; p=0.30; no means or F values reported) |
| **Massee L. A., et al., 2015** | 250mg catechin (cocoa tablet) | 4 weeks | No (effects of cocoa flavanol on Stroop accuracy F=0.002, p=0.96) |
| **Mastroiacovo D., et al., 2014** | High Flavanol: 993mg, Intermed Flavanol: 520mg | 8 weeks | Yes (Trail making A and B scores significantly improved, p<0.0001, for high and intermediate doses compared to low) |
| **Neshatdoust S., et al., 2016 (Study 2)** | 494 mg total flavanols | 28 days | No (yes in global cognition, greater following high flavanol intake compared to placebo p<0.01) |

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| **Pase M. P., et al., 2013** | 500 mg total polyphenol (high dose) 250 mg (lower dose) | 30 days | No (no significant effect of cocoa flavanol on any of the tasks. No p and F values reported) |
| **Sorond F. A., et al., 2013** | 609 mg flavanol twice a day=1,218 mg/day | 30 days | Yes (improved Trail B times compared to placebo: 116±78s vs 167±110s; p=0.007) |
| **Sumiyoshi E., et al., 2019** | 540mg total polyphenol | 30 days | Yes (Significant effects of dark chocolate on modified Stroop task, post intake (t (9) =2.93, p=0.017) compared to no effects of white chocolate t(7)=0.27, p=0.80) |
| **Sloan R., P., et al., 2021** | 260, 510 or 770 mg Cocoa Flavanols | Daily for 12 weeks | No (no effect of flavanol consumption on list sorting task; p = 0.172) |

### *Study Quality*

The total Jadad scores demonstrate a broad range of quality trials, scored from 1 to 5, and a mean score of 3.3. Table 3 shows the quality assessment and Jadad scores for all

studies included in the systematic review. Although blinding was generally well reported, six out of 20 studies, did not mention the method of blinding used. Only seven out of 20 studies reported details on the fate of all study participants. 18 out of 20 studies mention randomisation, only six of which provide details on the method of randomisation.

Table 1. 3 Quality assessment for risk of bias: Jadad scores

| Paper | Randomisation (max 2) | Blinding (max 2) | Account of all participants (max 1) | Total Score |
|---|---|---|---|---|
| **Crews W. D., et al., 2008** | 2 | 2 | 1 | 5 |
| **Decroix L. et al., 2016** | 1 | 2 | 0 | 3 |
| **Decroix L. et al., 2018** | 1 | 1 | 0 | 2 |
| **Desideri G., et al., 2012** | 1 | 2 | 0 | 3 |
| **Field D. T., et al. 2011** | 1 | 2 | 0 | 3 |
| **Francis S. T. , et al., 2006** | 1 | 1 | 0 | 2 |
| **Grassi D., et al., 2016** | 1 | 1 | 0 | 2 |
| **Karabay A., et al., 2018** | 1 | 2 | 0 | 3 |
| **Massee L. A., et al., 2015** | 2 | 2 | 1 | 5 |
| **Mastroiacovo D., et al., 2014** | 1 | 2 | 1 | 4 |
| **Neshatdoust S., et al., 2016** | 2 | 2 | 0 | 4 |
| **Pase M. P., et al., 2013** | 2 | 2 | 1 | 5 |
| **Saunders C. J., et al., 2011** | 1 | 1 | 0 | 2 |
| **Scholey A. B., et al., 2010** | 1 | 2 | 0 | 3 |
| **Sorond F. A., et al., 2013** | 1 | 1 | 1 | 3 |
| **Tsukamoto H., et al., 2018** | 0 | 1 | 0 | 1 |
| **Decroix L., et al., 2019** | 0 | 2 | 1 | 3 |
| **Sumiyoshi E., et al., 2019** | 2 | 2 | 0 | 4 |
| **Gratton G., et al., 2020** | 1 | 2 | 0 | 3 |
| **Sloan R., P., et al., 2021** | 2 | 2 | 1 | 5 |

*Intervention treatment*

The flavanol content of the cocoa based treatments used across studies varied. The doses of cocoa flavanol ranged from 172mg/day to 1,218mg/day. Five studies tested two different doses, a high and an intermediate one, compared to placebo. One study used three arms (low, medium and high intake) compared to placebo. Ten studies used a high flavanol dose (over 600mg a day), 13 studies used an intermediate dose (lower than 600mg a day). 12 studies used single dose acute intervention, orally administered at an average of 105.5 minutes before cognitive testing. Nine studies tested for chronic/sub-chronic effects, with an average duration of 5.6 weeks. Table 2 summarises the dose and duration of the interventions in the 20 included studies. Further, the majority of the studies (13 out of 19) used treatments in the form of cocoa flavanol rich drink. Three studies used flavanol rich chocolate bars, three used cocoa flavanol capsules and one used both a chocolate bar and a drink.

*Cognitive testing*

The cognitive tasks used to measure executive function varied greatly across trials. The Stroop test was the most used test (9 out of 19 studies), followed by the Trail Making test (5 out of 19 studies). Table 4 below shows the cognitive tests used across the 20 studies and the main cognitive processes involved. Those include processing speed and working memory, as well as other cognitive processes that fall under the executive function umbrella.

Table 1. 4 Cognitive tests and processes used in the included studies

| Paper | Cognitive Tests | Cognitive Processes |
|---|---|---|
| **Crews et al., 2008 [46]** | Trail Making Test (A and B); Stroop Word-Colour Test | Processing Speed, Selective attention, Inhibition, Working Memory, Updating. |
| **Decroix et al., 2016 [39]** | Stroop test | Processing Speed, Inhibition, Attention. |
| **Decroix et al., 2018 [40]** | Stroop test, n-back | Processing Speed, Inhibition, Attention, Working memory, Short term memory. |
| **Desideri et al., 2012 [19]** | Trail Making Test (A and B); Verbal Fluency | Processing speed, Long term retrieval, Executive control ability, Working Memory, Updating. |
| **Field et al., 2011 [20]** | Visual Spatial Working Memory Task | Visuospatial working memory, Inhibition. |
| **Francis et al., 2006 [21]** | Letter-Digit Switch Task | Working Memory. |
| **Grassi et al., 2016 [41]** | 2-back | Working Memory. |
| **Karabay et al., 2018 [29]** | RSVP, Visual Search Task | Interference control, Processing Speed. |
| **Massee et al., 2015 [22]** | Stroop; Spatial Working Memory; Serial 3's & 7's | Processing speed, Inhibition, Attention, Working memory, Updating. |
| **Mastroiacovo et al., 2014 [30]** | Trail Making; Verbal Fluency | Processing speed, Long term retrieval, Executive control. |
| **Neshatdoust et al., 2016 [23]** | Go-NoGo, Stroop, plus-minus, trail making, letter memory, serial sevens, digital symbol substitution, RVIP | Inhibitory control, Processing speed, Shifting, Updating, Working memory, Sustained attention. |

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|---|---|---|
| **Pase et al., 2013 [43]** | Spatial and Numeric Working Memory Task, Choice reaction time, digit vigilance | Working Memory, Sustained attention, Inhibition. |
| **Saunders et al., 2011 [24]** | GoNogo, Stroop, Letter memory | Processing speed, Inhibitory control, Updating, Working memory, Attention. |
| **Scholey et al., 2010 [25]** | Serial 3's and 7's, RVIP | Working memory, Updating, Sustained attention. |
| **Sorond et al., 2013 [26]** | Trail Making | Processing speed, Working Memory, Updating. |
| **Tsukamoto et al., 2018** | Stroop | Processing speed, Inhibition |
| **Decroix et al., 2019 [18]** | Flanker | Executive and Inhibitory control, Attention. |
| **Sumiyoshi et al., 2019 [32]** | Stroop, Digital cancellation | Processing speed, Inhibition, Attention. |
| **Gratton G., et al., 2020** | Modified Stroop | Processing speed, Inhibition, Attention. |
| **Sloan R., P., et al., 2021 [45]** | List-Sorting task (NIH Toolbox Cognition Battery) | Working memory. |

## Discussion

This systematic review shows that nine out of the 20 studies that met our inclusion criteria reported statistically significant beneficial effects of flavanol rich cocoa interventions on executive function. Five out of 20 showed significant acute effects and four out of 20 significant chronic effects. Overall, studies were characterised by relatively low sample sizes and high to intermediate flavanol doses. The overall quality of the studies was high. All studies were placebo controlled and blinded (single or double). The majority of the studies were randomised, and they reported adequate details on the study duration, participant characteristics, protocol design and methods used for the assessment of

executive function. There were various sources of heterogeneity; health status, age, treatment, intervention duration and methods of assessing executive function, as discussed below.

The age, health and cognitive status varied across the studies. Most studies recruited young, healthy participants of both sexes. One study recruited older adults with mild cognitive impairment and showed significant beneficial effects of high and intermediate doses of cocoa flavanol on executive performance [9]. Although subjects with mild cognitive impairment may be more responsive to the interventions used, or any observed effects might be more noticeable given their baseline cognitive status, it is debatable whether they can be compared with cognitively intact subjects. Mild cognitive impairment is a state of abnormal cognitive function in one or more domains, usually occurring after the age of 60 [35]. In clinical practice, mild cognitive impairment is a spectrum of cognitive states that is seen between normal ageing and dementia [36]. Therefore, mild cognitive impairment could also affect executive function. As such, although the results of Desideri and colleagues [9] are encouraging, future research is required to assess the effects of cocoa flavanols on executive function (as well as other cognitive domains independently) in subjects with mild cognitive impairment and compare them with those in healthy individuals.

Another study that recruited individuals with a medical condition was that of Decroix and colleagues who investigated the effects of cocoa flavanols on cognitive function in individuals with type 1 diabetes and their matched healthy controls, and concluded that cocoa flavanols improved executive function in both patients with type 1 diabetes and healthy controls [47]. Even though the two groups were matched, there is no mention of matched cognitive statuses; therefore, it cannot be assumed that they were both cognitively healthy. As such, and considering that type 1 diabetes mellitus has been linked to cognitive decline in adults [48], for the purposes of this systematic review we focused on the healthy control group, and not the T1D group, who may or may not have some level of cognitive deterioration, as it is not mentioned in the study. Counting that as a study that recruited healthy individuals, we could argue that the majority of the studies included in this review (19 out of 20) recruited physiologically and cognitively healthy subjects.

The participants' age ranged between 18 and 75 years, however the majority of the studies investigated the effects of cocoa flavanols on executive function in younger adults (18 to 45 years old), where some significant effects were evident. Nonetheless, given the importance of executive functioning in the aging brain and the urgency to establish whether cocoa flavanols could have potential effects on delaying cognitive decline, further research with older adult subjects is required. One study [23] reported statistically significant improvements on global cognition and, although they used a selection of tasks that measure executive function, executive data was not reported. Global cognition includes an executive element, but it is a generalised impression of cognitive function. Therefore, improvements in executive function in that study cannot be assumed.

The intervention treatments also varied across the 20 studies. The majority of the studies that reported significant effects of cocoa flavanol interventions on executive function used high flavanol doses (≥600mg a day), whilst most of the studies that failed to show any significant effects used lower doses (<600mg a day), which could suggest that higher doses may be more effective. This appears to be in agreement with the systematic review by Barrera-Reyes et al., (2020), who state that significant effects on cognitive functions (including but not limited to executive function) were observed after administration of higher doses of epicatechin (>50 mg per day). Furthermore, they mention that past a certain dose (750 mg of cocoa flavanols) there are no further improvements in cognitive performance [49]. An issue that needs to be highlighted here is the fact that there are inconsistencies in the way the dosage is measured and reported, as some studies measure 'total polyphenols', others 'total flavanol' or 'total flavonoid', and others mention specific types of flavanol, such as epicatechin, terms which are not interchangeable. Since the polyphenolic constituents of cocoa are predominately flavanols (monomeric forms: (−)-epicatechin, (+)-epicatechin, (−)-catechin, and (+)-catechin), and procyanidins, which are derivatives of flavanols, and, to some extent, anthocyanins, [50], it would be wrong to assume that 'cocoa polyphenols' is the same as 'cocoa flavanols'. Also, the term 'cocoa flavanols' includes both the monomeric forms and the oligomeric procyanidins [50], therefore 'cocoa flavanols' and 'total epicatechin' should not be used interchangeably either.

In addition, the effects of flavanol rich cocoa treatments on both cognitive behavioural and physiological changes cannot be attributed to a single compound or group

of compounds, i.e. flavn-3-ols, present in cocoa, as there are other bioactive compounds naturally occurring in cocoa, such as methylxanthines, which also appear to have psychoactive properties [51]. Caffeine and theobromine, two methylxanthines naturally present in cocoa, appear to have higher absorption rates than flavanols, therefore a more immediate stimulating effect [51], whilst flavanols seem to have slightly slower effects and plasma level peak time, usually observed between one and two hours post ingestion, [50]. Therefore, it has been suggested that cocoa flavanols and methylxanthines could act synergistically [52].

Finally, the methods used to assess executive function also varied. 21 different cognitive tasks that measure executive function were used in total across 20 studies, which highlights the lack of consistency in the selection of tasks. As executive function is a broad term including a variety of brain processes, there is some debate as to whether certain cognitive tests (such as serial threes and sevens) and functions (such as working memory and spatial working memory) fall under the executive function umbrella or not. Those are closely related to executive functioning, however, some non-executive functions, such as short-term memory are also implemented, highlighting the complicated nature of 'executive function' and the tasks used to measure it. Furthermore, various tasks that involve executive processes and are generally considered executive function tasks, are still subject to interpretation and often depends on the definition of an executive process, for example switching, updating or inhibition. Future research should aim to standardise the way executive function (and other cognitive functions) are measured as well as the way those outcomes are reported, to allow for more replicable and comparable results, and, importantly, quantitative analyses.

Moreover, physiological effects of the cocoa flavanol rich treatments have been also observed even when cognitive effects are not always present. For example, Decroix et al., (2018) showed that acute intake of cocoa flavanol enhanced the hemodynamic response in the prefrontal cortex but did not improve executive function [53]. Francis et al., (2006) observed an improvement in blood oxygenation level-dependent response after the consumption of a cocoa flavanol-rich drink for five days [21]. Grassi et al., (2016), showed that acute intake of a cocoa flavanol rich intervention had no effects on cognitive function but it had beneficial effects on vascular function [41], and Gratton et al., (2020) observed improvements in blood oxygenation responses to hypercapnia [44]. Interestingly,

Gratton et al., (2020) established a link between said improvements in blood oxygenation and improvements in executive performance observed following a single dose of cocoa flavanols, whilst the rest of the above-mentioned studies have failed to create such links between behavioural and physiological outcomes. All of those physiological parameters, i.e. neurovascular coupling, blood oxygenation response and arterial function/stiffness have been linked to cognitive function and impairment [54], [55], [56]. This confirms the biological activity of the treatments but also highlights the need for future research to perform such physiological tests along with cognitive testing.

Another limitation that needs to be taken into consideration is the data extraction and the appropriate meta-analytical methods for this review. Because of the multifaceted nature of executive function, as mentioned above, each study reported more than one outcome for the same measure, resulting in multiple non-independent effect sizes per paper. The use of multiple non-independent effect sizes poses a risk to the interpretation of meta-analytical results generated using conventional meta-analytical methods, as averaging them out without accounting for the within-study correlations, which in most cases are not known, results in conservative over-estimates [57]. As such, Robust Variance Estimation (RVE) was considered as the most appropriate meta-analytical method for this review. However, out of the 20 papers included in the systematic review only 10 provided sufficient data for extraction. In Robust Variance Estimation at least 10 studies, and ideally more than 40, are needed to produce valid statistical conclusions [58]. Therefore, conducting a RVE meta-analysis is beyond the breadth of this systematic review.

## Conclusions

In conclusion, this systematic review shows that there is some evidence supporting that cocoa flavanol interventions can have beneficial effects on executive function, both acutely and chronically; however, these findings should be interpreted with caution, as studies report different executive function outcomes, based on different cognitive tasks that are not always comparable. The studies that met our inclusion criteria did not necessarily present cognitive domain specific results but, instead, most of them focus on individual cognitive test battery scores, or global cognition using different methods and tests. This highlights the need to test different cognitive domains separately using

standardised and consistent across studies methods, which is not the case as yet. We conclude that higher doses (≥600mg total cocoa polyphenols per day) could be more effective, and we highlight the importance of investigating those effects in older adults, since they have been more extensively observed in young adults so far. That will shed more light on the potential effects of those compounds on neuropathological conditions related to brain ageing. Finally, further research and more published data are required to address and minimise the sources of heterogeneity and conduct a quantitative analysis with robust and meaningful results.

## Author's Contribution

M.G. contributed to the following: inception and design; literature search; result interpretation; PROSPERO registration.

CHAPTER 2

# The chronic effects of cocoa flavanols on cognitive and vascular function in healthy older subjects: A double blind randomised controlled trial

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## Abstract

Flavonoid-rich cocoa has been shown to have beneficial effects on cognitive function and mood. High flavonoid intake has been shown to result in increases in serum BDNF, improvements in arterial function and glucoregulatory mechanisms. We tested the hypothesis that 24 weeks of daily intake of a cocoa extract supplement can result in improved cognitive performance and mood. Additionally, we explored the possibility of changes in serum Brain Derived Neurotrophic Factor, flow-mediated dilation, and serum fasting glucose and insulin underpinning said beneficial effects of flavonoid-rich cocoa on mood and cognition. We conducted a double-blind, randomised, controlled, parallel arm, chronic intervention trial with healthy older adults (n=80), 40 male and 40 female, aged between 60 and 75 years to determine the effect of a flavanol-rich supplement (900mg total cocoa flavanol) taken daily for 24 weeks on various domains of cognitive function, self-rated mood, peripheral arterial health and brain mechanisms, compared to a matched low-flavanol control (10mg total flavanol). Linear Mixed Model with baseline as a covariate was used to analyse primary (global cognitive function, memory and executive function)

and secondary outcomes (mood, FMD and biochemical parameters) at baseline, post-intervention (24 weeks) and after a 12-week washout. A tendency towards an improvement in vascular function indicated by increased FMD response (mean difference = 1.38, df = 59.77, p = 0.079) and reduced heart rate (mean difference = -2.39, df = 64.83, p = 0.019) following 24 weeks of cocoa flavanol supplementation, as well as a trend towards a reduction in plasma concentration of NEFA (mean difference = -53.14, df = 60.10, p = 0.059) at follow-up were observed. There were no effects on any of the cognitive outcomes, however both the active treatment and the control resulted in improved cognitive function over time, which could potentially be attributed to another cocoa constituent present in both treatments. Further investigation is required to test such speculations.

## Introduction

Diet is one of the main lifestyle factors that can strongly influence brain function, and mental health [12] as well as the onset of vascular [16] and neurodegenerative diseases [13]. As cognitive decline and endothelial dysfunction occur during age advancing, establishing ways of delaying or preventing them is of pivotal importance. In recent years, various naturally occurring bioactives, in the form of dietary supplements, have been investigated extensively, to better understand their health promoting and potentially preventative attributes. Flavonoids, a group of polyphenolic compounds naturally occurring in many plant-based foods and beverages, like berries, grapes, tea, cocoa and chocolate products, have been shown to have such properties according to recent research [16, 49, 59-62]. Flavonoids consist of two aromatic rings and a third oxygenated heterocycle ring and they are divided into seven subclasses: flavonols, flavones, flavanones, flavanonols, anthocyanidins, isoflavones and flavanols [6]. Much attention has been drawn to the latter subclass, flavanols (also known as flavan-3-ols) as they are the most abundant flavonoids in our diets [14, 15]. More specifically, cocoa flavan-3-ols have been brought into focus, since cocoa is a high source of flavanols and important part of the western diet [63-65]. Flavanols are the main polyphenolic constituents of cocoa and they include monomeric forms: (−)-epicatechin, (+)-epicatechin, (−)-catechin, and (+)-catechin), and oligomeric procyanidins, which are derivatives of flavanols [50].

The neuropsychological effects of cocoa flavanols have been investigated in the last years and there is a growing body of evidence suggesting that cocoa flavanols have beneficial effects on various aspects of cognitive function [18-27] and potentially the ability to delay age related cognitive decline [28]. Dark chocolate consumption has been linked to acute improvements in episodic memory two hours post ingestion, suggesting that single portions of commercially available dark chocolate can exert cognitive benefits in healthy individuals [27]. Other cocoa flavanol rich treatments in the form of a drink or capsule have been shown to have beneficial effects on cognition, such as chronic [19] and acute [24] improvements in executive functioning, and it has further been linked to improvements in mood outcomes, such as calmness and contentment [43].

The effects of cocoa flavanols on mood is of particular interest as it is a common perception that chocolate consumption has such properties [66]. Although this may be due to the taste and palatability, as well as its carbohydrate and fat content that results in pleasure [67-69], a lot of attention has been drawn to the chemical constituents of cocoa that may be accountable for those effects and the potential biochemical mechanisms behind them. Since cocoa contains compounds that, as mentioned above, have been shown to have psychoactive properties, and the ability to cross the blood brain barrier [70] and induce beneficial neurological changes [6], it is conceivable that some of those effects on neurocognitive function and mood could be attributed to them. Previous research on the effects of cocoa flavonoids on mood has shown both chronic and acute improvements in self-rated mood, specifically improvements in calmness and contentment following 30 days of daily intake of a cocoa flavanol [43] and reduction in mental fatigue following a single dose of cocoa flavanol [71]. Furthermore, cocoa (−)-epicatechin has been shown to reduce oxidative stress and attenuate the development of cortisol resistance [72], which could be one of the mechanisms underlying the effects of cocoa flavanols on mood, since cortisol levels have been linked with certain psychophysical pathologies, including mood disorders [73].

Several other mechanisms have been suggested to describe the processes behind the beneficial effects of cocoa flavanols on brain function. Firstly, there appears to be a link between cognitive and vascular function, as age related cognitive dysfunction occurs with cardiovascular disease and other comorbidities, including reduced cerebral blood flow [74]. Previously established beneficial effects of cocoa on vascular function were seen as

increases in peripheral blood flow, flow-mediated dilation (FMD) of the brachial artery and reductions in blood pressure [16], as well as improvements in cerebral blood flow, which may be linked to the beneficial effects of cocoa flavanols on cognitive function [17]. These vascular effects have been shown to be mediated by a (NO)-dependent mechanism [75], whereby cocoa flavanols increase endothelial nitric oxide production which causes vasodilation and, therefore, reduction in blood pressure [76] and increase in blood flow, including cerebral blood flow [75].

Another proposed mechanism whereby cocoa flavanols exert physiological effects related to their neurocognitive benefits is by increasing insulin sensitivity and glucoregulatory control [77]. Since cognitive (dys)function can be mediated by alterations in the insulin signalling pathway [78, 79] and hyperinsulinemia is linked to increased risk of neurocognitive impairments such as dementia [80], recent research has been aiming to investigate whether cocoa flavanols have any involvement in those mechanisms. Indeed, there is a great deal of evidence suggesting that long term (1 to 8 weeks) polyphenol-rich cocoa intake can significantly increase insulin sensitivity [16, 19, 81, 82] and that those effects can play an influential role in modulating cognitive performance [19]. These effects on insulin resistance can further be linked to the vascular effects mentioned above, since evidence suggests that insulin can stimulate production of NO in human endothelial cells, indicating that certain insulin-signalling pathways are involved with NO production [83].

On a neurobiological level, the effects of flavonoids on neurocognitive function have been shown to be underpinned by their ability to interact with and modulate neuronal signalling pathways that promote synaptic plasticity [84]. One of those pathways involves the activation of extracellular receptorkinase (ERK), Protein kinase B (Akt) and cyclicAMP-response element-binding protein (CREB) by flavonoids, which leads to increases in BDNF expression, resulting in improvements in synaptic plasticity [84, 85]. Increased BDNF release in the brain plays a crucial role in memory function (memory acquisition and consolidation) [9] and peripheral BDNF levels have also been linked to mood and mood disorders such as depression [86, 87]. Improvements in cognitive function caused by the intake of dietary flavonoids has been explained by increases in serum BDNF [23], suggesting that flavonoid-rich interventions, can upregulate BDNF and improve cognitive function. However, this theory has not yet been confirmed by determining the BDNF levels in the human brain or cerebrospinal fluid following, cocoa, or other flavonoid-rich treatment,

ingestion [32]. Considering the beneficial effects of cocoa flavanols on mood, and that BDNF has been suggested as a biomarker of mood and mood disorders, it is speculated that BDNF may have some involvement in those effects [87].

As such, and building on existing research previously conducted by our research group [23], this study is designed to determine how a supplement with high cocoa-flavanol content may enhance cognitive function in older adults over multiple weeks of daily supplementation. We focus on neuropsychological outcomes and attempt to explore some physiological parameters related to the potetial underpinning mechanisms described above. Specifically, we investigate how cocoa flavanols enhance arterial function in the periphery, measured as changes in FMD response, and in the brain, assessed with magnetic resonance imaging (MRI). Further, we determine the levels of fasting insulin and glucose as well as the serum BDNF and cortisol levels. The outcomes of this study aim to allude to the potential effects of dietary flavanols on counteracting the decline in human cognitive function, and improving mental wellbeing during normal and abnormal ageing.

## Methods

### *Study outline*

A double-blind, randomised, controlled, parallel arm chronic intervention trial with healthy older adults, aged between 60 and 75 years, was conducted to determine the effect of a cocoa flavanol-rich supplement on cognitive function, mood, peripheral arterial health and biochemical markers related to vascular and brain function. The ethical principles for medical research with human subjects were followed at all stages of the trial in accordance with the Declaration of Helsinki and the GCP/ICH guidelines. The trial was given the favourable opinion for conduct by the Reading University Ethics Committee (Reference number: 1548, final amendment dated 9 August 2017, clinical trial identifier number: NCT03030053).

On enrolment into the study, participants were invited to the University on three separate occasions. They were asked to arrive at the Hugh Sinclair Unit of Human Nutrition in the Department of Food & Nutritional Sciences where a fasted blood sample was taken. Following this, blood pressure was recorded and a FMD measurement taken. Volunteers

were then escorted to the Department of Psychology for cognitive testing and brain imaging. After the initial visit, participants received a 24-week flavonoid intervention or matched control, which they were instructed to consume daily. Changes in diet and mood were monitored between visits. Participants could collect further doses of the intervention at the Hugh Sinclair Unit of Human Nutrition in the Department of Food and Nutritional Sciences or the Department of Psychology throughout the study period if needed. Figure 2.1 below shows an overview of the study design.

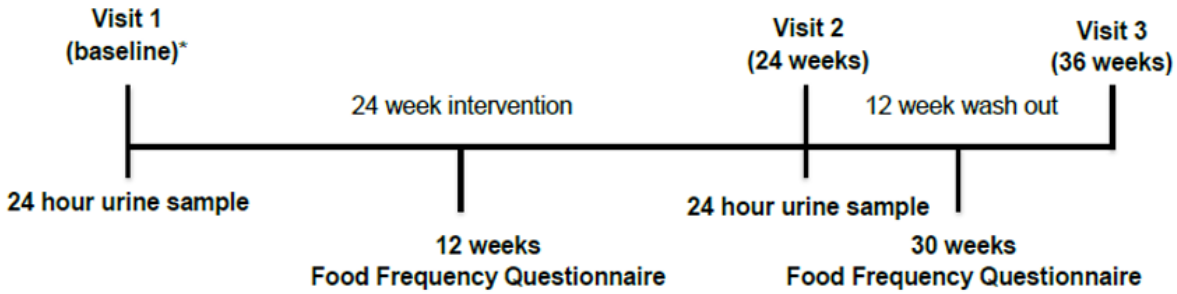


Figure 2. 1 Study Timeline

Participants were recruited from the Hugh Sinclair Unit of Nutrition patient database, the Psychology Department's Older Adult Panel and public advertisements. Potential volunteers showing interest in participating in the study were sent a study information sheet. Potential volunteers were also contacted via telephone and during this conversation the study was explained and the volunteers were invited to ask any questions about participation in the study or any of the specific procedures involved. If they remained interested in participating, they underwent preliminary screening for eligibility during this telephone call. If eligible, the study participants were invited to attend a screening visit at the Hugh Sinclair Unit of Human Nutrition to confirm eligibility. A letter was sent to the volunteers' GP informing them that their patient is interested in participating in the study (a copy of the letter was also sent to the volunteers).

### *Screening*

On arrival to the Hugh Sinclair Unit of Human Nutrition, the experimenter reiterated the purpose and procedures of the study based on the information sheet and answered

any additional questions. Once the participant understood the purpose and procedures involved in the study and was happy to take part, his/her eligibility to participate was confirmed according to the following inclusion/exclusion criteria:

- Males and females aged 60-75 years
- No general global cognitive impairment (MMSE score ≥24, assessed during screening)
- English as primary language, able to understand the study information sheet, follow instructions in English and give informed consent
- No un-corrected vision or hearing problems
- No speech or communication difficulties
- Not currently suffering from depression (BSI score of <11, assessed at screening)
- Should not be diagnosed with any learning difficulty such as Dyslexia or Dyspraxia
- Non-smoker
- Not sensitive/allergic to the intervention or the study foods (ask Experimenter for details of study foods)
- Should not suffer from any form of clinically diagnosed disease, including:
    - major mental illness (current or previous episode with hospitalization)
    - chronic fatigue syndrome
    - liver disease o diabetes mellitus
    - heart disease or myocardial infarction
- Should not be taking blood pressure medication, anticoagulants, anti-platelet medication or antidepressants
- Should not be on a weight reducing dietary regimen or taking any dietary supplements (including dietary fatty acids), unless willing to temporarily refrain from taking dietary supplements for the duration of the study
- Subjects should be consuming no more than seven portions of fruit and vegetables a day
- Subjects should be consuming no more than five cups of tea a day
- Alcohol consumption should be within the current NHS recommendation - women: ≤21 units per week (max 3 per day), 1 large 250mL glass of wine (Alcohol By Volume 12%) is 3 units; men: ≤ 28 units per week (max 4 per day), 1 pint of strong lager/beer/cider (Alcohol By Volume 5.2%) is 3 units
- Men should not be taking part in more than 10.5 hours of moderate to vigorous exercise per week and women should not be taking part in more than 7 hours of moderate to

vigorous exercise per week (assessed on an individual basis to avoid recruitment of people who exercise too vigorously)

- Must not be taking illegal substances
- BP <150/90 (determined at screening)
- BMI <30 (determined at screening)
- Full blood count parameters within the normal range to be determined from screening blood sample, specifically:
    - Haemoglobin to check for anaemia (males >12.5 g/dL, females >11.5 g/d)
    - Total white cell count (3.6-11.0 x109/L)
    - Differential count:
        - Neutrophils (1.8 - 7.5 x109/L)
        - Lymphocytes (1.0 - 4.0 x109/L)
        - Monocytes (0.2 - 0.8 x109/L)
        - Eosinophils (0.1 - 0.4 x109/L)
        - Basophils (0.02 - 0.1 x109/L)
    - Normal platelet function (platelet count 140-400 x109/L)
    - Red cell count (4.50-6.50 x1012/L for males; 3.80-5.80 x1012/L for females)
    - Haematocrit (0.40-0.54 L/L for males; 0.37-0.47 L/L for females)
    - Mean Cell Volume (80-100 fL)
    - Mean Cell Haemoglobin (27-32 pg)
    - Reticulocyte Count (0.2-2.0 %)
- The following blood parameters within the normal range: Liver function (gamma GT level < 80 IU/L, ALT < 30 U/L, ALP < 320 U/L), kidney function (total bilirubin ≤ 22 µmol/L, creatinine ≤ 106 µmol/L, uric acid < 506 µmol/L), fasting blood glucose level (< 7 mmol/L), triglycerides (< 2.2 mmol/L) and plasma cholesterol (< 8 mmol/L), determined from screening blood sample
- Participation in other research trials within the last 6 months needed to be declared as it could affect the start date for participation in this trial.

If participants were suitable to take part and are happy to be enrolled in the study, it was explained that their eligibility to participate would be confirmed upon completion of the screening visit once criteria such as BMI, BP and screening blood parameters have been checked against the eligibility criteria. Participants were told that they would be informed

of their eligibility as soon as this has been determined (blood test results from the Royal Berkshire Hospital were usually obtained within 7-10 days).

If participants are happy with the procedure, they were asked to give their consent to participate in the study by completing and signing the study specific consent form. Following this, height and weight (from which BMI can be derived), and waist circumference were measured. Participants then rested in a sitting position and their blood pressure was taken.

A trained phlebotomist took a fasted blood sample. A small EDTA tube was sent to the Royal Berkshire Hospital (5mL) for full blood count analysis to ensure that the study participant is in generally good health before taking part in the study and is not anaemic. The full blood count reported haemoglobin level, total number of platelets, red blood cells, white blood cells (including for each of the different types of white blood cell e.g. lymphocytes), haematocrit level, mean cell volume, mean cell haemoglobin and reticulocyte count. An additional screening blood sample (4mL) was also taken for clinical-chemical measurements to check that the fasting cholesterol, triglyceride and glucose levels are within the range we have specified and that the volunteer does not have impaired liver or kidney function. As mentioned previously, the volunteer and their GP were informed of the full blood count results from the Royal Berkshire Hospital as well as other screening results.

Participants were then given breakfast and asked to complete some screening questionnaires. These were a set of short questionnaires to assess participants' general health and lifestyle, screen for depression, and to assess their habitual diet and exercise patterns. Participants also completed an initial screening form for MRI scanning at the CINN in accordance with CINN standard procedure. Following this, participants underwent cognitive behavioural testing. Volunteers were asked to complete a measure of global cognitive function (the Mini Mental State Examination), crystallized IQ (National Adult Reading Test), and fluid IQ (Block Design Task form the Wechsler Adult Intelligence Scale – Revised). Figure 2.2 below shows an overview of the screening visit (timings are approximate).

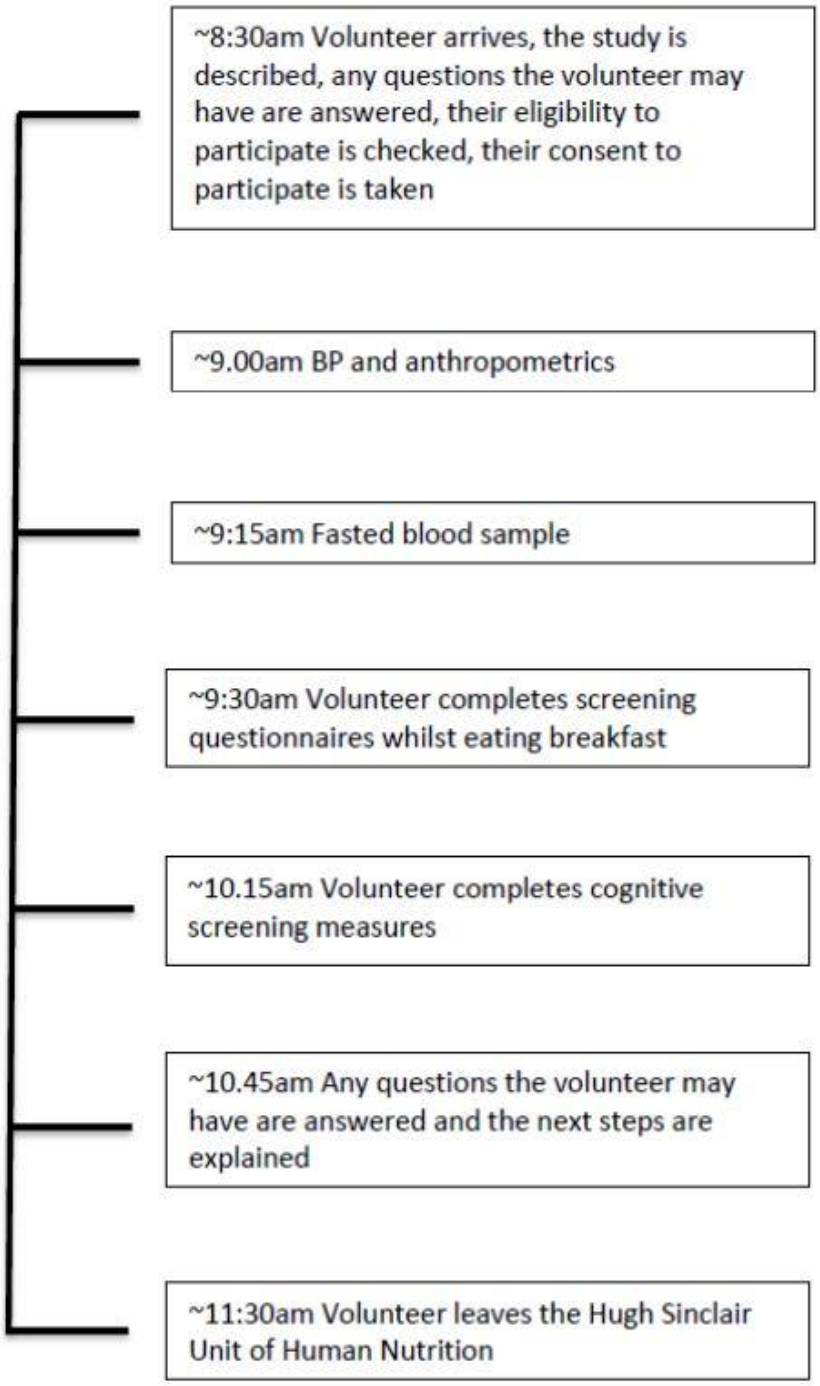


Figure 2. 2 Screening visit timeline

### *Familiarisation*

Once eligibility was confirmed, participants were informed and invited back to the Nutrition Unit for a familiarisation visit. On these visits participants were asked to practice each of the cognitive tasks they will complete during the study visits. Participants practiced all tasks performed during the cognitive test battery (Letter Memory, RVIP, Corsi Blocks,

CPT, Verbal Fluency and AVLT) and self-reported mood assessments, as well as the three tasks that were performed in the MRI scanner (two cognitive tasks with approximately 10 minutes length each and a mood Visual Analogue Scale). On completion of the familiarisation visit, the Experimenter explained what would happen on the volunteer's next visit and reminded the volunteer to arrive fasted for their study visit days.

### *Testing*

#### Phase one of study visit (location: Hugh Sinclair Unit of Human Nutrition)

For each visit, participants arrived at the Hugh Sinclair Unit of Human Nutrition in a fasted state (12-hour overnight fast: only water allowed, starting after dinner the evening prior to each visit). Water was available throughout the study day. On arrival at the Unit, the experimenter confirmed that the volunteer was happy to proceed with their participation in the study. If not, the volunteer was thanked and asked to complete a study payment form to receive remuneration for any main study visits previously attended. If the volunteer was happy to proceed with their participation, the study procedure outlined in Figure 2.3 below was performed on each study day.

Compliance with the requirements for overnight fasting were established and if participants brought in their 24-hour urine sample, this was taken and processed in the sluice room in the Hugh Sinclair Unit and in the laboratory. In cases where more than 8 weeks had elapsed between the participant's screening visit and first study visit, Haemoglobin levels were checked using the Haemocue (according to standardised procedure, involving a small finger prick) in accordance with the revised Hugh Sinclair Unit procedure. Anthropometrics were then recorded. The volunteer then rested for ten minutes before having blood pressure measured.

Participants then underwent the FMD procedure (by which time the volunteer had been resting for ~15 minutes). The FMD measurement involved an ultrasound measure of the brachial artery before and after a 5 minute blood flow restriction. After completion of the FMD procedure, a fasted blood sample was taken by a trained phlebotomist (combined volume of <45mL). Blood samples were processed and stored to be later on analysed for biomarkers indicating increased risk of cardiovascular disease and markers associated with cognitive function and mood as well as compounds of interest in terms of potential

mechanisms of action of flavonoids. The participant was then provided with a standardised study breakfast.

The next phase of the study day took place in the Department of Psychology. Participants were either driven by the experimenter or took their own car. In exceptional circumstances, the second part of the study visit needed to be performed at a later time the same day or during the previous or next day. In this case, participants were met by a researcher in the reception of the Psychology Department.

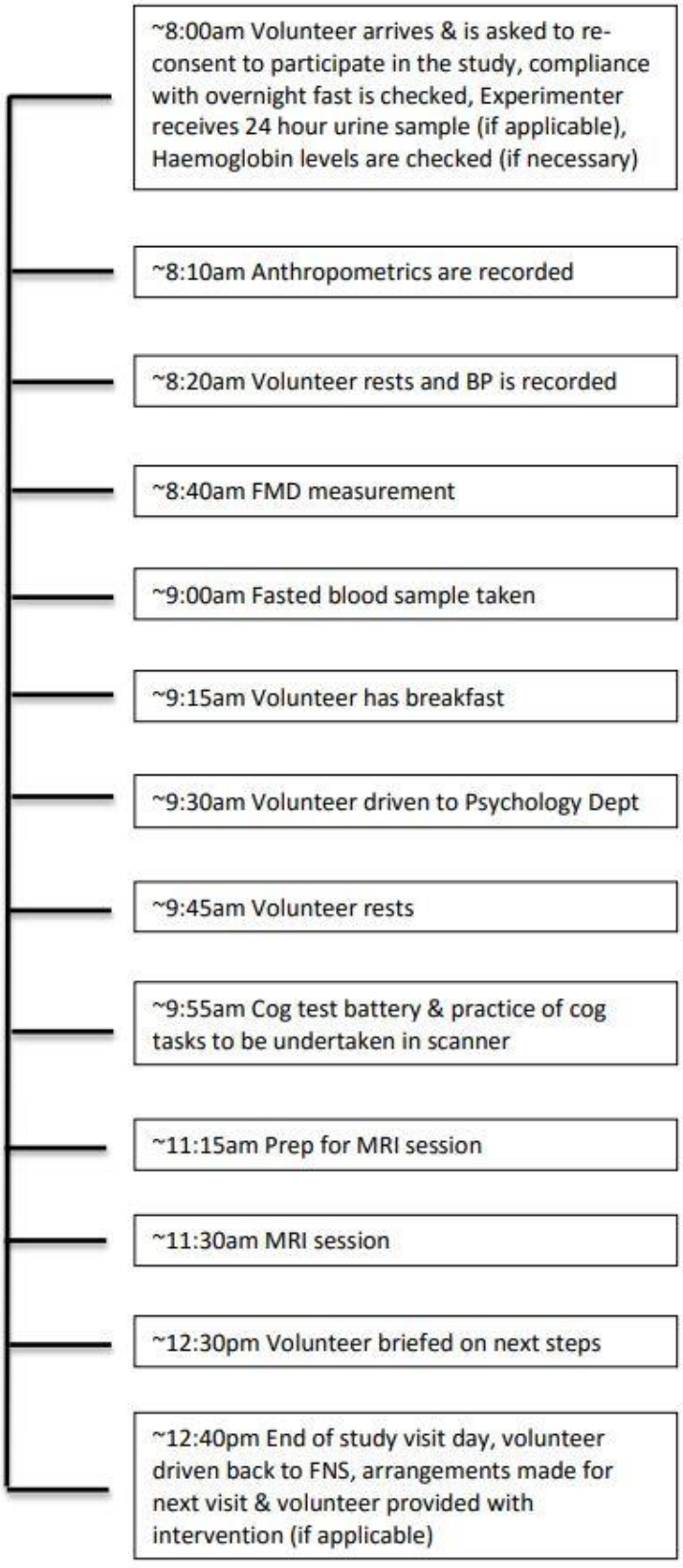


Figure 2. 3 Main study visit timeline

### Phase two of study visit (location: Department of Psychology)

Participants arrived at the reception of the Psychology Department and were escorted to a cognitive testing room, where they had a short rest (~10 minutes) before completing the cognitive test battery. The cognitive test battery included the following tests: Letter Memory Task (testing executive functioning, mainly 'updating'), Rapid Visual Information Processing (RVIP; measuring sustained attention and processing speed), Rey Auditory Verbal Learning Test (RAVLT; measuring verbal learning and memory), Corsi block tapping test (measuring visuospatial working memory), Verbal Fluency test and a Continuous performance task (CPT; measuring sustained attention and inhibitory control). Participants also performed a short set of mood measures (Bond-Lader VAS scale & Spielberger State Anxiety Inventory), all within approximately 1 hour. Volunteers then practiced the cognitive tasks that they would later perform in the MRI scanner (lasting no longer than 15 min in total). Following this, participants were taken to the CINN for their MRI session.

Upon arrival at the CINN, the participant was asked to revisit their initial screening form and to advise the researchers if any of the answers have changed. If so, they were asked to complete a new initial screening form. In accordance with the CINN operating procedure, they were subsequently asked to complete a second screening form in the presence of an appointed person (AP), who checked the answers with them in order to ensure that participants are safe to enter the scanner environment. Copies of the screening forms and consent form were held securely for 10 years by the investigator in the School of Psychology, and in the MRI unit.

Participants were introduced to the scanner environment and were made as comfortable as possible in the scanner. A respiratory belt round their chest and a pulse oximeter on their finger, were used to measure their heart rate via a finger clip, which was explained to them and applied in a way that was comfortable for the participant. Participants also received a button box in order to give responses whilst in the scanner. Participants then underwent MRI scanning whilst completing the two computer based cognitive tasks they had practiced prior to entering the scanner environment. Whilst in the scanner, participants were also asked to complete a short mood measure (Bond-Lader VAS scales and SSAI) up to three times in order to track any mood changes. When not

performing a task, participants were instructed to relax, but remain still throughout each individual scan whilst high-resolution images of their brain, resting blood flow measures, fieldmaps and other calibration scans, as well as diffusion tensor images to highlight white-matter changes were obtained. The scanning session took approximately 1 hour to complete.

Following completion of the scanning session, participants were offered a snack before leaving the Department. At their first study visit (baseline), they were also given the intervention to be taken daily for the next 24 weeks. If participants were not able to take all of the intervention packs necessary for 24 weeks, an additional appointment was made to collect further doses. After the last study visit, participants completed an 'End of study' Questionnaire. Interim visits were scheduled halfway throughout the intervention period, to take anthropometric measurements, complete questionnaires, pick up a further supply of intervention capsules and report any concerns, potential adverse events or ask questions.

### *Intervention Treatment*

The study supplement was a capsule of cocoa-extract containing ~300mg of flavanol per capsule. This supplement was provided by Mars. To ensure double-blinding, both high and low flavanol interventions were coded and encapsulated in opaque capsules which ensured the treatments were indistinguishable as they were identical in taste, texture and appearance. The interventions were matched for macro and micronutrients, a full breakdown of which can be seen in table 2.1 below. Participants were asked to take 3 capsules a day (1 capsule, 3 times a day) for 24 weeks, in addition to their normal diet. In order to monitor compliance, volunteers were asked to record the time capsules were taken in a log book along with any doses of the intervention that were not taken. The high flavanol cocoa extract capsules contained 300mg flavanols per capsule and therefore consumption of 3 capsules a day delivered a dose of 900mg of flavanols a day. The low flavanol cocoa extract capsules contained only 10mg of flavanols per capsule and hence consumption of 3 capsules a day delivered a daily dose of 30mg flavanols.

Table 2. 1. Composition of the cocoa treatment and matched control

| Component | High Flavanol Cocoa | Low Flavanol Cocoa |
|---|---|---|
| Total cocoa flavanols (mg) | 900 | 10 |
| (-)-epicatechin (mg) | 138 | <1 |
| Calories | 100 | 90 |
| Total fat (g) | 1 | 1.2 |
| Saturated fat (g) | 0.6 | 0.6 |
| Cholesterol (mg) | 4 | 4 |
| Total carbohydrates (g) | 14 | 14 |
| Dietary fiber (g) | 2 | 4 |
| Sugars (g) | 8 | 6 |
| Protein (g) | 6 | 6 |
| Caffeine (mg) | 16 | 14 |
| Theobromine (mg) | 172 | 156 |
| Sodium (mg) | 70 | 76 |
| Potassium (mg) | 412 | 512 |
| Calcium (mg) | 174 | 178 |
| Iron (mg) | 4 | 4 |
| Phosphorus (mg) | 208 | 200 |
| Magnesium (mg) | 68 | 64 |
| Zinc (mg) | 2 | 2 |
| Copper (mg) | 0.2 | 0.4 |
| Manganese (mg) | 0.4 | 0.4 |

### *Interim questionnaires & feedback forms*

Any changes in participants' diet and mood were monitored by sending out short questionnaires via email or post to be completed on up to 5 occasions within the 36-week study period.

Feedback forms were given to participants after completion of their first visit day, at their interim visit and upon completion of the final visit of the study. These forms were

completed anonymously and provided feedback from the volunteers on how the study was being run and whether any improvements could be made. A similar form was also given to any volunteers who dropped out of the study in order to see if there were ways the number of drop outs could be reduced.

### *Adverse events*

No adverse effects were expected with the supplement. As a precaution however, participants had the number of the PI and the study mobile phone, which they could in case of adverse effects or general questions. All adverse events were reported through the procedure for adverse events of the Hugh Sinclair Unit of Human Nutrition.

### *Cognitive Function and Mood*

The primary interest of this study was to determine whether 24 weeks of daily supplementation with 900mg cocoa flavanols would result in better cognitive function and self-rated mood compared to following a control intervention. This was assessed using a battery of computerised cognitive tests. E-prime version 2 (Psychology Software Tools, Sharpsburg, PA, USA) was used to display stimuli and record participants responses, with audio presented through noise cancelling headphones. The cognitive battery measured attention, executive function and memory through a number of tasks. The measure of global cognitive function consisted of the primary dependant variable from all six cognitive tasks, whilst the executive function measure consisted of five tasks (Letter Memory, Rapid Visual Information Processing, Corsi Blocks, Continuous Performance Task and Verbal Fluency) and the episodic memory measure consisted of three variables obtained from one task (Rey Auditory Verbal Learning Test). For the measurement of self-assessed mood, volunteers completed the Bond-Lader Visual Analogue Scale (VAS) to determine three different composite measures of mood: sedation, contentment and relaxation, as well as the SSAI to determine self-rated state anxiety. The complete battery was approximately one hour in length.

Scores from each cognitive test were converted to Z scores and then combined to generate several composite measures: global cognitive function, executive function, episodic memory. These were then entered as outcomes in the statistical analysis.

Responses with a reaction time <200ms were excluded as is standard practice to discount responses that may have been to a prior stimulus.

### *Blood samples*

A fasting blood sample (total <45mL) was collected at the beginning of each study visit. Specifically, two 4mL EDTA tubes, two 9mL serum separator tubes and two 6mL lithium heparin tubes were used to collect blood for analysis of biomarkers associated with cognitive function as well as those associated with increased risk of cardiovascular disease and compounds of potential interest in terms of mechanisms of action of flavonoids. Samples were centrifuged and stored at -80°C.

A number of clinical chemical parameters were determined using enzyme-based colorimetric tests on an ILAB600 clinical chemistry analyser (Instrumentation Laboratories Ltd., Warrington, UK). Glucose, triglycerides, total cholesterol, HDL cholesterol and non-esterified fatty acids (NEFA) were measured. LDL cholesterol was subsequently calculated using the triglyceride, total and HDL cholesterol levels measured. Insulin levels were determined using the Crystal Chem Insulin ELISA (Crystal Chem Inc., USA) according to manufacturer's protocol.

Mature BDNF levels were analysed in serum samples using the Biosensis Mature BDNF Rapid ELISA kit (Biosensis Pty Ltd, Thebarton, Australia) according to the manufacturer's instructions. Preliminary tests were carried out in order to check for the optimal dilution which was found to be 1 in 100. Standards and samples were assayed in triplicate. Similarly, Cortisol levels were analysed in serum using the Crystal Chem Cortisol ELISA (Crystal Chem Inc., USA) and serum Insulin levels using the Crystal Chem Insulin ELISA kit (Crystal Chem Inc., USA), according to manufacturer's kit instructions.

### *Flow mediated dilation (FMD)*

A sphygmomanometric cuff was placed just under the elbow and inflated using a pressure of 220 mm Hg for 5 minutes to induce a restriction of blood flow in the brachial artery. The pressure cuff was then released to induce reactive hyperemia and hence vasodilatation of the brachial artery. The procedure uses an ECG-gated trigger and image-grabbing software (MIA-llc) to collect images at 0.25 frames/s. Doppler-derived velocity

was measured for 1 min prior to commencing each FMD measurement. Baseline images were taken for 1 min, after which the blood pressure cuff inflated to 220 mmHg to occlude blood flow. After 5 min of occlusion, the pressure was rapidly released, allowing reactive hyperemia to occur; measurement collection continued for 5 min post release. Analysis of the images was performed using wall-tracking software (MIA-llc). Image files were analysed by a single researcher who remained blinded to the measurement details, and peak diameter was defined as the largest diameter obtained after the occlusion was released. FMD response was calculated using change from baseline to peak diameter divided by baseline and reported as a percentage value. Velocity analysis was performed over a minimum of 5 cardiac cycles and averaged, then converted to flow by multiplying by the cross-sectional area of the artery.

### *Location*

The study took place on Whiteknights campus of the University of Reading in both the Hugh Sinclair Unit of Human Nutrition in the Department of Food and Nutritional Sciences and in the School of Psychology and Clinical Language Sciences at the University of Reading.

1) Hugh Sinclair Unit of Human Nutrition:

The Head of school and member of staff concerned (Principle Investigator) were responsible for the safety of all procedures undertaken throughout the study. Venous blood samples were taken only by a) medically qualified staff, b) an approved member of staff following attendance and assessment on an accredited course or c) a member of staff who has been assessed by an approved trainer.

Health & Safety considerations at the Hugh Sinclair Unit of Human Nutrition:

•All necessary acts and procedures were followed stringently throughout the course of a human study.

• Only members of the research team are able to enter the vascular suites during the study day period.

• Each member of the research team had been previously trained in health and safety procedures of the University Department, and have also undertaken a Good Clinical Practice (GCP/ICPH) course.

• The Nutrition Unit is supported by a team of qualified first aiders in the case of a medical emergency or accident.

• Formal SOP's exist for various procedures that take place in the unit, e.g. the SOP for the correct disposal of urine in the sluice room.

• Researchers who use the kitchen and prepare any food for volunteers are qualified in basic food hygiene and safety. Hand washing is a strict and compulsory requirement whilst preparing any food in the kitchen.

• There is a designated fire safety warden who is trained and responsible for directing staff and students out of the building via the nearest safety exit.

• All adverse events were documented using the Unit's case forms and procedures.

2) School of Psychology and Clinical Language Sciences

Cognitive testing took place in the School of Psychology and Clinical Language Sciences and a brain scan using functional magnetic resonance imaging (FMRI) will be performed at the Centre for Integrative Neuroscience and Neurodynamics (CINN) housed within the School of Psychology and Clinical Language Sciences. During MRI scanning, all protocols and standard procedures were followed at CINN in order to minimize risk to participants in accordance with the Risk Assessment completed for MRI at CINN.

### *Sample Size and Statistical Analysis*

A power calculation determined that in order to detect an effect of size 0.25, with an α error probability of 0.05 for a study design with two parallel groups and three repeated measurements, a total sample of 62 volunteers would be required to achieve power (1 - β error probability) of 0.80. The power calculation was based on global cognition as the primary outcome from previous research in our group. A total of 80 volunteers were recruited and randomised to allow for drop-outs and non-compliance.

Primary and secondary outcomes were analysed per protocol using SPSS v25 (IBM, Armonk, NY, USA). LMM with baseline as a covariate was carried out for each endpoint. Intervention group and Visit were fixed factors in the model. Bonferroni corrected pairwise comparisons were examined regardless of the significance of the overall F test statistic.

*Recruitment*

A total of 80 healthy older adults were recruited via existing volunteer databases and public advertisements. Volunteers were recruited through the Nutrition Unit's volunteer database (approx. 2000 volunteers that have participated in studies previously run in the Unit) and from the Ageing Research Panel at the School of Psychology and Clinical Language Sciences. The panels have a range of volunteers who have been tested previously on single or multiple occasions. They were contacted using previously supplied contact information, via telephone, post or email, offering them the opportunity to participate in the current research project. They received the study information sheet by post or email before attending the screening session at the University. If they were interested in taking part, volunteers were given the opportunity to ask any questions about the study and were initially screened for eligibility over the phone and during the screening visit, before giving informed consent.

Additionally, new volunteers were recruited via emails circulated within the University of Reading and within public groups that explicitly agree to distribute these, as well as via advertisements placed in local newsletters/leaflets and posters placed within the University as well as public locations in the local community. Leaflets were distributed in public locations or at public events. After initial expression of interest, participants were recruited as described above. If participants are not able to perform the MRI part of the experiment for medical reasons, they were still allowed to take part and perform all other elements of the study.

Before the study visit, volunteers were randomised to one of the two intervention groups (high or low flavanol cocoa extract) using software (Minim, Evans et al., 2017) based on the method of minimisation, balancing the two groups in terms of sex and age. Figure 4 below outlines the recruitment, screening, randomisation and fate of all study participants.

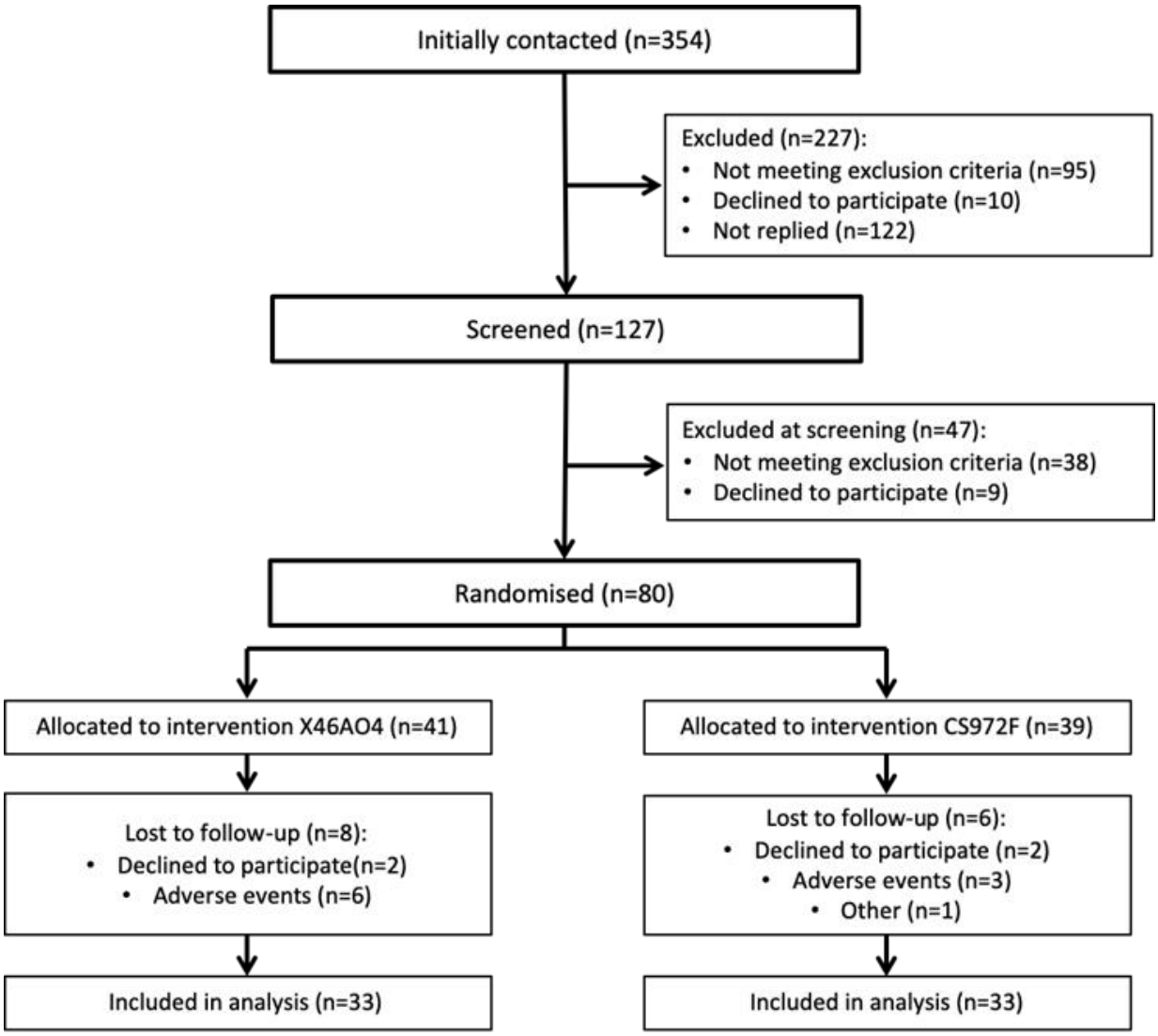


Figure 2. 4 Recruitment, screening, randomisation and fate of study participants

## Results

There was no significant difference between the two intervention groups ($p > 0.05$) for any of the baseline characteristics measured. Table 2.2 below shows the baseline characteristics of the population sample.

Table 2. 2 Baseline characteristics of the two arms

| | Placebo | Cocoa |
|---|---|---|
| **Age (on visit 1)** | 66.97 ± 0.80 | 66.94 ± 0.79 |
| **Height (m)** | 1.69 ± 0.02 | 1.70 ± 0.02 |
| **Weight (kg)** | 70.90 ± 2.08 | 71.82 ± 2.25 |
| **BMI (kg/m$^2$)** | 24.40 ± 0.48 | 24.31 ± 0.48 |
| **Waist Circumference (cm)** | 85.34 ± 1.79 | 85.59 ± 1.90 |
| **Hip Circumference (cm)** | 100.99 ± 1.12 | 101.17 ± 1.04 |
| **WHR** | 0.84 ± 0.01 | 0.85 ± 0.02 |
| **Systolic BP (mmHg)** | 127.90 ± 2.79 | 127.69 ± 2.11 |
| **Diastolic BP (mmHg)** | 72.69 ± 1.48 | 72.36 ± 1.03 |
| **Heart Rate (bpm)** | 58.48 ± 1.74 | 60.79 ± 1.55 |
| **Chol (mmol/L)** | 5.84 ± 0.16 | 5.86 ± 0.18 |
| **TRIG (mmol/L)** | 1.01 ± 0.06 | 1.13 ± 0.09 |
| **HDL (mmol/L)** | 1.80 ± 0.07 | 1.76 ± 0.07 |
| **LDL (mmol/L)** | 3.59 ± 0.14 | 3.60 ± 0.16 |
| **Glucose (mmol/L)** | 5.52 ± 0.07 | 5.46 ± 0.09 |
| **CRPUS (mg/L)** | 1.72 ± 0.76 | 1.77 ± 0.43 |
| **NEFAHR (mmol/L)** | 548.87 ± 32.26 | 578.33 ± 40.63 |
| **MMSE** | 28.24 ± 0.23 | 28.58 ± 0.26 |
| **NART** | 41.41 ± 0.69 | 40.41 ± 1.16 |
| **Block Design** | 35.67 ± 1.20 | 33.09 ± 1.36 |
| **Years in Education** | 14.78 ± 0.56 | 15.00 ± 0.57 |
| **HFI (mg)** | 907.79 ± 62.78 | 951.73 ± 79.11 |
| **PASE** | 165.20 ± 11.58 | 163.31 ± 10.68 |

*Values represent Mean ±SE*

*BMI Body Mass Index; WHR Waist to Hip Ratio; Chol Total Cholesterol; HDL High Density Lipoprotein; LDL Low Density Lipoprotein; CRPUS Ultra Sensitive C-Reactive Protein; NEFAHR Non-Esterified Fatty Acids measured with the HR enzymatic colorimetric assay; MMSE Mini Mental State Examination; NART National Adult Reading Test; HFI Habitual Flavonoid Intake; PASE Physical Activity Scale for the Elderly.*

*Cognitive Function*

## Global Cognitive Function

Main effects of visit (F(2, 66.32) = 3.01, p = 0.056) and intervention (F(1, 66.10) = 3.62, p =0.062) on global cognitive function approached significance, which appears to be driven by improved performance at follow up compared to baseline (mean difference = 0.095, df = 65.32, p = 0.075). Better performance overall in the placebo group (mean = 0.11) compared to the active treatment (mean = - 0.11) respectively (mean difference = 0.22, df = 66.10, p = 0.062), but it was not significant. There was no significant interaction between visit and intervention, however, an examination of the pairwise comparisons revealed a trend towards better global cognitive function at post intervention (mean difference = 0.22, df = 66.50, p = 0.082) and follow up (mean difference = 0.23, df = 66.00, p = 0.073) for the placebo group compared to the active treatment group.

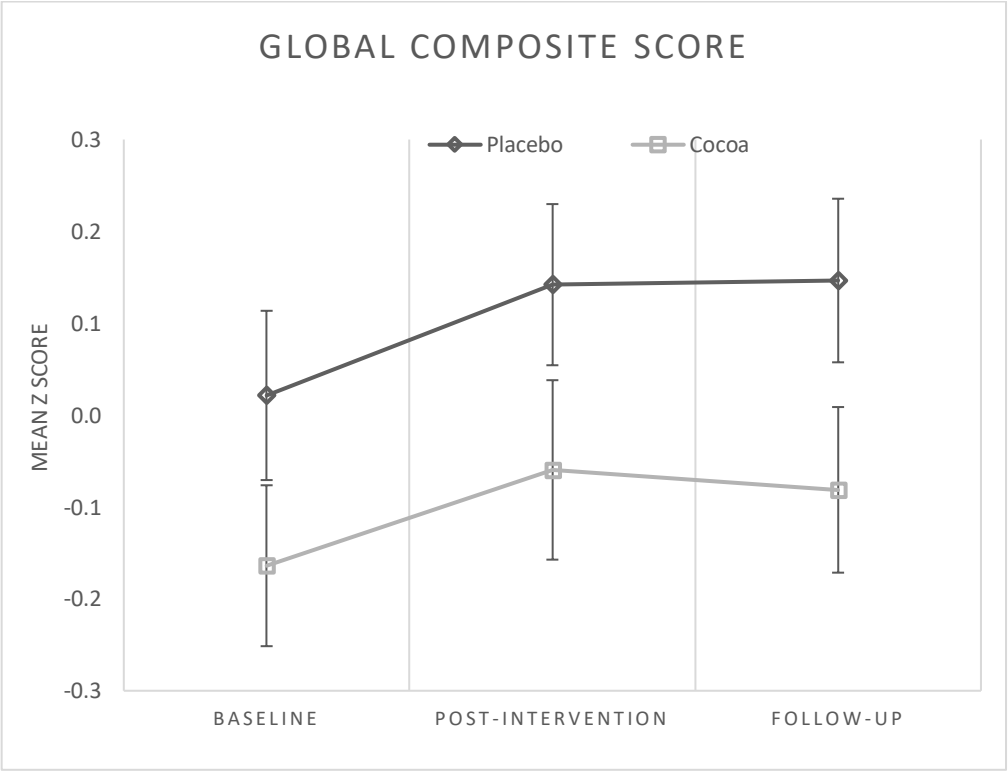


Figure 2. 5 Mean composite scores for global cognitive function at baseline, post-intervention (24 weeks) and after a 12 week wash-out.

## Executive Function

There was a trend towards a main effect of intervention (F(1, 66.02) = 3.02, p = 0.087) on executive function with greater performance in the placebo group (mean = 0.10) compared to active treatment (mean = -0.10).

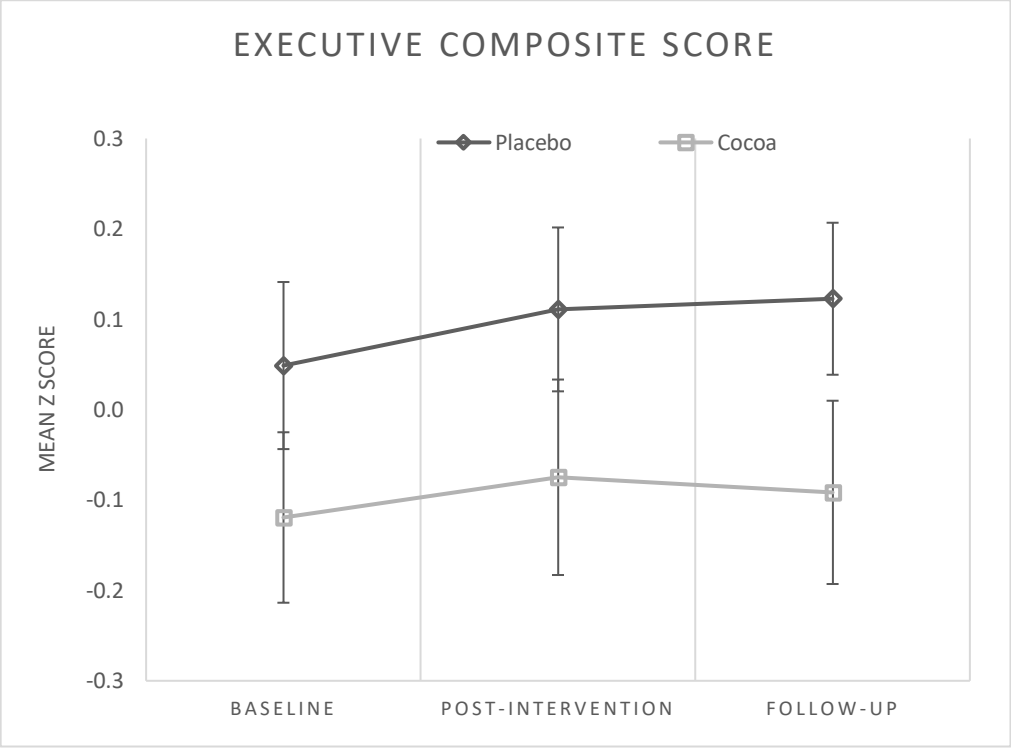


Figure 2. 6 Mean composite scores for executive function at baseline, post-intervention (24 weeks) and after a 12 week wash-out.

## Memory Function

There was a significant main effect of visit on memory function (F(2, 65.85) = 10.43, p < 0.001) with improved performance at post intervention (mean = 0.13) and follow up (mean = 0.086) compared to baseline (mean = - 0.217; mean difference = 0.35, df = 65.94, p < 0.001 and mean difference = 0.30, df = 66.00, p = 0.001 respectively).There was no main effect of intervention on memory function and the interaction between visit and intervention was not significant.

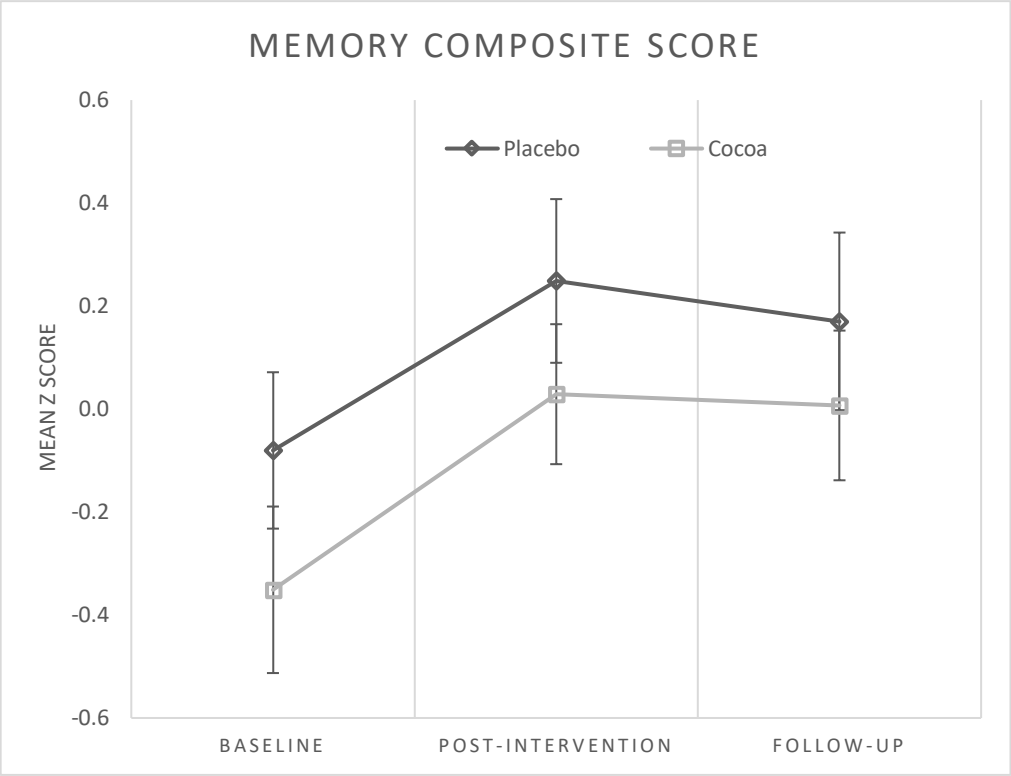


Figure 2. 7 Mean composite scores for memory function at baseline, post-intervention (24 weeks) and after a 12 week wash-out.

Table 2. 3.Average scores for individual cognitive test battery tasks

| | **Placebo** | | | **Cocoa** | | |
|---|---|---|---|---|---|---|
| | Baseline | Post-Intervention | Follow-Up | Baseline | Post-Intervention | Follow-Up |
| **RAVLT Overall score** | 65.13 ±11.57 | 69.19 ±11.90 | 69.26 ±12.80 | 62.95 ±12.12 | 65.89 ±10.52 | 65.74 ±10.94 |
| **Letter Memory Task (%correct)** | 60.90 ±17.43 | 66.54 ±19.76 | 63.07 ±17.64 | 54.73 ±15.85 | 60.80 ±14.37 | 59.28 ±15.40 |
| **RVIP (%correct)** | 92.14 ±8.86 | 88.48 ±16.03 | 92.92 ±11.73 | 90.79 ±8.78 | 88.90 ±16.47 | 87.43 ±12.28 |
| **Corsi Blocks (%seq. correct)** | 53.54 ±12.23 | 54.78 ±13.73 | 53.69 ±10.67 | 49.47 ±9.51 | 48.86 ±10.49 | 49.91 ±10.92 |
| **Verbal Fluency Test Overall score** | 59.23 ±12.04 | 61.06 ±10.35 | 59.39 ±9.81 | 58.95 ±13.92 | 58.19 ±12.16 | 58.67 ±11.95 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **CPT (%targets correct)** | 88.46 ±10.21 | 89.31 ±9.46 | 88.38 ±10.02 | 88.46 ±8.87 | 87.17 ±8.37 | 88.18 ±8.92 |

*Values indicate mean ± SD*

### *Mood*

#### State Anxiety

There were no significant main effects, or significant interaction between intervention and visit for self-assessed state anxiety. However, pairwise comparisons revealed a trend towards a difference between the two groups with greater state anxiety in the cocoa group (mean difference = 1.545, df = 66, p = 0.075).

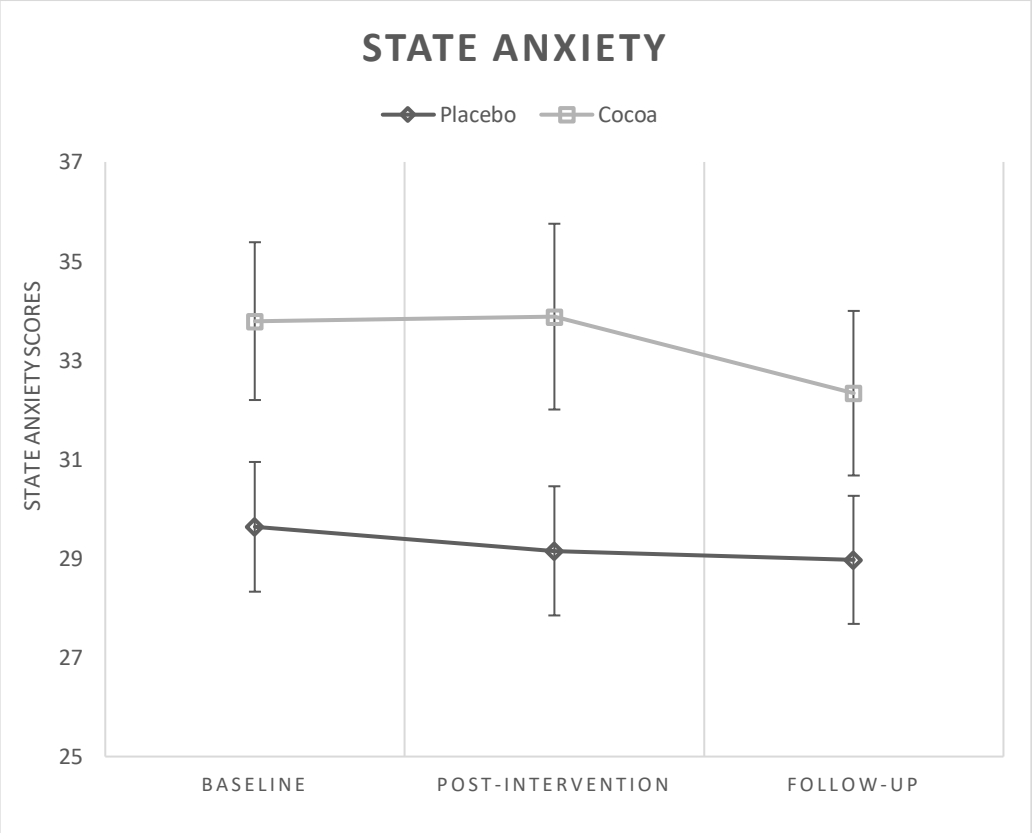


Figure 2. 8 Mean composite scores for self-assessed state anxiety on the 3 visits: baseline, post-intervention (24 weeks) and after a 12 week wash-out.

## Sedation

Whilst there were no significant main effects or a significant interaction between intervention and visit, pairwise comparisons revealed a trend towards a difference between the two groups at baseline with greater sedation in the Cocoa group (mean difference = 67.31, df = 65, p = 0.077). Also, within the cocoa group, self-reported sedation was significantly reduced at follow-up compared to baseline (mean difference = -45.47, df = 64.09, p = 0.048).

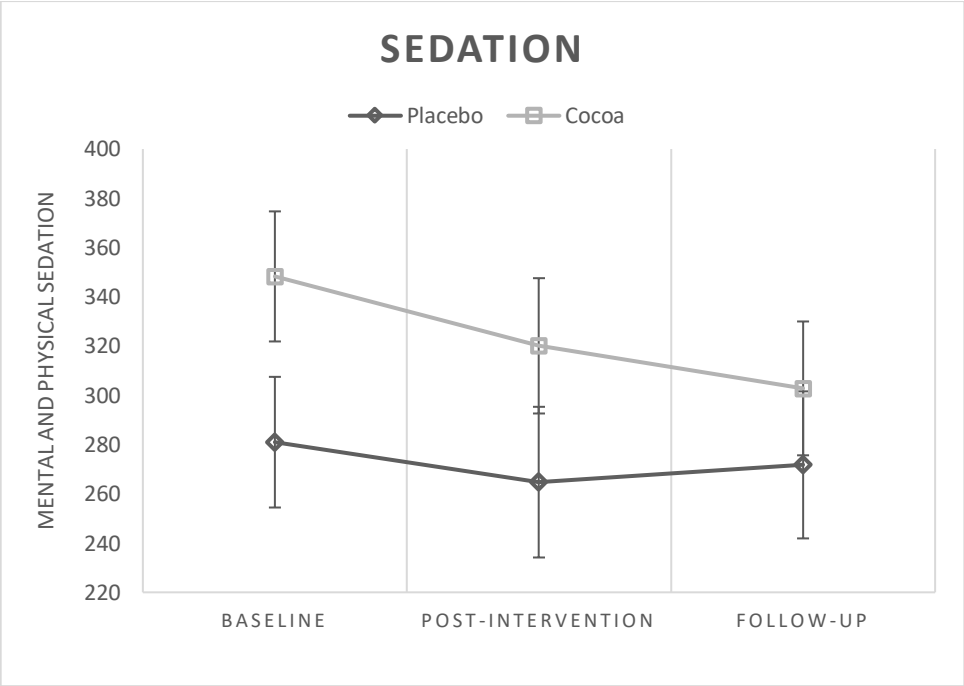


Figure 2. 9 Mean composite scores for mental and physical sedation on the 3 visits: baseline, post-intervention (24 weeks) and after a 12 week wash-out.

## Contentment

There were no significant main effects or an intervention by visit interaction. However, pairwise comparisons revealed a trend towards reduced contentment post-intervention (main difference = -22.012, df = 64.94, p = 0.084) and follow-up (main difference = -26.17, df = 66.19, p = 0.084) compared to baseline in the group consuming the cocoa treatment.

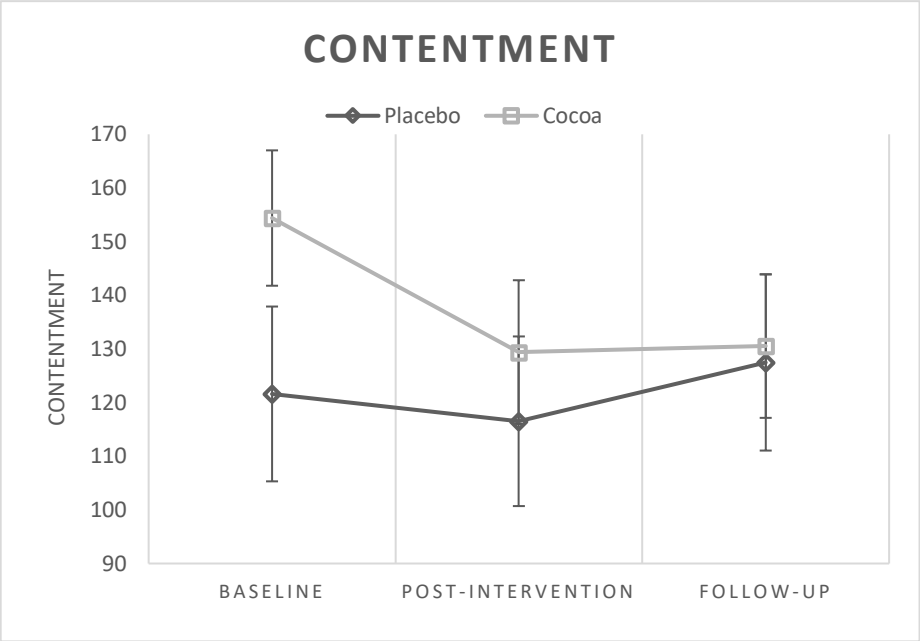


Figure 2. 10 Mean composite scores for Contentment on the 3 visits: baseline, post-intervention (24 weeks) and after a 12 week wash-out.

## Relaxation

There were no significant main effects or a significant interaction between intervention and visit.

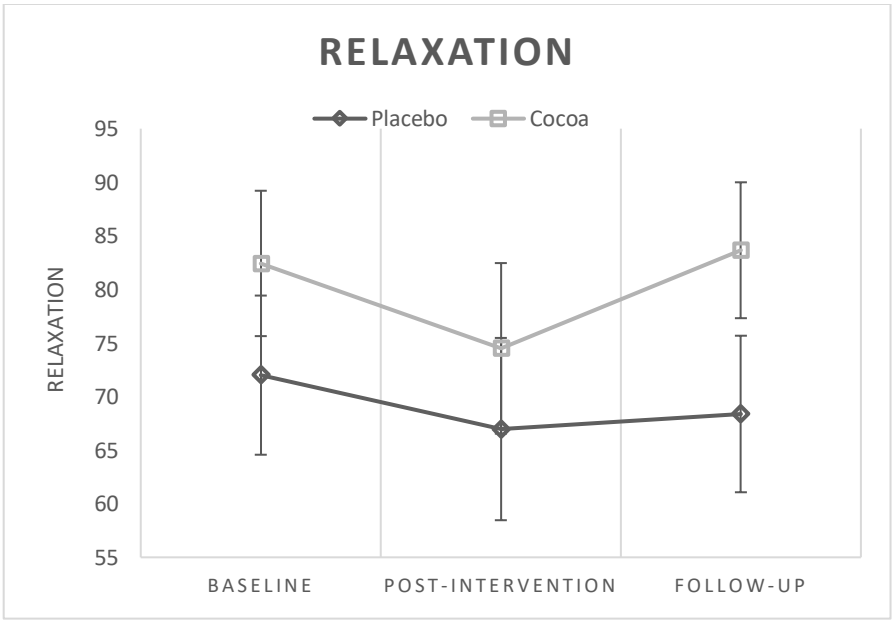


Figure 2. 11 Mean composite scores for Relaxation on the 3 visits: baseline, post-intervention (24 weeks) and after a 12 week wash-out.

*Flow Mediated Dilation, Blood Pressure and Heart Rate*

There was a trend towards a significant intervention by visit interaction (F(2, 59.26) = 2.59, p = 0.084) driven by improved FMD response post-intervention (mean = 4.68) compared to baseline (mean = 3.30) in the group consuming the cocoa treatment (mean difference = 1.38, df = 59.77, p = 0.079). There were no significant main effects or an intervention by visit interaction for systolic or diastolic blood pressure.

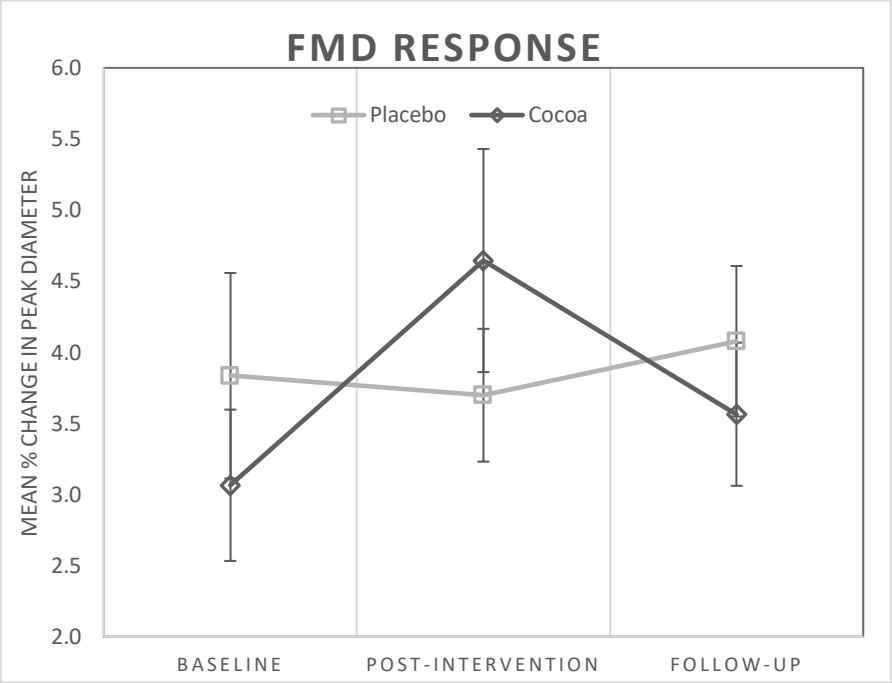


Figure 2. 12 Mean change FMD response measured as change in peak diameter

Whilst there were no significant main effects or an intervention by visit interaction for pulse pressure, there was a trend towards a significant interaction between intervention and visit for heart rate (F(2, 65.14) = 2.99, p = 0.057) with pairwise comparisons revealing reduced heart rate post-intervention (mean = 58.40) compared to baseline (mean = 60.79) in the cocoa group (mean difference = -2.39, df = 64.83, p = 0.019).

*Biochemical parameters*

## Cortisol

There were no significant main effects or a significant interaction between intervention and visit. A noticeable yet not significant difference was observed at 24 weeks for the cocoa group compared to placebo (pairwise comparisons: mean df=0.496, df=62.677, p=0.316).

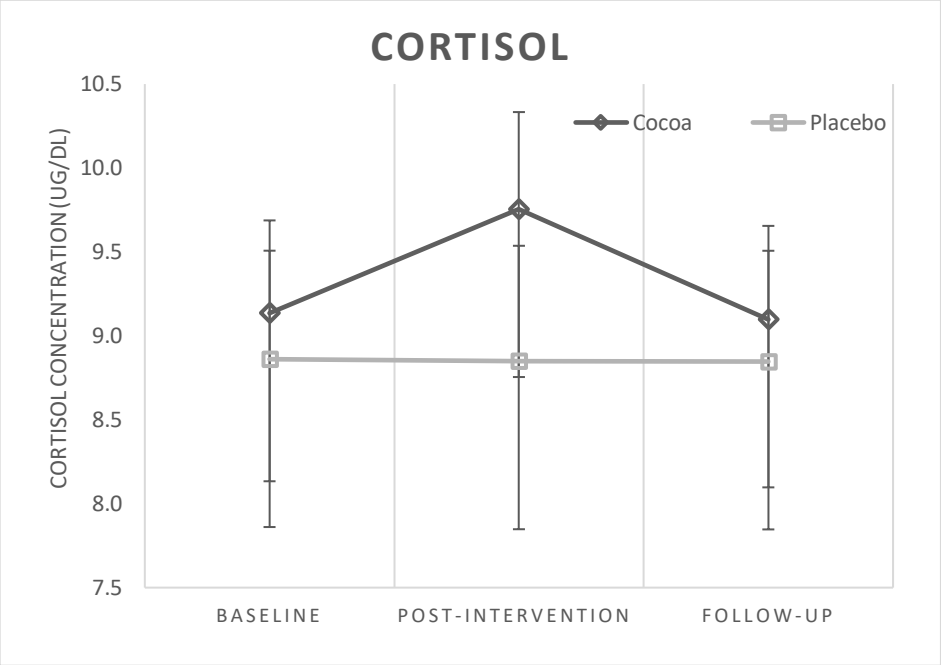


Figure 2. 13 Serum cortisol levels at baseline, post-intervention (24 weeks) and after a 12 week wash-out

## BDNF

There were no significant main effects or a significant interaction between intervention and visit, however, pairwise comparisons revealed a trend towards reduced BDNF concentration (mean difference = -1.44, df = 63.93, p =0.096) post-intervention (mean = 28.66) compared to baseline (mean = 30.10) for the cocoa group compared to placebo (F(2, 62.89) = 2.43, p = 0.096).

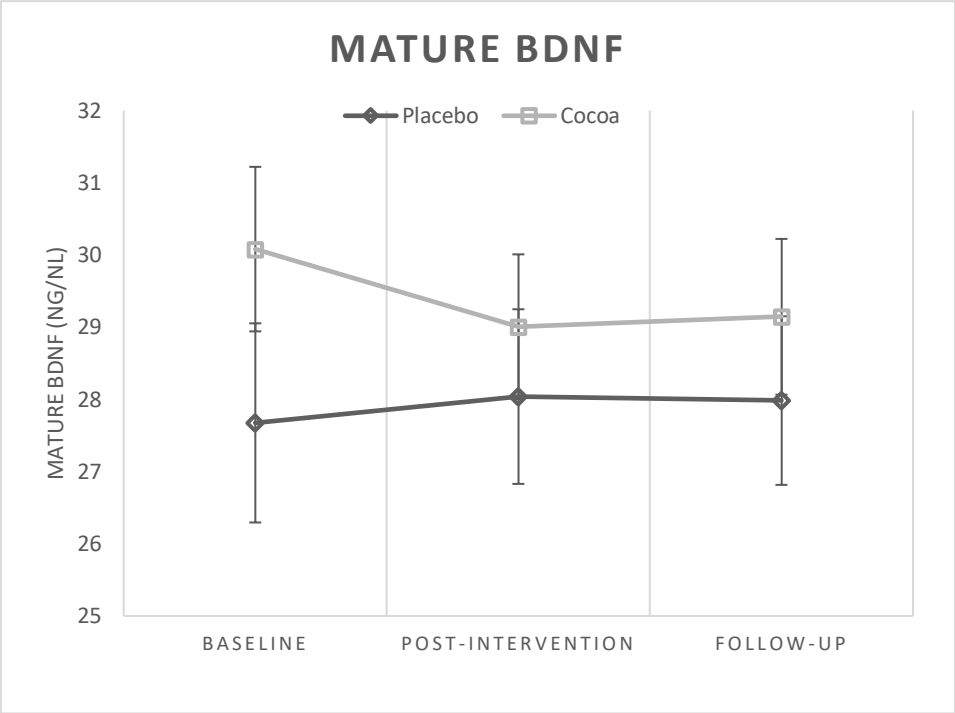


Figure 2. 14 Plasma BDNF levels at baseline, post-intervention (24 weeks) and after a 12-week washout

## Glucose and Insulin

There was a significant main effect of visit (F(2, 61.26) = 4.53, p = 0.015) with reduced glucose concentration (mean difference = -0.11, df = 61.52, p = 0.012) at follow up (mean = 5.38) compared to baseline (mean = 5.49). Pairwise comparisons revealed this was likely driven by a significant reduction (mean difference = -0.16, df = 62.28, p = 0.018) at follow-up (mean = 5.37) compared to baseline (mean = 5.52) for the placebo group (F(2, 61.05) = 4.24, p = 0.019). There were no significant effects of, or interactions between, visit and intervention, for the serum insulin concentrations. An increase in serum insulin was observed at follow-up for the cocoa group, compared to placebo, but it was not significant (F[1,62.1]=0.24, p=0.240).

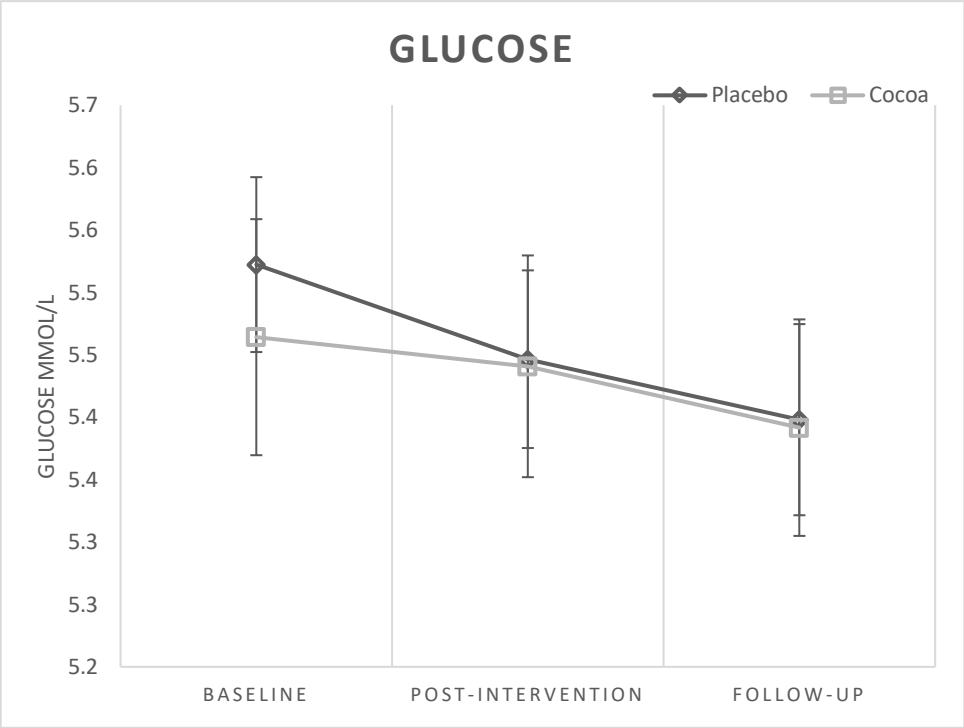


Figure 2. 15 Serum glucose levels at baseline, post-intervention (24 weeks) and after a 12 week wash-out

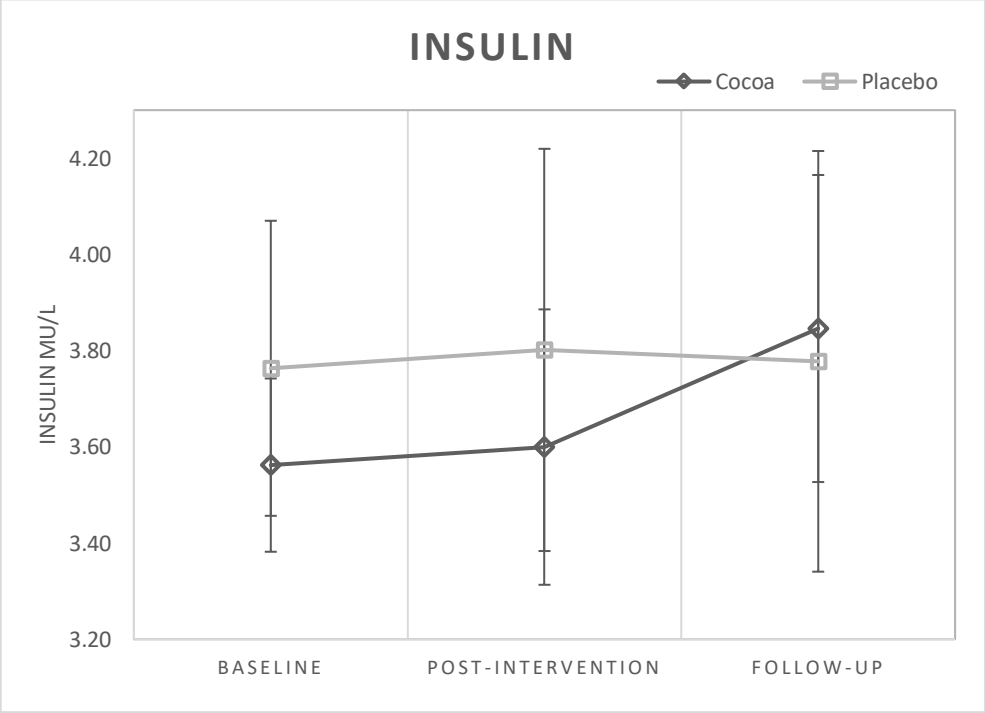


Figure 2. 16 Serum insulin levels at baseline, post-intervention (24 weeks) and after a 12 week wash-out

### C-Reactive Protein

Whilst there were no significant main effects or an intervention by visit interaction, pairwise comparisons revealed a significant reduction in CRP (mean difference = -1.05, df = 64.82, p = 0.042) at follow-up (mean = 1.00) compared to post intervention (mean = 2.05), for the placebo group (F(2, 65.68) = 3.75, p = 0.029).

### NEFA

There was a significant main effect of visit (F(2, 61.85 = 3.21, p = 0.047) with a significant reduction in NEFA (mean difference = -53.14, df = 60.10, p = 0.059) post intervention (mean = 515.43) compared to baseline (mean = 568.56). In addition, whilst there was no significant interaction between intervention group and visit, pairwise comparisons revealed a significant reduction in NEFA (mean difference = -71.05, df = 57.69, p = 0.046) at follow-up (mean = 518.30) compared to baseline (mean = 589.35) for the cocoa group (F(2, 63.94) = 3.50, p = 0.036).

### Cholesterol, Triglycerides, HDL and LDL

There were no significant main effects or an intervention by visit interaction for these parameters.

## Discussion

In this double-blind, randomised, controlled, parallel arm chronic intervention trial we endeavoured to assess the effects of daily consumption of a cocoa flavanol rich supplement for 24 weeks on cognitive performance, self-rated mood and biochemical parameters related to those effects and their potential underlying mechanisms, on healthy and cognitively intact older adults. We found that chronic cocoa supplementation can improve vascular function, seen as an increase in FMD response and decrease in heart rate, as well as decrease in serum NEFA levels. We further observed differences in sedation and contentment, with greater scores in the active treatment arm. Although we failed to demonstrate any improvements in any of the cognitive outcomes, we saw an overall increase in cognitive performance for both treatment conditions, which could be attributed

to another cocoa constituent present in both treatments. The theory that those effects might be attributed to the methylxanthines, such as theobromine and caffeine that naturally occur in cocoa and were present in both treatment capsules, seems unlikely as discussed below, however we have no evidence to support either of those bases.

Our findings are in line with those of previous studies reporting beneficial effects of cocoa flavanol treatments on endothelial function in healthy adults [88-91], as well as individuals with hypertension [81] and mind cognitive decline [19], following chronic or sub-chronic daily supplementation. The significant difference in serum NEFA that we observed at follow-up compared to baseline for the cocoa group might be supportive of the theory that flavonoid rich cocoa can improve lipemia induced endothelial dysfunction [92]. Such improvements have previously been reported following acute supplementation of high flavanol cocoa powder, though the authors claimed that changes in triglycerides or free fatty acids do not represent mechanisms whereby cocoa flavanols exert endothelial benefits [92]. We speculate that this might not be the case with chronic supplementation, since our data showed reduced serum NEFA at follow-up, and not post-intervention, compared to baseline, but this requires further investigation. The fact that our data showed a reduction in heart rate and increase in FMD response at post-intervention, as well as a significant reduction in serum NEFA at follow-up, confirms the biological activity of the cocoa flavanol treatment, which would have otherwise been questioned due to the lack of any significant cognitive effects. Though these physiological effects are positive, we have not been able to establish whether they underlie any improvements in brain function. Better and more clinically significant effects of cocoa flavanols on the vascular endothelium would have likely been seen in subjects in high risk of heart disease, such as patients with hypertension, as reported by Grassi and colleagues [81].

Other parameters related to cardiovascular health and the risk of cardiovascular disease measured in this study were metabolic markers such as fasting serum insulin and glucose levels. Although we failed to demonstrate any significant effects of the cocoa treatment on fasting glucose and insulin, there is a body of evidence suggesting that cocoa flavanol intake, in doses similar to our intervention treatment, has beneficial effects on fasting glucose, fasting insulin and HOMA-IR (for meta-analysis see Lin and colleagues [93]). It is likely that our data did not show any effects on those metabolic parameters due to the remarkably good health status of our participants. To support that, Grassi and colleagues

[81] have reported improvements in insulin sensitivity following 15 days of dark chocolate supplementation in patients with hypertension. Similarly, Desideri and colleagues [19] showed evidence of beneficial effects of cocoa flavanol supplementation daily for eight weeks on insulin resistance in patients with MCI, which, further, mediated improvements in cognitive function. It is, therefore, speculated that such effects might be more noticeable in patients with higher risk of cardiometabolic conditions, and possibly individuals with diabetes mellitus.

In addition, we showed that self-reported sedation was significantly reduced at follow-up compared to baseline for the cocoa arm, and a non-significant trend towards a difference between the two treatment conditions, with overall greater scores in the cocoa arm. This is in agreement with previous research in our lab that showed significant acute increases in the change in the self-rated mental and physical sedation after the administration of a grapeseed based flavonoid-rich drink (see Chapter 3). However, this should be interpreted with caution as the overall interaction was not significant and the decrease could be due to greater reported sedation at baseline in this arm. We also observed a trend towards reduced contentment at post-intervention and follow-up, compared to baseline, with overall higher contentment in the cocoa arm. This may be suggestive of beneficial effects of flavonoids on mood and perhaps clinical symptoms of mood disorders such as depression and anxiety. Supportive of this basis, in their recent research, Pase and colleagues [94] showed that 30 days of daily cocoa flavanol supplementation (500mg total polyphenols) resulted in significant increases in self-rated calmness and contentment. In the same study, such effects were not established following an acute dose of cocoa flavanols, implying that cocoa flavanol treatments might be more effective in the long term.

Although there were no significant effects of the cocoa treatments on mood, we saw a noticeable but not significant increase in serum cortisol at post intervention for the cocoa arm, which appears to be in agreement with the slight difference in the overall self-rated state anxiety for the cocoa group. However, those changes are not of clinical significance or enough to reject the hypothesis that flavonoids can have anxiolytic effects, resulting in improvements in self-rated mood. Given their known ability to cross the BBB and wield modulatory influence on $GABA_A$ receptors [95, 96], we are inclined to believe that this is a more likely mechanism behind their anxiolytic effects and not by causing

changes in cortisol response. However, further clinical trials are required to investigate those effects on mood from a neurochemical point of view, to better understand the mechanisms underlying them. In addition, given that our population sample were in good health, both mental and physical, such mood effects may have been hard, or impossible to detect. As such, further investigation in subjects with clinical anxiety or other mood disorders, may reveal more about the potential mood enhancing effects of cocoa flavonoids.

Consistent with Crews et al., Francis et al., and Pase et al. [21, 43, 97], we failed to demonstrate any effects of the cocoa treatment on cognitive performance. However, given that there is a growing body of evidence suggesting that cocoa flavanol supplementation does have effects on cognitive function (as reviewed by Scholey et al., and Barrera-Reyes et al. [49, 77]), we speculate that our results might be explained by the fact that our sample was high-functioning (average baseline MMSE scores 28.2 and 28.58 out of 30 for the placebo and cocoa group respectively). Therefore the cognitive tasks that we used might not have been sensitive enough. As such, investigating the effects of the cocoa flavanol treatment in patients with lower baseline cognitive status, or even with MCI, may be more suitable. Indeed, Desideri et al., [19] showed that chronic supplementation of cocoa flavanol resulted in improved cognitive performance in subjects with MCI, although there is no indication as to how that would compare with healthy and cognitively intact subjects. Future research should aim to address this question by investigating the neurocognitive effects of cocoa flavanol on MCI subjects and their healthy controls. In addition, whilst there were no clear beneficial effects on global cognitive function for either of the two treatments, both led to improved performance. Considering performance does not continue to increase substantially between post-intervention and follow-up, it is unlikely that this effect is due to practice.

The fact that both interventions resulted in improved cognitive function over time is encouraging and suggests a role of cocoa in improving cognitive performance. However, it also implies that effects may not be driven by the flavanol content but, rather, an active ingredient common to both interventions. Both the placebo and active treatment contained methylxanthines (caffeine and theobromine), a class of stimulants that could account for the cocoa-induced cognitive benefits in this study. Smit and colleagues [51] have previously substantiated the psychopharmacological properties of chocolate

methylxanthines. They showed that chocolate methylxanthines (theobromine and caffeine) are the cocoa constituents accountable for the improvements in cognitive performance and mood, but they used higher theobromine doses (approximately 100mg higher than our interventions). On that basis, one could argue that higher methylxanthine doses are required to exert cognitive benefits. However, Sansone and colleagues [41] in their recent research showed that similar doses of cocoa methylxanthines and flavanols as our treatments, taken together, can have beneficial effects on physiological parameters related to cardiovascular function. Even though we cannot extrapolate any conclusions on cognitive performance from those results, the cardiovascular effects confirm the biological activity of those doses. Whether those effects could coincide with improvements in cognitive performance or not, needs to be further investigated, since our results failed to demonstrate any signs supportive of that theory. It is worth noting, that according to Sansone and colleagues, those effects appeared to be enhanced when flavanols and methylxanthines were taken together, suggesting a synergistic effect between methylxanthines and flavanols [52]. Said effects were acute, therefore it is speculated that cocoa methylxanthines and flavanols may have more pronounced effects when they are in the circulation, which is in line with several studies that have reported evidence of acute effects of cocoa flavanols on cognitive function [20, 22, 24, 25, 27, 41]. As such, further investigation is required to establish the effects of methylxanthines on cognitive and vascular function, as well as the potential interactions of methylxanthines with flavanols in synergistically improving vascular function, and indirectly, cognitive function as a result, a hypothesis that seems generally accepted but cannot be supported by our data. Since methylxanthines have been linked with beneficial long-term effects in Aβ peptide pathology and, therefore the progression of Alzheimer's disease in animal models [98], future research should aim to explore the clinical effects of methylxanthines in relation to these pathways.

Our results contradict those of Neshatdoust and colleagues who found significant increases in serum BDNF following 12 weeks of cocoa flavnol supplementation. This increase in serum BDNF appeared to coincide with significant improvements in global cognition, suggesting that serum BDNF could potentially be used as a biochemical marker of cognitive function [23]. Considering that Neshatdoust et al. recruited study participants from the same cohort as our study (participants from the greater Reading area from the

University of Reading patient database), of similar age and health and cognitive status, there is no rational explanation as to why our results are inconsistent. It could, therefore, be due to type 1 error, time of the day or year effects and other non-identifiable factors.

Although the role of BDNF in the CNS has been investigated by many researchers and it has been largely linked to memory consolidation and synaptic plasticity as reviewed by Borodinova et al. [99], there are other aspects of brain function and neurochemistry were BDNF plays a pivotal role. In fact, serum BDNF has been proposed as a biomarker for mood and mood disorders such as depression, (for reviews see Hashimoto, 2010 [87] and Polyakova et al., 2015 [100]), and considering the effects of cocoa flavanols and methylxanthines on mood, as discussed above, future research should explore the possibility of serum BDNF increases mediating the effects of cocoa interventions on mood and mood disorders, and the possible link between mood and cognitive performance.

## Future Directions

The next steps of this study include flavanol bioavailability and MRI data analyses. This will aim to shed light on the effects (or the absence thereof) of our treatments on cognitive function and mood and the potential underlying biochemical mechanisms.

## Conclusion

We showed that chronic cocoa supplementation can improve vascular health, seen as increases in FMD response and decreases heart rate, as well as a decrease in serum NEFA levels and we further observed some trends towards differences in sedation and contentment, with greater scores in the active treatment arm. Although we failed to demonstrate any improvements in any of the cognitive outcomes, we saw an overall increase in cognitive performance for both treatment conditions, which could be attributed to the methylxanthines, such as theobromine and caffeine that naturally occur in cocoa and were present in both treatment capsules. Further research is required to investigate the effects of cocoa flavanol rich treatments in participants of different health and cognitive statuses, where any changes might be more prominent, and aim to segregate effects caused by methylxanthines and flavanols.

## Author's Contributions

M.G. contributed to the following: collection and processing of all biological samples and the analysis of blood samples; Scoring, reviewing and entry of the cognitive behavioural results; overall data processing and entry; subject recruitment and overall trial conduct and coordination.

CHAPTER 3

# The Acute Effects of a Polyphenol-rich Grape Extract on Cognitive Function, Mood, Salivary Cortisol and Glucose Metabolism in Healthy Older Adults

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## Abstract

Research has shown that berry flavonoid supplementation can enhance cognitive function by modulating glucose metabolism and insulin sensitivity. Furthermore, flavonoids have been shown to have beneficial effects on mood and can also moderate the effect of oxidative stress in reducing the effectiveness of cortisol in response to inflammation. This study aimed to investigate whether flavonoid supplementation has beneficial effects on cognitive function and whether those are mediated by the effects of flavonoids on glucose metabolism and stress response. A double-blind, randomised, controlled, cross-over, acute intervention trial with healthy older adults (n=30), 18 female and 12 male, aged 55-75 years, was conducted to assess the effect of flavonoids on cognition, mood, glucose metabolism and stress response before and approximately 150 minutes after the consumption of a polyphenol-rich grapeseed beverage (600mg of total polyphenols) and a matched control (0mg of total polyphenols), along with a standardised meal, which, together, delivered a fixed glucose load (75g of total carbohydrate). Linear Mixed Model with baseline measurements as a covariate was carried out for primary (global cognition, memory and executive function) and secondary outcomes (blood glucose, salivary cortisol,

mood). Statistically significant increase in the change of mental and physical sedation after the completion of a cognitive test battery post-intervention for the grapeseed drink condition was observed, compared to placebo. Despite this increase in mental and physical sedation, there was no attenuation of global cognition and executive function, which may be suggestive of a protective effect of grapeseed polyphenols against cognitive decline due to fatigue. There was a statistically significant increase in the postprandial blood glucose peak time for the grapeseed drink compared to placebo, suggesting that grapeseed polyphenols may have a delaying effect on glucose uptake. No effects of the polyphenol-rich drink on cortisol levels and anxiety were established. Further investigation is required to assess the potentially beneficial effects of an acute, high-dose grapeseed polyphenol supplementation on glucose uptake, cognitive fatigue and mood demonstrated in this study.

## Introduction

Flavonoids are a group of compounds with health promoting properties, found in many plant-based foods and beverages, such as berries, grapes, tea, cocoa, chocolate products and red wine. Flavonoids share a common structure that consists of two aromatic rings and a third oxygenated heterocyclic ring and they are divided into seven subclasses: flavonols, flavones, flavanones, flavanonols, flavanols, anthocyanidins, and isoflavones [6]. Anthocyanidins are the most abundant flavonoids found in berries (Blackcurrant, Blueberry, Cherry, Cranberry, Grape) [101].

Recent research has shown that flavonoid consumption can lead to improvements in cognitive performance, especially in older adults, suggesting that foods containing flavonoids could have cognitive enhancing benefits [22] and, to a certain extent, prevent cognitive decline [30]. Chronic polyphenol-rich grape and blueberry extract supplementation has been shown to have positive effects on episodic memory in healthy older adults with lower memory performance [61], signifying that polyphenols might be particularly beneficial for older people with age related memory decline. Moreover, previous research has shown that flavonoid supplementation can result in cognitive benefits [59], mediated by the effects of flavonoids on glucose metabolism and insulin sensitivity [19]. Great attention has been drawn to berry anthocyanidins, in particular,

which have been shown to have beneficial effects on various aspects of cognition, namely, delayed recognition and executive function in older adults [102]. In addition, berry flavonoids have been shown to improve cognitive performance in healthy older adults, seen as improved performance on letter memory and immediate word recognition tasks respectively [60], helping maintain and enhance cognitive performance and prevent cognitive decline across the day in both young [103] and older adults [60]. Further, acute supplementation of polyphenol-rich grape and blueberry extracts has been shown to enhance working memory and attention in healthy young adults [104]. Watson and colleagues, [105] have concluded that blackcurrant extract can improve attention compared to a matched control in young adults. Interestingly, they observed that blackcurrant juice (as opposed to blackcurrant extract powder) attenuated a decline in blood glucose over the duration of the cognitive test battery, suggesting that blood glucose metabolism might be a potential mechanism mediating the positive cognitive effects.

Although there is evidence to suggest that polyphenols can decrease glucose uptake [106], to date, the underpinning mechanisms are still unclear and little is known about whether the beneficial effects of flavonoid supplementation on cognition are mediated by those glucoregulatory mechanisms in healthy older adults. In addition, research suggests that apple and blackcurrant polyphenols decrease post-prandial glycaemia [107] and that cocoa polyphenols can improve glucose metabolism by reducing insulin resistance [30]. It is also evident that poorer glucose tolerance in individuals with glucose levels within the normal range can lead to impaired cognitive performance [108], suggesting a link between cognitive performance and glucose metabolism. As such, if flavonoids can increase the ability of the body to metabolise glucose, this could have a protective effect against decline in cognitive performance. Therefore, within the context of this study, it is anticipated that flavonoid supplementation may attenuate any decline in performance after the consumption of a high-carbohydrate breakfast along with the grapeseed drink and that this will be associated with reduced plasma levels of glucose determined via incremental area under the curve [iAUC]. Thus, this study aimed to investigate whether glucose metabolism may be a potential mechanism whereby an acute dose of grape polyphenols can induce beneficial effects on cognitive function, mood and stress response in older adults.

Finally, self-rated mood scores seem to be positively affected by flavonoid intake which could be linked with both brain function and glycaemia. Specifically, previous studies

have shown that flavonoids have beneficial effects on mood, such as the study by Scholey and colleagues [109], who found that an acute dose of epigallocatechin gallate (EGCG) induced an alert, yet calm, state in healthy younger adults. Moreover, Pase et al. [43] found a positive effect on mood, seen as an increase in self-rated calmness and contentedness, after a 30 day supplementation with cocoa polyphenols compared to placebo.

Furthermore, recent research suggests that EGCG can moderate the effect of oxidative stress in reducing the effectiveness of glucocorticoids in response to inflammation [72]. However, the effects of polyphenols on mood and stress response, and whether those are linked to cortisol levels and glucose metabolism, still remains unclear. As increased stress is, intuitively, associated with a decline in cognitive performance and flavonoids have been shown to positively affect mood, it is expected that any beneficial effects of the flavonoid-rich drink could be associated with reduced stress as measured using salivary cortisol and self-reported anxiety.

## Objectives

This project aims to address two main research questions:

1) Can grapeseed flavonoid supplementation lead to cognitive benefits in healthy older adults and are these benefits driven by the effect of flavonoids on glucose metabolism?
2) Can grapeseed flavonoid supplementation result in positive effects on the stress response and does this mediate their beneficial effects on cognitive performance in a sample of healthy older adults?

## Materials and Methods

A double blind, randomised, controlled, cross-over acute intervention trial with healthy older adults was conducted to determine the effect of a flavonoid-rich beverage on cognitive function, stress response and glucose metabolism. 18 women and 12 men were recruited via poster and email advertisements and were identified based on eligibility criteria from the Hugh Sinclair Unit volunteer database. After showing initial interest in the study, potential volunteers were provided with an information sheet about the study and

underwent initial telephone screening for study eligibility. They were then invited to attend a screening visit at the Hugh Sinclair Unit of Human Nutrition.

### *Screening*

On arrival, the study and procedures involved were explained and participants were given the opportunity to ask any questions about the study. Once participants understood the purpose and procedures involved, and if they were still interested in taking part in the study, eligibility was confirmed according to the following inclusion/exclusion criteria:

- Healthy males and females aged 55-75
- Achieve a score ≥24 on the MMSE (Mini Mental State Examination), a measure of global cognitive function (assessed during screening)
- English as primary language, must be able to understand the study information sheet, follow instructions in English and give informed consent
- No significant vision, hearing or language problems
- No history of depression
- Non-smokers
- Able to consume the beverages and not sensitive to blueberries or dairy products
- Should not suffer from any form of disease, including:
    - major mental illness
    - chronic fatigue syndrome
    - liver disease
    - diabetes mellitus
    - myocardial infarction
- Should not be on medication for the treatment of:
    - hypertension
    - elevated lipids
    - diabetes (type I and II)
    - medication known to impact on endothelial function such as statins, hypotensive medication or anticoagulants
- Should not be suffering gall bladder problems or gastrointestinal abnormalities

- Should not be on a weight reducing dietary regimen or taking any dietary supplements (including dietary fatty acids), unless they are willing to temporarily refrain from taking their dietary supplements for the duration of the study
- Should not follow a vegetarian or vegan diet
- Should not be consuming more than 15 units (120g) of alcohol per week
- Should not be vigorous exercisers (>3 times/week, 20 minutes each session)
- Must not be taking illegal substances
- Should not have taken antibiotics within the last 8 weeks
- BP <150/90 (determined at screening)
- BMI <30 (determined at screening)
- Normal full blood count, specifically: haemoglobin to check for anaemia (> 125 g/l for men and > 115 g/l for women), gamma GT for liver impairment (< 80 IU/l), normal platelet function (determined from screening blood sample)
- Normal fasting blood glucose level (3.9 to 5.5 mmols/l) (determined from screening blood sample)

If the volunteers considered they were eligible according to the criteria above and they were willing to participate in the study, informed consent was taken. Participants were told that taking part in the study would be subject to eligibility and that they would be informed whether or not they were suitable to participate as soon as possible after the screening visit i.e. once screening data had been checked against the eligibility criteria (i.e. BMI has been calculated and blood pressure checked against the accepted range) as well as on receipt of the full blood count results from the hospital. If the participants were happy to proceed, height and weight (from which BMI can be derived), and waist circumference were taken. Blood pressure was recorded following 10 minutes of rest, after which a fasted blood sample was taken. A small EDTA tube was sent to the Royal Berkshire Hospital (5mL, approximately) for full blood count analysis to ensure the subjects were in generally good health before taking part in the study and would report the total level of platelets, red blood cells and white blood cells (including levels of the individual types of white blood cell e.g. lymphocytes). The results were used to check that the volunteers were not anaemic and did not have any liver impairment. In cases where taking a venous blood sample from a volunteer did not seem feasible to a nurse or trained phlebotomist from the Hugh Sinclair

Unit of Human Nutrition (e.g. due to status of veins), they could decide that haemoglobin level, the most important criterion of general health for this study, would be checked via finger prick using the HemoCue according to Hugh Sinclair Unit procedure in order to ensure that participants were not anaemic and hence eligible for the study. The volunteers were also asked to undergo a finger prick to determine fasting glucose level to ensure this is within the normal range. They were then given breakfast and asked to complete the screening questionnaires, specifically, a Health and Lifestyle Questionnaire, the Brief Symptom Inventory, to screen for depression, and a Food Frequency Questionnaire. The cognitive element of the screening visit was performed on the same day after the physiological screening procedure. Volunteers taken by an experimenter to a quiet testing room where they were asked to complete the Mini Mental State Examination (MMSE) to determine their global cognitive function and the Spielberger Trait Anxiety Inventory to determine their general anxious tendencies. They also completed two tasks to determine their crystallised and fluid IQ (the National Adult Reading Test (NART) and Block Design Task respectively). Volunteers then had a familiarisation session, where they practiced each of the cognitive tasks as well as the self-rated mood assessments that they would be asked to complete during the main study visits. On completion of the screening visit, the Experimenter explained what would happen on the volunteers' next visit and provided them with the materials they needed prior to their first visit day, specifically, dietary and lifestyle restrictions prior to the study visit, which included avoiding alcohol, vigorous exercise and following a low polyphenol diet. They were also provided with the evening meal they were to consume the day prior to the visit (this was a low polyphenol meal and the volunteers were asked to consume a similar meal prior to their second visit). The volunteers were asked to arrive fasted for their study visit days. Both study visits were performed with a minimum of 60 hours in between, so that participants had a chance to recover from their first visit for at least 24 hours before starting to follow the low-polyphenol diet for 24-hours before coming in for their second visit.

Tables 3.1 and 3.2 below show the demographic, anthropometric and dietary information and the cognitive and mood status of the population sample respectively, as determined at screening. Figure 3.1 shows the study day timeline and design and Figure 3.2 shows a flow chart of the recruitment, screening and randomisation process.

Table 3. 1. Participant Demographic, Anthropometric and Dietary Information

| | |
|---|---|
| **Mean age (range)** | 63.77±0.94 (55-75) years |
| **Mean BMI** | 23.88±0.50 kg/m$^2$ |
| **Mean fasting Plasma Glucose** | 5.22±0.075 mmol/L |
| **Mean Systolic Blood Pressure** | 120.07±2.34 mmHg |
| **Mean Diastolic Blood Pressure** | 72.22±1.34 mmHg |
| **Mean Vegetable Intake** | 6.73±0.45 portions/week |
| **Mean Salad Intake** | 3.87±0.35 portions/week |
| **Mean Fruit Intake** | 8.87±1.11 portions/week |

*Values represent mean ± SE*

Table 3. 2 Population Sample Cognitive/Mood Status

| | |
|---|---|
| **Mini Mental State Examination** | 27.67±0.25 (out of 30) |
| **National Adult Reading Test** | 37.83±0.81 (out of 50) |
| **Block Design** | 31.27±1.45 |
| **Spielberger Trait Anxiety Inventory** | 31.93±1.53 |
| **Brief Symptom Inventory** | 1.27±0.47 |

*Values represent mean scores ± SE*

### *Study testing visits*

On arrival at the Department of Nutrition, the experimenter confirmed that the volunteers were happy to proceed with their participation in the study. Volunteers were reminded that they could withdraw at any time. If the volunteers were happy to proceed with their participation, the same study procedure was performed on each study day as shown in Figure 3.1 below.

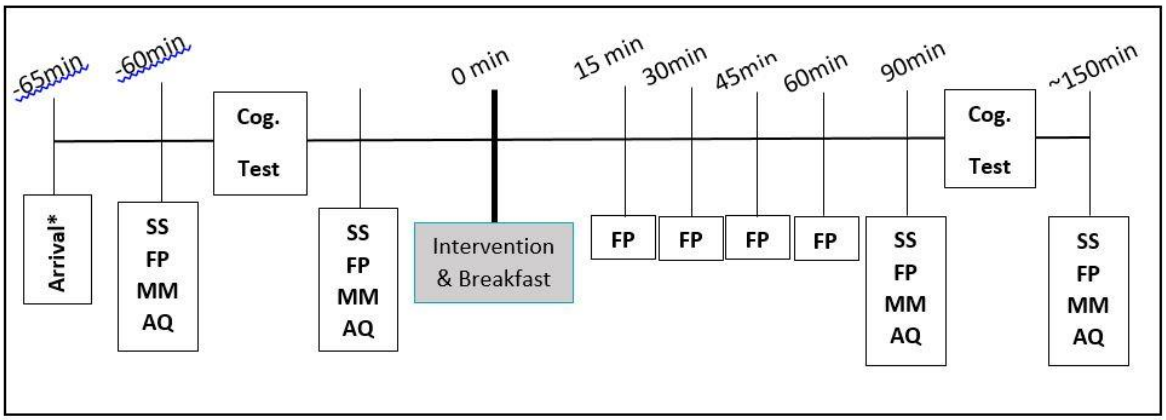


Figure 3. 1 Study visit day timeline

*48 h Food recall to check compliance with the demand for fasting and adherence to the diet and lifestyle restrictions. *SS=Saliva sample, FP=Finger prick, MM=Mood measure, AQ=Anxiety questionnaire, Cog. Test=Cognitive Test Battery.*

Compliance with the requirement to fast and adherence to the diet and lifestyle restrictions were checked with a 48-hour dietary recall. A blood glucose reading was obtained via finger prick and the volunteers were asked to provide a saliva sample from which cortisol levels were determined. The volunteers then completed the cognitive test battery. Each cognitive test battery session was approximately 60 minutes long and consisted of the following tasks: Rey Auditory Visual Learning Test (RAVLT), Letter Memory Task, Corsi Block Tapping Test, Verbal Fluency Task and Rapid Visual Information Processing (RVIP). Before and after each cognitive task session volunteers were asked to complete the Spielberger State Anxiety Inventory and the Bond-Lader Visual Analogue Scales, to assess self-reported anxiety and mood. Following, a subsequent finger prick blood sample and a saliva sample were taken. The volunteers were then asked to consume the study breakfast and the study drink (either the flavonoid-rich drink or the nutrient matched control drink - drink order was randomly assigned), which together delivered the glucose load of 75g of carbohydrate. Volunteers were asked to consume the breakfast and study beverage within 15 minutes. A finger prick blood glucose reading was taken at 15 minutes post consumption of glucose load and study drink, then subsequently every 15 minutes until 60 minutes post consumption, after which a sample was taken at 90 minutes with a final sample, taken at 135-150 minutes post intervention, which coincided with the completion of the cognitive test battery. Saliva samples were taken pre and post completion of the

cognitive test battery which occurred at baseline and 90 minutes post consumption of the glucose load and study drink (specifically, samples were taken at -45-60 minutes, 0 minutes then 90 minutes and 150 minutes). The saliva samples were analysed to determine salivary cortisol levels after the completion of testing, using Salimetrics® High Sensitivity Enzyme Immunoassay Kits. With each of the four saliva samples on each study day, volunteers also completed a mood measure (the Bond-Lader Mood Scale), in order to determine their mood, and an anxiety questionnaire (SSAI) to specifically assess state anxiety. At approximately 90 minutes post drink, the volunteers were asked to complete the cognitive test battery for a second time. This time was chosen to reflect the approximate peak plasma concentration of grape seed polyphenols [110], which largely consist of catechins ($T_{max}$=1.3-1.6h), gallic acid ($T_{max=}$0.9-1.3h) [110] and anthocyanins ($T_{max}$=1-3h) [111]. On completion of the study visit, volunteers were offered a snack before leaving, that would be prepared in the Hugh Sinclair Unit of Human Nutrition, and they were given the supplies for the next and final study visit day. Upon completion of their final study visit, volunteers were asked to complete the end of study questionnaire and to fill in the form to receive remuneration.

### *Intervention Drink*

The study intervention drinks were grape seed extract powders, formulated by ActivInside, reconstituted with water to form juice drinks. The high-polyphenol drink consisted of 1g of grape extract (>20% monomers, >1.3% anthocyanins) delivering 600mg of polyphenols and 30g of sugar (total sugar load of 75g when consumed together with the standardised study breakfast). The control drink was matched in colour, taste, appearance and nutrient content (30g of sugar), except it had no polyphenols. Both drink powders came in individual sachets and were coded as drink 43 or 62 for blinding purposes and the order in which they were administered to volunteers was determined based on a randomisation schedule. The sachets were stored in the freezer and prepared a few minutes before the 'breakfast time' on visit days by a non-affiliated researcher. The drink (powder mixed with 150ml of Buxton water) was prepared and served in a lidded, opaque container with an opaque straw, to ensure that subjects were also blind. All experimenters remained blind until after the completion of data processing and analysis.

Table 3. 3 Nutritional contents of the intervention powder and matched control.

| | Grapeseed | Control |
|---|---|---|
| **Saccharose (g)** | 30 | 30 |
| **Grapeseed Extract (g)** | 1 | 0 |
| **Citric Acid (g)** | 0 | 0.214 |
| **Orange Artificial Flavour (g)** | 0.1 | 0.05 |
| **Artificial Sweetener (Sucralose) (g)** | 0.075 | 0.075 |
| **Thickener (Xanthan Gum) (g)** | 0.066 | 0.066 |
| **Artificial Colour E124 (g)** | 0.03 | 0.037 |
| **Artificial Colour E151 (g)** | 0.01 | 0.012 |
| **Total Polyphenols (mg)** | 600 | 0 |

**The powders were dissolved in 150 ml of Buxton natural mineral water before administration.*

### *Statistical Analysis*

Primary and secondary outcomes were analysed using SPSS v25 (IBM, Armonk, NY, USA). Linear Mixed Model with baseline measurements as a covariate was carried out for each outcome. Intervention drink and Visit were fixed factors in the model and Bonferroni corrected pairwise comparisons were also used. Drop out data were included in the intention-to-treat analysis (1 volunteer dropped out during their second visit due to an adverse event). In all mood, anxiety and salivary cortisol outcomes, where 4 measurements were taken (baseline-before cognitive test battery, baseline-after cognitive test battery, post intervention-before cognitive test battery and post intervention-after cognitive test battery) the two baseline and the two post-intervention measurements were collapsed and, therefore were modelled as 'difference at post-intervention' compared to 'difference at baseline', with baseline measurements as a covariate.

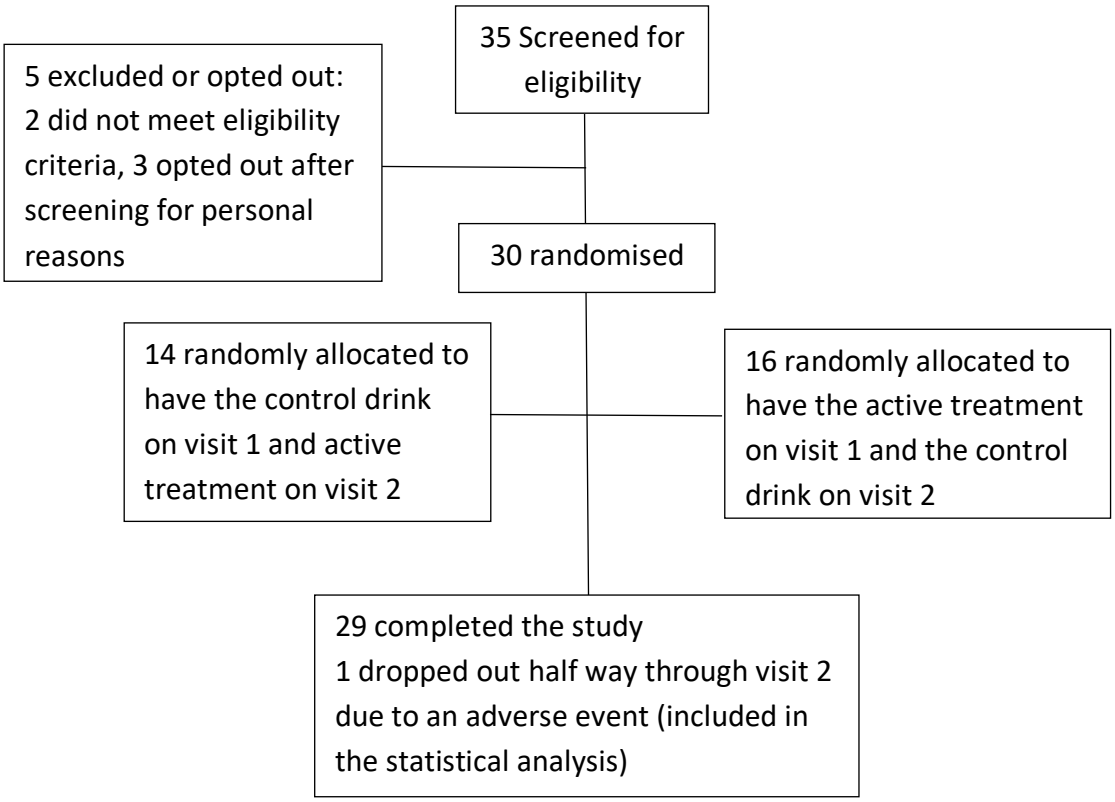


Figure 3. 2 Flow chart of full account and fate of all participants screened and enrolled in the study.

## Results

### *Cognitive function*

No significant differences were observed between the two drink conditions at baseline and post-intervention for any of the dependent variables; global cognition (F[1,3]=0.223 p=0.641, [-0.124, 0.197] 95% Confidence Interval), executive function (F[1,30]=0.563, p=0.460, [-0.129, 0.275] 95% Confidence Interval) and memory (F[1,30]= 0.043, p=0.838, [-0.318, 0.389] 95% Confidence Interval).

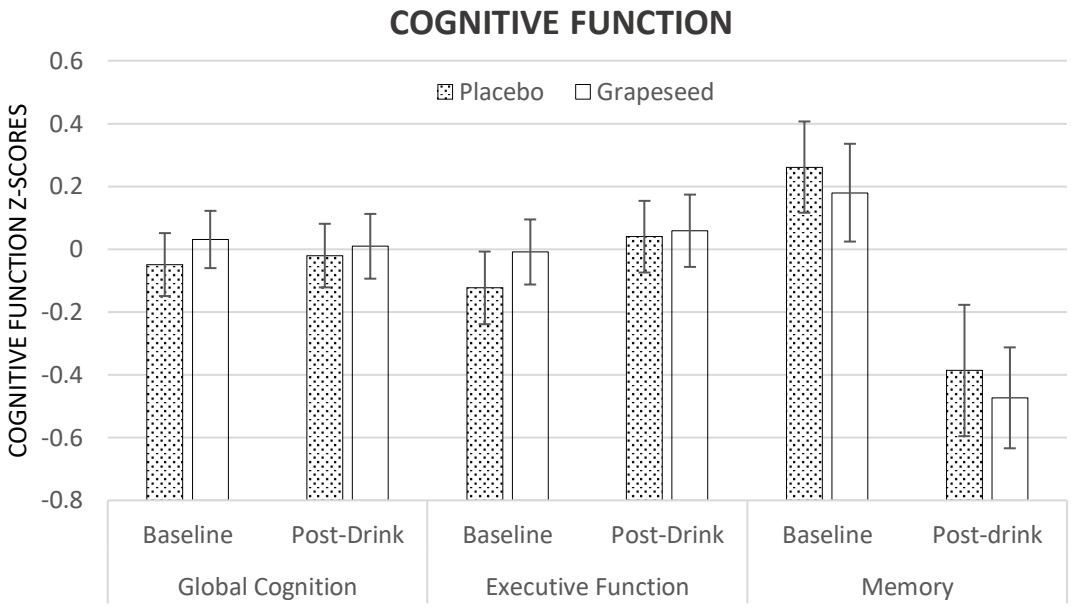


Figure 3. 3 Cognitive function composite scores at baseline and post-intervention for the two drink conditions.

Table 3. 4 Average scores for individual cognitive test battery tasks

| | **Placebo** | | **Grapeseed** | |
|---|---|---|---|---|
| | Baseline | Post-Intervention | Baseline | Post-Intervention |
| **RAVLT Overall score** | 72.20±2.6 | 62.63±2.5 | 70.83±2.6 | 63.86±2.7 |
| **Letter Memory Task (% correct)** | 32.54±2.2 | 31.88±1.9 | 34.58±2.35 | 33.62±2.4 |
| **RVIP (% correct)** | 91.67±1.95 | 94.82±1.39 | 93.05±1.58 | 95.31±1.17 |
| **Corsi Blocks (% sequences correct)** | 49.79±2.27 | 50.83±1.89 | 49.69±1.84 | 49.68±2.19 |
| **Verbal Fluency Test Overall score** | 60.85±1.97 | 62.82±2.01 | 62.38±2.36 | 63.72±1.76 |
| **CPT (% targets correct)** | 86.90±2.51 | 88.56±3.96 | 86.67±2.24 | 87.24±2.72 |

*Values indicate mean ± SE*

*Mood*

There was a significant effect of the drink condition on the changes in 'Mental and Physical Sedation' after the administration of the drink and breakfast, measured as difference in the Bond-Lader Visual Analogue Scale means (after minus before cognitive testing) ($F[1,30]=7.045$, $p<0.05$, [13.497, 102.81] 95% CI). Average self-reported anxiety scores at baseline were significantly increased after the completion of the cognitive test battery compared to before ($p<0.001$, [-5.8, -2.23] 95% CI, correlation=0.883), which demonstrates the anxiety inducing capacity of the cognitive test battery. No significant differences were observed between the two drink conditions at baseline and post-intervention, measured and modelled as mean score differences between before and after cognitive test battery (mean scores after cognitive testing minus mean scores before cognitive testing) at both baseline and after treatment, for the dependent variables: contentment ($F[1,30]=0.543$ $p=0.467$, [-22.046, 47.024] 95% Confidence Interval), relaxation ($F[1,30]=0.557$, $p=0.458$, [-10.721, 23.475] 95% Confidence Interval), anxiety ($F[1,30]= 0.663$, $p=0.422$, [-4.125, 9.575] 95% Confidence Interval) (Table 3.5). Regardless of the non-significance, a noticeable drop in the mean differences for the 'relaxation' and 'contentment' scores was observed after the completion of the cognitive test battery (compared to before) post-intervention for the placebo, but not for the grapeseed drink condition (Table 3.5), suggesting that the polyphenol-rich treatment may have helped prevent any attenuation in 'relaxation' and 'contentment' following a mentally fatiguing cognitive test battery.

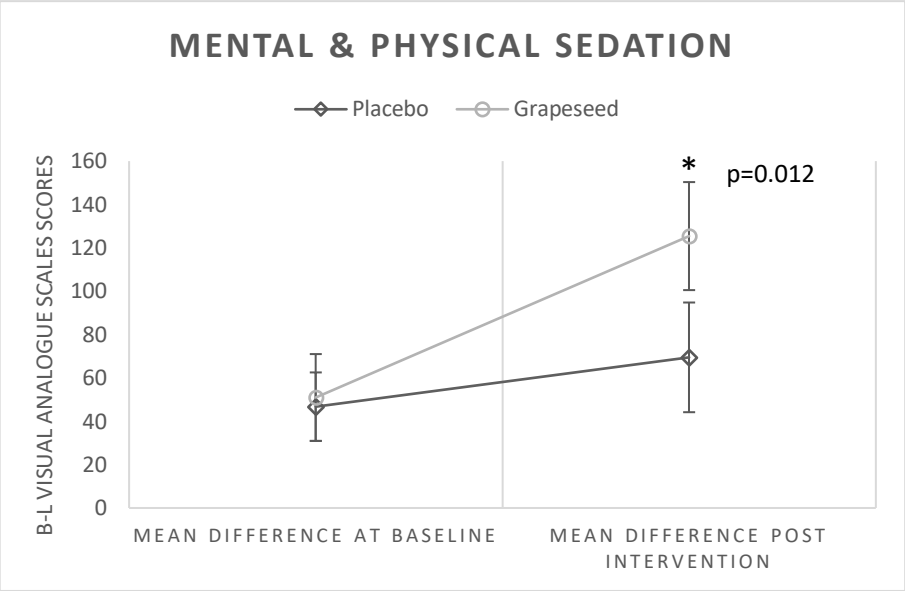


Figure 3. 4 Changes in Mental and physical sedation at baseline (baseline means are collapsed to reflect difference between the two baseline mood tests, 'before' minus 'after' cognitive testing) and post-treatment (post-intervention means collapsed, similarly to baseline) for the two drink conditions (error bars indicate standard error).

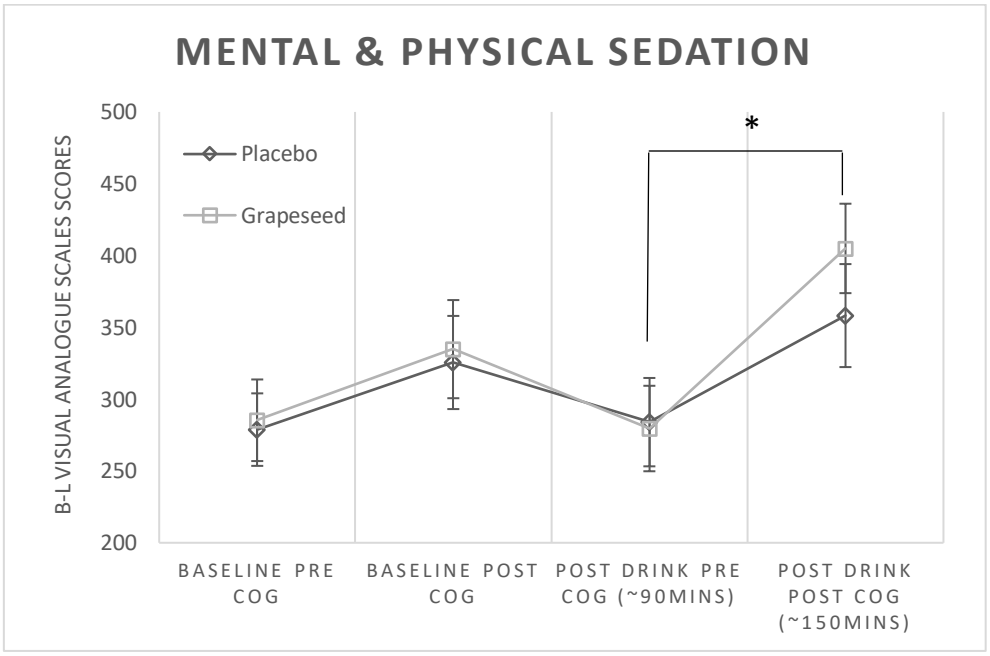


Figure 3. 5. Changes in Mental and physical sedation at all time points: baseline before cognitive testing, baseline after cognitive testing, post intervention (~90 mins) prior to cognitive testing and post intervention (~150 minutes) after cognitive testing. Change

between post drink pre-cognitive testing and post drink after cognitive testing, statistically significant (*p=0.012*) (error bars indicate standard error).

Table 3. 5 Bond-Lader VAS mood scores for the two drink conditions

| | **Grapeseed** | | **Placebo** | |
|---|---|---|---|---|
| | Baseline | Post-Intervention | Baseline | Post-Intervention |
| **Mental & Physical Sedation** | 50.97±20.03 | 125.41±24.9 ** | 46.73±15.8 | 69.48±25.3 |
| **Contentment** | 32.66±12.14 | 32.34±7.76 | 32.57±8.27 | 21.17±11.1 |
| **Relaxation** | 19.72±5.68 | 20.52±6.47 | 16.2±4.7 | 10.55±4.63 |

** Statistically significant effect on Mental & Physical Sedation was observed after the administration of the flavonoid-rich intervention compared to placebo (F[1,30]=7.045, p=0.012, [13.497, 102.81] 95% CI). Mean Differences indicate collapsed means (± SE) for the Bond-Lader Visual Analogue Scales Scores (before minus after the completion of a cognitive test battery) at baseline and post-intervention for the two drink conditions.

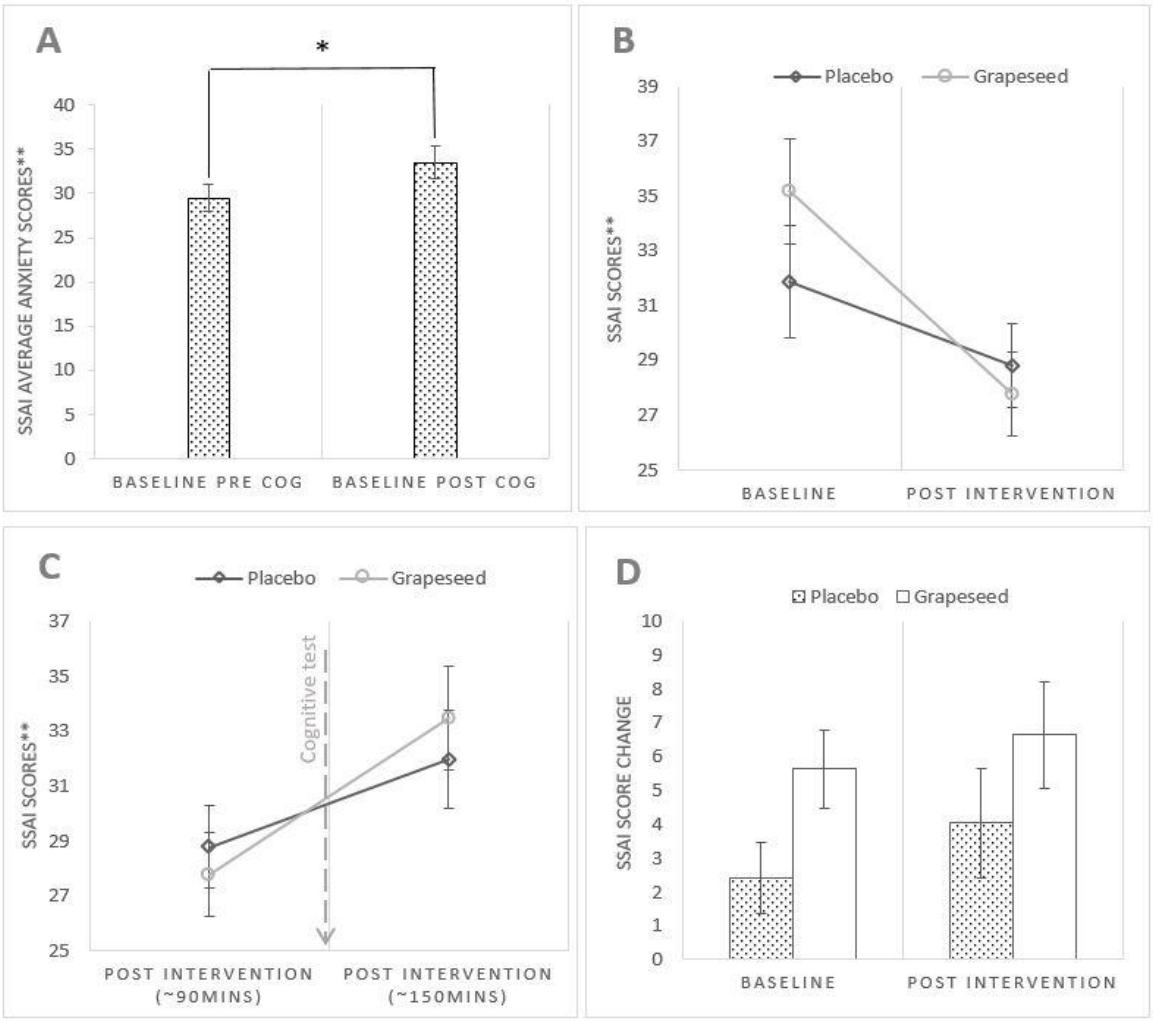


Figure 3. 6 Effects of the polyphenol-rich grapeseed extract on anxiety.

***Panel A:*** *Anxiety inducing effects of the cognitive test battery: Significant increase ($p<0.001$) of the average SSAI scores after the completion of the cognitive test battery at baseline.* ***Panel B:*** *SSAI scores drop substantially (but not significantly) approximately 90 minutes after the administration of the grapeseed extract, compared to placebo.* ***Panel C:*** *SSAI scores increase after the completion of the cognitive test battery for both treatment conditions.* ***Panel D:*** *Changes in the SSAI scores (after cognitive testing minus before cognitive testing) at baseline and post intervention.*

***SSAI; Spielberger State Anxiety Inventory. Higher SSAI scores indicate higher anxiety levels. A general cut-off point of 39-40 has been suggested as an indicator of clinically significant symptoms [112]. Normative values for different age groups can be found in the Manual for the State-Trait Anxiety Inventory* [113].

*Salivary Cortisol*

No significant differences in salivary cortisol were observed between the two different drink conditions (F[1,30]=0.482, p=494, [-0.027, 0.055] 95% Confidence Interval), analysed as cortisol level change between before and after cognitive testing, at baseline and post-treatment. Figure 7 presents the mean salivary cortisol levels (error bars indicate standard error), at four different time points across the testing visit days: (1) Baseline, (2) Baseline after cognitive testing, (3) Post-Intervention, (4) Post-intervention after cognitive testing.

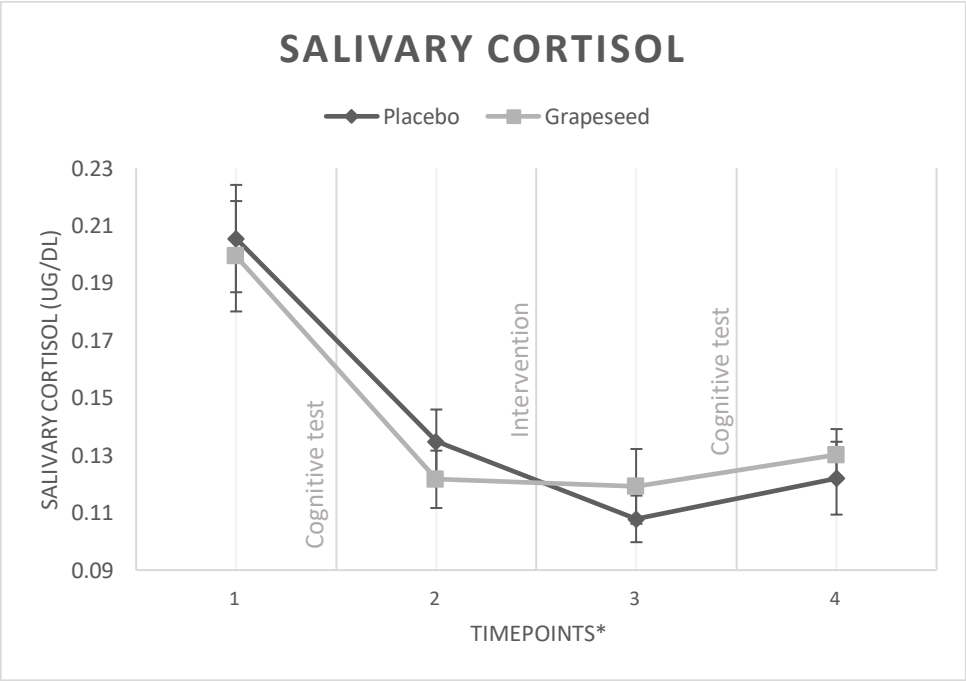


Figure 3. 7 Mean salivary cortisol levels across the study visit days for the two drink conditions.

** Time point 1: Baseline (before cognitive testing); Time point 2: Baseline after cognitive testing; Time point 3: Post-intervention (before cognitive testing); Time point 4: Post-intervention after cognitive testing.*

*Blood Glucose*

A paired sample t-test was performed to analyse the incremental areas under the curve (iAUC) for the glucose response. No significant difference in the iAUC was observed between the two treatment conditions (p=0.569, [-85.1, 47.7] 95% Confidence Interval) (mean blood glucose levels and area under the curve shown in Figure 3.7). However, the average blood glucose peak time was significantly higher (p=0.002) for the grapeseed drink compared to placebo, as shown in Figure 3.7, which may suggest a slower glucose uptake for the polyphenol-rich treatment condition. Figure 3.8 further highlights the fact that glucose response curves across the population sample appear to be noticeably smoother for the grapeseed drink condition compared to placebo, suggesting a more effective metabolic regulation of glucose.

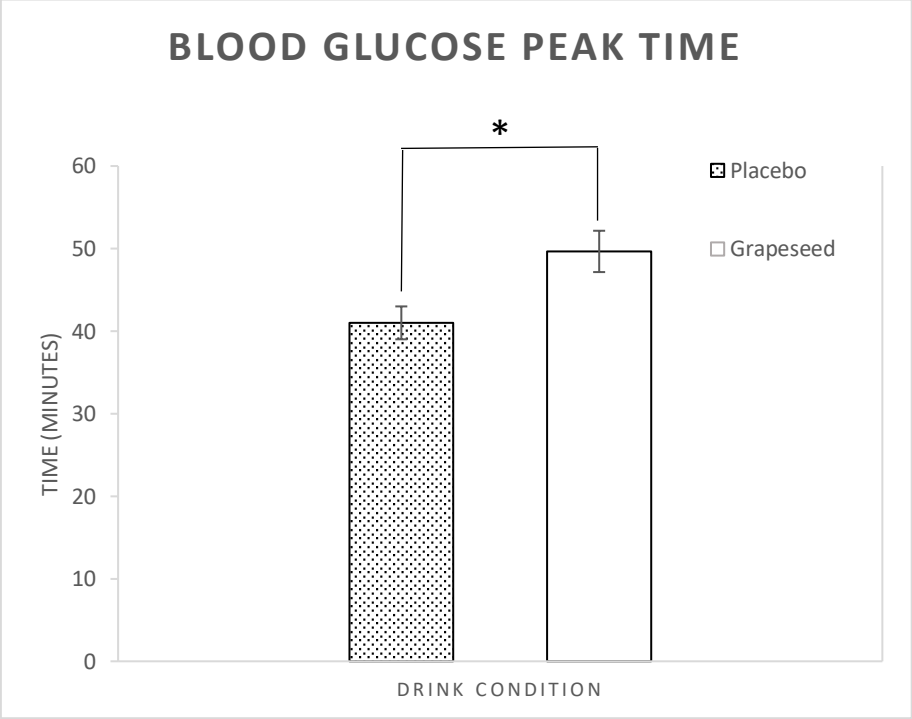


Figure 3. 8 The mean blood glucose peak time point was significantly higher for the grapeseed drink condition compared to placebo (p=0.002). Error bars indicate standard error.

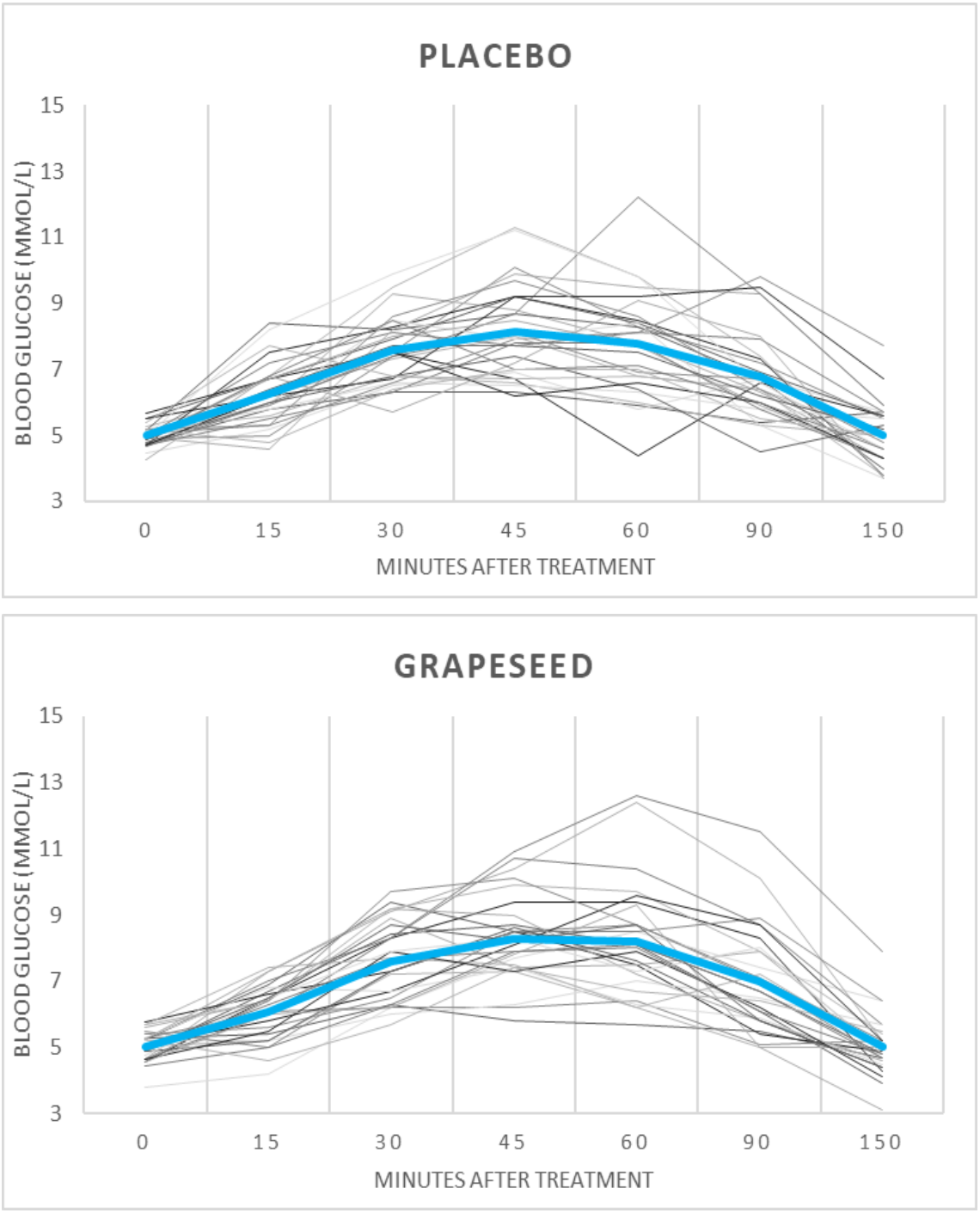


Figure 3. 9 Postprandial blood glucose levels (mmol/L) of the population sample (N=30) at different time points post-intervention for the two drink conditions. Mean levels are shown in blue.

## Discussion

This study shows evidence of effects of a high flavonoid grapeseed drink on mood. Specifically, a greater change (increase) in mental and physical sedation scores on the Bond-Lader visual analogue scales was observed after the cognitive test battery, following the administration of the grapeseed drink compared to placebo in a sample of healthy older adults. This suggests that the subjects were feeling more sedated after the completion of the second round of cognitive testing (post-intervention) following the administration of the flavonoid-rich grapeseed extract drink, compared to a matched control. No statistically significant changes were observed in any other mood outcomes, such as anxiety, relaxation and contentment. A slight drop in 'contentment' and 'relaxation' was seen after the completion of the cognitive test battery post-intervention for the placebo, but not for the grapeseed condition, suggesting that the active treatment may have helped maintain the levels of 'relaxation' and 'contentment' after a fatiguing cognitive test battery, however those changes were not statistically significant.

Even though mood was affected to some extent, cognitive performance did not change significantly at any of the time points for either of the drink conditions, which is consistent with Pase et al. [43], who showed that acute, high-dose cocoa polyphenol supplementation enhanced mood but not cognitive function in healthy, middle-aged adults, however, those mood improvements were seen as increases in 'calmness' and 'contentment', which could be because of the different treatment used and its different polyphenolic profile. Improvements in mood but not in cognitive performance is a pattern hard to explain that requires further investigation, however we speculate that there might be an negative relationship between cognitive performance and mood in the short term, as discussed below.

Although we failed to demonstrate any statistically significant improvements on cognitive performance, we showed that global cognition and executive function remained practically unchanged, despite the fact that there was a significant increase in sedation after the completion of a mentally fatiguing cognitive test battery at post intervention, which may suggest a protective effect of the active treatment on cognitive decline due to fatigue. This, however, was not the case for memory function, where decreases in performance were observed for both drink conditions, likely due to retroactive

interference, across the four cognitive testing sessions that each participant underwent over the span of a week. Cognitive performance for some of the tasks such as RVIP, CPT and Verbal Fluency Task, showed a slight, non-significant increase at post intervention, for both drink conditions, which could arguably be due to practice effect, though there was a separate 'familiarisation' session devoted to cognitive testing practice, in order to eliminate practice effects as much as possible.

The results of this study are also in agreement with Hendrickson et al. [114] who found no acute effects of grape juice consumption on memory and mood in younger adults, as well as Crews et al. [115] who also failed to demonstrate any evidence of cognitive benefits of cranberry juice in cognitively intact older adults. Nonetheless, there is a significant body of evidence, which has shown that polyphenol rich foods have beneficial effects on cognitive function in older adults [60, 61, 102]. Given the link between stress and cognitive performance, the low levels of stress and high levels of calmness observed in this trial should have, logically, led to an increased cognitive performance, however it has been suggested that induced acute stress can have a facilitating effect on cognitive performance, especially executive functions [116] and response inhibition [117] as well as working memory [118]. As such, moderate levels of induced acute stress can have a facilitating effect on cognitive performance. Therefore, the relatively high levels of sedation observed in this trial could have, in theory, had a detrimental effect cognitive performance. The lack of such effects in this study could potentially be attributed to the high-flavonoid grapeseed drink, however further investigation is required to establish the role of polyphenol-rich nutritional interventions on cognition and mood, as well as the link between them.

Further, there is evidence suggesting that polyphenol-rich foods can positively modulate glucose metabolism. Desideri and colleagues [19] have shown that flavonoids have a beneficial effect on cognitive function in older adults with mild cognitive impairment and that they can reduce insulin resistance. Moreover, recent research suggests that flavonoids could attenuate glucose absorption [119] which may be one of the potential mechanisms of action mediating the effects of flavonoids on cognitive performance. This study failed to show any significant changes in glucose response, expressed as iAUC, between the two treatment conditions. However, the average blood glucose peak time was significantly higher for the grapeseed drink compared to placebo, which may be suggestive of slower glucose uptake for the polyphenol-rich treatment condition, although it could be

argued that this may be of little or no clinical importance in healthy/non-diabetic subjects. According to recent research, the beneficial effects of polyphenols on glucose metabolism and insulin resistance are evident on high fat diets [120]. Therefore, we speculate that the effects of the flavonoid-rich grapeseed extract in this experiment might have been more pronounced in a state of post-prandial dysmetabolism, following a glucose and fat challenge, as instead of just a glucose challenge. Further research is required to explore this possibility. Moreover, the measurement of plasma glucose with glucose monitors that utilise capillary blood samples, could arguably be a limitation, since venous blood samples analysed by clinical chemistry analysers, can provide more accurate and precise results. The coefficient of variation for our baseline blood glucose measurements using the above mentioned method was 7.3%, which suggests a relatively good, but not high, performance of this method, however a CV% of 5% or less would be preferrable. As such, using more robust methods of measuring blood glucose is recommended.

The clinical relevance of these results as well as a number of limitations need to be taken into consideration. First, the health status of our subjects does not match that of the average older adult in the UK population. Most participants had a healthy lifestyle and were following a healthy diet, which could explain the high baseline scores in some of the measures and the non-significance of some of the results. Lack of significant results in healthy populations has also been reported by previous studies, such as Masse and colleagues [22], however they used a considerably lower polyphenol dose with a much younger population sample. Further, chronic berry flavonoid supplementation has been shown to have beneficial effects in older populations with mild cognitive impairment [59], as well as acutely, with similar beneficial effects, attenuating age-related memory decline in older adults with cognitive impairments [61]. This suggests that there might be a 'ceiling effect', thus cognitively healthy individuals cannot have any further improvement in cognitive performance, as they are already high functioning, especially considering that many of our participants achieved maximum scores in some tasks (for example delayed recall scores of 15 out of 15). Moreover, the 20-year age range of the participants could potentially be considered as too wide and result in substantial differences in cognitive performance between different subgroups within this range. Although we cannot substantiate this, as there was no correlation between age and cognitive performance, we

observed that some participants on the upper end of the age range struggled to understand and perform certain tasks.

In addition, various other parameters such as differences in glucose metabolism, circadian rhythms, sleep pattern and quality of sleep the night before the testing day between individuals should, ideally, be taken into account. Habitual and long-term polyphenol consumption should also be taken into consideration and used as an exclusion/ inclusion criterion, despite the fact that participants were asked to follow a low polyphenol diet for two days prior to each study visit day. Another limitation of this study could have been the short wash out period (two days). Other studies have used longer wash out periods. For instance, Watson et al. [105], in a similar study investigating the acute effects of blackcurrant juice on cognitive function allowed one week or more as a wash out period, which helps reduce the risk of carryover effects, since it is not firmly known whether flavonoids and their metabolites are still available in the body two days post-consumption or not.

The flavonoid dose is also a point of discussion. Previous studies have shown significant effects with much higher flavonoid doses, for example Brickman et al. [121] found beneficial effects on a cognitive task following a dose of 900mg of cocoa flavanols. Based on that, the lower dosage in our study (600mg) could be considered as a potential limitation. However, previous studies that used lower polyphenol doses have also demonstrated significant results, such as Scholey et al. [71], who found improved performance in a cognitive task following a dose of 520mg of cocoa flavanols, therefore it cannot be assumed that the lack of significant results in our study was due to the fact that the polyphenol dose was not high enough. It has been suggested that future studies should aim to administer varying doses in the same study design to allow comparisons [122] or even different doses according to participants' bodyweight as suggested by Krikorian and colleagues [59].

This study did not investigate the effects that acute flavonoid supplementation may have on neurochemical and physiological parameters related to brain health. Previous research has suggested that physiological changes in the brain can be shown even without any behavioural cognitive changes [123]. Therefore, future studies investigating the effects of flavonoid supplementation on cognitive function should look at the potential brain changes using brain imaging, alongside behavioural effects. On the other hand, the

expectations of acute flavonoid supplementation having immediate physiological and/or structural effects may be unrealistic. Changes in neurochemical parameters, such as levels of neurotransmitter and their metabolites should also be considered in future research.

Finally, it is debateable whether one single acute dose of flavonoids is enough to result in major cognitive benefits compared to chronic supplementation, and, more so, reverse or delay the onset of age related cognitive decline. Chronic supplementation trials might be a more accurate way of investigating the effects of flavonoid supplementation on cognitive performance and their underpinning mechanisms, since chronic studies are more representative of habitual, long-term polyphenol intake, which might be a potential long-term intervention strategy for the prevention of neurodegenerative diseases.

## Conclusion

This randomised, double-blind, placebo controlled, cross-over trial has demonstrated that an acute dose of a flavonoid-rich grapeseed drink could potentially prevent any attenuation in global cognition and executive function, in a state of increased mental and physical sedation, following a mentally fatiguing cognitive test battery, in healthy older adults. This study has also shown that blood glucose peak time is greater after the administration of a flavonoid-rich grapeseed drink, compared to placebo, which may suggest that grapeseed flavonoids may have the ability to slow down glucose uptake. Further investigation is required to establish the effects of acute polyphenol supplementation on cognition, mood and glucose metabolism, as well as the underlying mechanisms of action.

## Author's Contribution

M.G. contributed to the following: hands-on experimental conduct and data collection; recruitment; neuropsychological testing; data entry and processing; statistical analysis; biological sample collection, processing and analysis.

CHAPTER 4

# The effects of a hop-rich beer on mood on healthy young adults with common mental disorders: a study protocol for a randomised controlled trial

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## Abstract

Hops (*Humulus lupulus*) have a long history of herbal and medicinal use and are known to have sedative and anxiolytic effects. According to recent research, chronic daily supplementation of hops extract can reduce self-reported anxiety, depression and stress levels. Moreover, alcohol-free beer containing hops has been shown to improve night sleep quality and decrease anxiety. Pre-clinical research findings are in line with those claims, suggesting interactions of hops extracts with specific neurotransmitter receptors as potential mechanisms whereby active compounds in hops induce beneficial effects on mood. Those effects have been attributed to the ability of the prenylated flavonoids that naturally occur in hops, to positively modulate Gamma-Aminobutyric Acid receptors in the brain, as well as interact with subtypes of serotonin and melatonin receptors, thus playing an important role in the regulation of mood, and circadian rhythms. Despite the fact that there is convincing evidence that hops extracts have beneficial effects on neurochemical markers of mood in animal and cell-culture models, to our knowledge, those effects have not been investigated in humans. Human trials using self-reported mood measures are supportive of those claims, however more investigation is required to substantiate the effects of hops on neurochemical parameters. As such, this study protocol has been developed to investigate the effects of a hop-rich beer on self-reported mood and

neurochemical markers of mood, compared to a matched control in a sample of young adults that are likely to have common mood disorders. A variety of biochemical parametrs will be examined: Plasma dopamine, serotonin, GABA and their metabolites, plasma monoamine oxidase, adrenaline, noradrenaline and cortisol and serum S100B proteins, to attempt to understand the biochemical mechanisms underlying the anxiolytic and antidepressant effects of hops.

## Introduction

The female inflorescences of hops (*Humulus lupulus*) have been traditionally used as a bittering and stabilising agent in brewing. However, hops also have a long history of herbal and medicinal use and are known for their sedative and anxiolytic effects. According to recent research, daily supplementation of as little as 0.4 g of hops extract for 4 weeks reduced self-reported anxiety, depression and stress scores in healthy young adults, compared to placebo [124]. Moreover, alcohol-free beer containing hops has been shown to improve night sleep quality and decrease anxiety in individuals with demanding and inconsistent work and sleep patterns, following 14 days of treatment with 330ml of alcohol-free beer a day [125]. Furthermore, pre-clinical studies support the above-mentioned hypotheses by suggesting interactions of hops extracts with specific neurotransmitter receptors as potential mechanisms whereby active compounds in hops have beneficial effects on mood.

More specifically, Benkherouf and colleagues [126] attributed the hops extract potency to the ability of 8-prenylnarigenin, a phenolic compound and natural phytoestrogen occurring in hops, to positively modulate GABA receptors in rat brain tissue. Given the already established links between polyphenol intake from various sources and their positive effects on mood in humans [94, 127-129], the modulation of $GABA_A$ receptors could be one of the potential molecular mechanisms by which phytochemicals like naringenin enhance positive mood states and reduce anxiety, a hypothesis that has been previously confirmed [130]. The link between GABA, mood and hop phytoestrogens is of particular interest in early adulthood and late adolescence, a period with the highest risk of low mood and common mental disorders [131] but also a period of rapid development of neurocircuitry and regional morphology of the brain, leading to brain maturation, which

is often not complete until the age of 25 [132]. Therefore, it is of great importance that we determine whether phytochemicals like the prenylated flavonoids found in hops could be used as a treatment for common mood disorders that very frequently occur in those age groups, and to explore the potential underlying neurochemical mechanisms.

Abourashed and colleagues [133] were the first to report hops interactions with 5-$HT_6$ and $ML_1$ receptors, subtypes of serotonin and melatonin receptors respectively, both of which play important roles in brain functions related to mood, cognition and circadian rhythms. Further, antidepressant properties of *Humulus lupulus* extracts in rats were reported by Zanoli and colleagues [134], who suggested a U shaped dose-dependent effect of hops extract on immobility time during a behavioural self-despair test in rats. Despite the fact that there is convincing evidence that hops extracts can have beneficial effects on neurochemical markers of mood in animal and cell-culture models, to our knowledge, those effects have not been confirmed by or tested in clinical trials. Human trials using self-reported mood measures are in link with that evidence, however self-reported mood measures can be subjective and difficult to interpret. Therefore, more investigation is required to establish the effects of hops on the levels of plasma neurotransmitter metabolites, as markers of mood, in humans.

As such, this project will aim to fill this knowledge gap by addressing two main questions.

1. Do hops extracts, in the form of a hop-rich beer, have the ability to beneficially change the levels of serum neurotransmitter and their metabolites associated with brain functions related to mood and mood disorders, in a sample of young adults likely to have common mood disorders?
2. Can beer containing high levels of hops reduce negative mood, based on self-reported measures, in those young adults, and is that in accordance with any observed neurotransmitter changes?

A variety of serum/plasma biomarkers related to mood will be examined. Plasma Dopamine metabolites, 3-O-methyl-Dopa and homovanillic acid (HVA) will be determined, as the dopamine system plays an important role in depression [135] and dopamine metabolites are indicators of dopaminergic activity in the brain. Ibero-Baraibar and colleagues in their recent research [127] showed that cocoa flavanol supplementation contributed to an increase of plasma HVA, which was further associated with the decrease

of depressive symptoms in obese subjects following a calorie-restrictive diet, a population sample highly prone to mood disorders such as depression. Several other polyphenolic copmpounds and sources, such as luteolin, tea polyphenols and Japanese hops extracts, have been shown to have implications in the dopaminergic system, with potential neuroprotective effects [136-138], it is, therefore, important that we explore the possible beneficial effects of hop phenolics on the dopaminergic system as a potential biochemical mechanism underpinning their effects on mood.

Another monoamine of interest is plasma serotonin (5-HT) and its metabolite 5-hydroxindole acetic acid (5-HIAA), as serotonin is shown to be involved in the aetiology of depression as well as the circadian regulation [139]. Monoamine Oxidase (MAO), a group of enzymes that catalyse the oxidation of the above mentioned neurotransmitters, in plasma will also be tested, since elevated levels of MAO are associated with anxiety and depressive symptoms, as MAO breaks down and therefore decreases the availability of serotonin, dopamine and other monoamines [140]. Stress response related neurotransmitters and hormones in plasma, such as epinephrine, norepinephrine and cortisol will also be tested. Finally, levels of serum S100b proteins, a group of glial marker proteins, will be determined, as they have been recently suggested as potential biomarkers for mood disorders [141], and, although historically described and used as a marker of brain damage, they are now believed to be good indicator of glial and BBB (dys)function, in humans, including adolescents, with mood disorders [141, 142].

## Strategic Relevance

There is a growing demand for novel foods and nutritional supplements that promote health and can potentially help prevent or treat mental illness and common medical conditions. Great attention has been drawn to brain function and mental health, as there is an ever-increasing prevalence of common mood disorders, such as depression and anxiety in the UK population, more pronounced in, but not limited to, young adult females, which compromises the quality of life of the population and poses a financial burden to the NHS. According to the most recent adult psychiatric morbidity survey on mental health and wellbeing in England [131], one adult in six has a common mental disorder (approximately one in five women and one in eight men) in England, with young

women being a high-risk group for common mental disorders. More people tend to seek medical help for the treatment of common mental disorders in the last seven years (approximately one in three), resulting in over prescription of antidepressant medication and long waiting lists for talking therapies and other mental health and wellbeing services in the NHS. Presently, the most effective and common treatments for adolescent depression are psychological therapies and SSRI medication, such as Fluoxetine. However, discontinuation rates appear to be high and relapse phenomena are common, as well as withdrawal symptoms after cessation of the treatment [143, 144].

As such, there is a growing need to develop alternative treatments and public health intervention strategies, as well as a shift towards lifestyle changes and better self-awareness. Given that most cases of common mental disorders, such as mild depression, can be treated, or alleviated, simply with informed lifestyle improvements (for instance regular exercise, balanced diet, healthier living environment etc.), one plausible way for the prevention of mood disorders is through diet and nutritional supplements. Healthy diets are associated with lower risk of mood disorders over the lifespan [145] and it has been suggested that the flavonoid-rich foods included in these diets could account for these benefits [146]. It is, therefore, conceivable that phytochemical-rich foods or food supplements may play an important role in the alleviation of mood disorders in certain groups of the population.

Polyphenols and polyphenol-rich foods have been shown to have beneficial effects on brain function and mood, with a great deal of research currently focused on their psychoactive and other health promoting properties. It has been established that higher flavonoid intakes, particularly flavanones, such as naringenin, were associated with lower depression risk [147]. In addition, flavonoids, in particular the prenylated flavanones found in hops, have been postulated to possess activity at benzodiazepine receptors and may thus act as natural anxiolytic compounds [126]. For all these reasons, we anticipate that this research will have implications in the developing of alternative intervention strategies for the treatment of common mood disorders.

## Methods

A double-blind, randomised, controlled, cross-over acute and chronic (3 weeks) intervention trial with healthy young adults aged between 18 and 26 years who experience 'low-mood' and are likely to have common mood disorders such as stress and anxiety will be conducted to determine the effects of a hop-rich beer on plasma and serum mood biomarkers and self-reported mood.

Participants, mainly University of Reading under- or post-graduate students will be recruited via email, poster and social media advertisements and will be identified based on eligibility criteria from the Hugh Sinclair Unit volunteer database. After showing initial interest in the study, potential volunteers will be provided with an information sheet about the study and will undergo initial telephone screening for study eligibility. They will then be invited to attend a screening visit at the Hugh Sinclair Unit of Human Nutrition.

### *Screening*

On arrival, the study and procedures involved will be explained and participants will be given the opportunity to ask any questions about the study. Once participants understand the purpose and procedures involved and if they are still interested in taking part in the study, eligibility will be confirmed according to the following inclusion/exclusion criteria:

- Healthy males and females aged 18-26
- Depression Anxiety and Stress Scales-21 (DASS-21) scores: >14 for depression, >10 for anxiety, >19 for stress (assessed during screening)
- English as primary language, or good English language skills-must be able to understand the study information sheet, follow instructions in English and give informed consent
- Non-smokers
- Able to consume the study beverages and not sensitive to yeast, grains or hops.
- Subjects should be consuming no more than 2 pints of beer a day other than the study beer.
- Should not suffer from any form of disease, including:
    - Major mental illness such as bipolar disorder and schizophrenia

  - Endocrine disorders such as hypo-thyroidism and chronic fatigue syndrome
  - liver disease
  - diabetes mellitus
  - heart disease
- Should not be on medication for the treatment of:
  - Depression or other mental health disorder
  - insomnia
  - diabetes mellitus
- Should not be suffering gall bladder problems or gastrointestinal abnormalities.
- Should not be on a weight reducing dietary regimen or taking any dietary supplements (including dietary fatty acids and St John's wort (Hypericum perforatum)), or CBD oil unless they are willing to temporarily refrain from taking them for the duration of the study
- For female participants: Should not be pregnant or lactating. If using any form of hormone regulating contraception they should adhere to it for the duration of the study
- Should not be on any specific diet (for instance keto, calorie-restrictive, vegan etc)
- Should not be consuming more than 14 units of alcohol per week
- Should not be vigorous exercisers (>4 times/week, 30-60 minutes each session)
- Must not be taking illegal substances
- Should not have taken antibiotics within the last 8 weeks
- BP <150/90 (determined at screening)
- BMI <30 and >19 (determined at screening)
- Normal full blood count, specifically:
  - Haemoglobin to check for anaemia (>12.5 g/dL for males and >11.5 g/dL for females)
  - Total white cell count (3.6-11.0 x109/L)
  - Differential count:
    - Neutrophils (1.8 - 7.5 $x10^9$/L)
    - Lymphocytes (1.0 - 4.0 $x10^9$/L)
    - Monocytes (0.2 - 0.8 $x10^9$/L)

        - Eosinophils (0.1 - 0.4 x$10^9$/L)
        - Basophils (0.02 - 0.1 x$10^9$/L)
    - Normal platelet function (platelet count 140-400 x$10^9$/L)
    - Red cell count (4.50-6.50 x$10^{12}$/L for males; 3.80-5.80 x$10^{12}$/L for females)
    - Haematocrit (0.40-0.54 L/L for males; 0.37-0.47 L/L for females)
    - Mean Cell Volume (80-100 fL)
    - Mean Cell Haemoglobin (27-32 pg)
    - Reticulocyte Count (0.2-2.0 %)
- Normal fasting blood glucose level (3.9 to 5.5 mmols/l) (determined from screening blood sample)
- Participation in other research trials within the last 6 months will need to be declared and may affect the start date for participation in the current trial.

If the volunteers are eligible according to the criteria above and they are willing to participate in the study, informed consent will be taken. Participants will also be asked to sign informed consent for providing venous blood samples. Participants will be told that taking part in the study will be subject to eligibility and that they will be informed whether or not they are suitable to participate as soon as possible after the screening visit, once screening data have been checked against the eligibility criteria (i.e. DASS-21 scores have been calculated, BMI has been calculated and blood pressure checked against the accepted range) as well as on receipt of the haematological results from the lab. Height, weight, and waist circumference measurements will also be taken on the screening visit.

Blood pressure will be recorded following 10 minutes of rest, after which a fasted blood sample will be taken. A small EDTA tube (20mL, approximately equivalent to a tablespoon) will be used for full blood count analysis in the Nutrition Research lab to ensure the subject is in generally good health before taking part in the study and will report the total level of platelets, red blood cells and white blood cells (including levels of the individual types of white blood cell e.g. lymphocytes). The results will be used to check that the volunteer is not anaemic and does not have any liver impairment. We will also take an additional screening blood sample (4mL) to check that the fasting cholesterol, triglyceride and glucose levels are within the range we have specified and that the volunteer does not have impaired liver or kidney function. In cases where taking a venous blood sample from

the volunteer does not seem feasible to a nurse or trained phlebotomist from the Hugh Sinclair Unit of Human Nutrition (e.g. due to status of veins), they can decide that haemoglobin level, the most important criterion of general health for this study, will be checked with an extra drop of blood via finger prick. Haemoglobin levels will be analysed with the HemoCue according to Hugh Sinclair Unit procedure in order to ensure that participants are not anaemic and hence eligible for the study. If taking venous blood samples from the volunteer is not feasible at all times, under normal circumstances, the participant will be excluded from the study as non-eligible, as blood samples will also be required on study visit days.

They will then be given breakfast and will be asked to complete the screening questionnaires, specifically, DASS-21 (for depression, stress and anxiety), a Health and Lifestyle Questionnaire, a Food Frequency Questionnaire and a Spielberger Trait Anxiety Inventory. On completion of the screening visit, the Experimenter will explain what will happen on the volunteer's next visit, provided they are eligible. The next visit will be confirmed and scheduled via email once the screening visit results (including full blood count from the hospital) have been assessed and deemed acceptable. If volunteers have scored very high on the DASS-21 questionnaire (i.e. within the range of 'extremely severe'), they will be advised to speak to their GP or be referred to the University Counselling and Wellbeing Services, if they are students at the University of Reading. The experimenter or a research nurse will have a discussion with the participants about the nature and severity of their mood disorder, and they will be advised not to enrol in the study.

On completion of the screening visit, the Experimenter will explain what will happen on the volunteer's next visit and will provide them with the information they need prior to their first visit day, specifically, dietary and lifestyle restrictions prior to the study visit, which includes avoiding alcohol, vigorous exercise and avoiding hops/beer. The volunteer will be asked to arrive fasted (12-hour overnight fast: only water allowed, starting after dinner the evening prior to each visit) for their study visit days. Both study visits will be performed with a minimum of 60 hours in between, so that participants will have a chance to recover from their first visit. Volunteers will be advised not to drive after the completion of the acute trial and information on alternative means of transport (e.g. local buses) will be provided if needed.

*Acute Trial*

## Study Visits

The first part of the study will be a double blind, placebo controlled, crossover, acute intervention trial that will take place in the Hugh Sinclair Unit of Human Nutrition. Upon arrival, the Experimenter will confirm that the volunteer is happy to proceed with their participation in the study. If not, the volunteer will be thanked and asked to complete a study payment form so that they can be provided with remuneration for any main study visits previously attended. If the volunteer is happy to proceed further with their participation, the same study procedure will be performed on each study day as detailed in Figure 1 below.

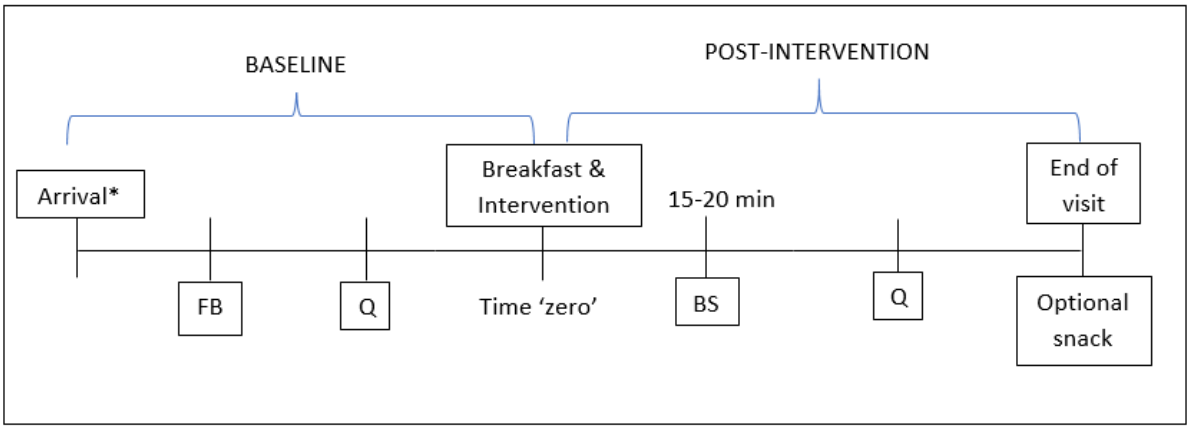


Figure 4. 1 Acute Study Visit Timeline

*Check consent, that the volunteer is fasted and compliance with the diet and lifestyle restrictions. Water available on arrival and throughout the visit.

FB=Fasted Blood Samples, Q=Questionnaires (SSAI, Hamilton Scales, DASS-21, Bond Lader Visual Analogue Scales for Mood), BS=Blood Samples.

Compliance with the demand for fasting and adherence to the diet and lifestyle restrictions will be checked at the beginning of each visit. Fasting blood samples will then be obtained (12 EDTA tubes and 2 serum tubes, which is a total of 60 ml of whole blood approximately). Volunteers will also be asked to fill in self-reported mood questionnaires, including Spielberger State Anxiety Inventory, Hamilton Scales for Depression and Anxiety and DASS-21 as well as Bond-Lader Visual Analogue Scales for self-rated mood.

The volunteer will then be asked to consume the study drink (either the hop-rich beer or the matched for alcohol level low-hop beer - drink order will be randomly assigned) along with some breakfast, similar to what they had on screening and a study snack (following the Hugh Sinclair Unit of Human Nutrition procedures) This will consist of toast for breakfast and plain crisps for snack. The volunteer will be asked to consume the Unit meal and study beverage 330ml of 5% ABV beer, either hop-rich or matched low-hop control, which is the equivalent of 1.6 units of alcohol, within ~15 minutes. Participants will be asked not to drive after the completion of the visit.

Blood samples will be taken post intervention (same as at baseline) and the self-reported mood measures (questionnaires and visual analogue scales) will also be repeated.

At the end of the first testing visit day, volunteers will be given a briefing for the next and final acute study visit day, which will be at least 60 hours after the first study visit to allow time for washout. On completion of their final acute study visit, which will be the exact same procedure except with a different drink, volunteers will be briefed on the procedure of the chronic trial that will follow and be provided with the intervention drinks (a non-affiliated member of staff or student will be responsible for handling and administration of intervention drinks for blinding purposes). Finally, their upcoming visits (for the chronic trial) will be scheduled.

### *Chronic trial*

After the completion of the acute trial, study participants will be given at least 3 days to recover before starting the chronic trial. For the chronic trial, provided they are happy to proceed, volunteers will be asked to consume one can of intervention drink (330ml, 5% ABV) a day, which translates to 11.2 units of alcohol per week, for 3 weeks (intervention or matched control). The intervention and control drinks will be the same as the ones used for the acute trial and the order in which they will be administered will be randomly assigned. A three-week washout period will be allowed between the two different chronic treatments. Study participants will be invited to visit the Hugh Sinclair Unit of Human Nutrition on 3 occasions: at the end of week 3 (i.e. after 3 weeks of daily consumption of the the study drink or matched control), between weeks 5 and 6 (during washout period) to be provided with intervention drinks (control or active accordingly) which they will be asked to consume for 3 weeks, and to report any concerns and confirm

they are happy to continue, and last, at the end of week 9 (i.e. after 3 weeks of daily consumption of the second study drink). The order in which the drinks will be administered will be randomly assigned and the packaging (cans) will be covered with opaque tape, for blinding purposes. At the end of each 3-week intervention period, volunteers will be asked to return the empty cans. A non-affiliated researcher or member of the Hugh Sinclair Unit will be responsible for handling the administration of the intervention drinks for both trials, acute and chronic.

### Testing Visit day procedure (week 3 & week 9)

For each of the two testing visits, volunteers will be asked to arrive at the Hugh Sinclair Unit of Human Nutrition in a fasted state (12-hour overnight fast: only water allowed, starting after dinner the evening prior to each visit). Water will be available throughout the study day. On arrival at the Unit, the experimenter will confirm that the volunteer is happy to proceed with their participation in the study. If not, the volunteer will be thanked and asked to complete a study payment form to receive remuneration for any main study visits previously attended. If the volunteer is happy to proceed with their participation, the study procedure outlined in Figure 2 below will be performed on each study day (timings are approximate).

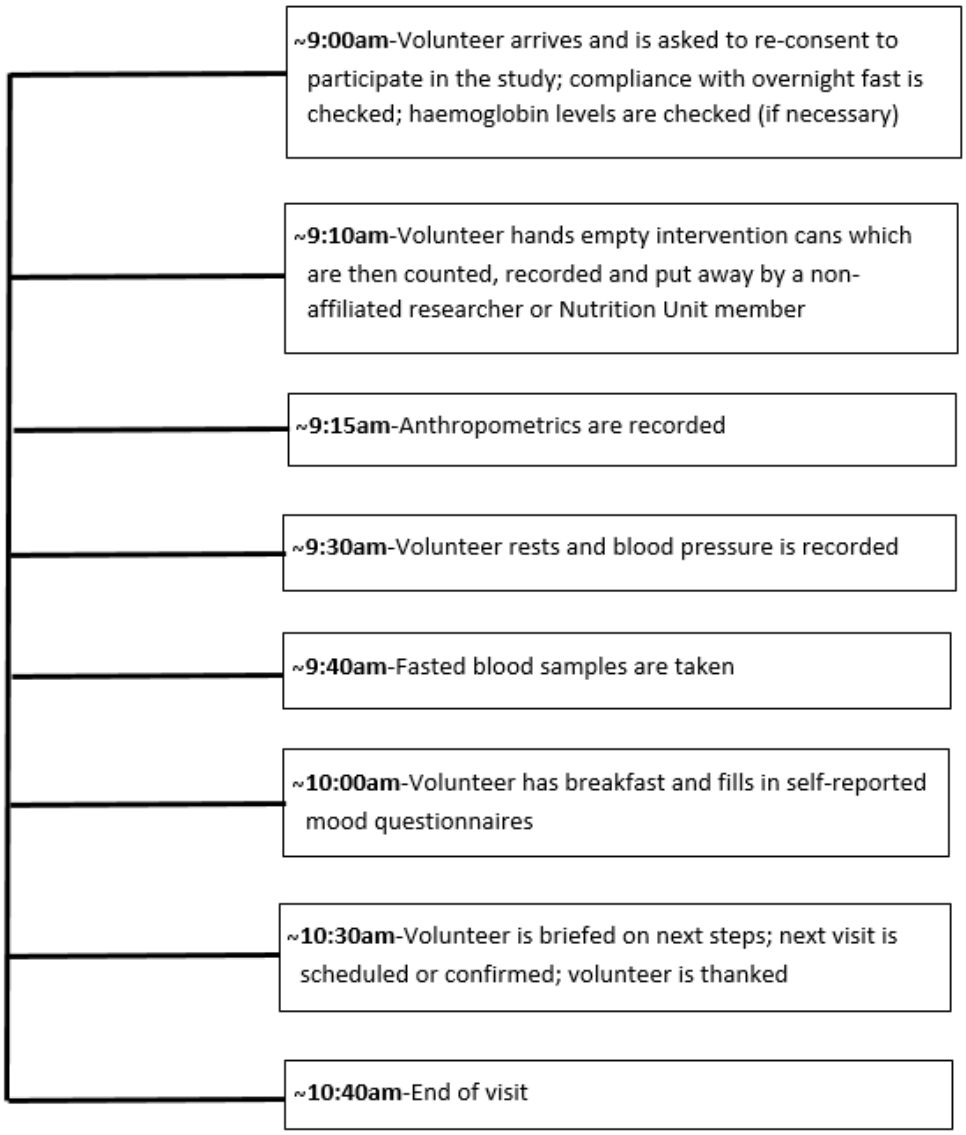


Figure 4. 2 Chronic trial testing visit timeline (Timings are approximate)

Compliance with the requirements for overnight fasting will be checked. Empty intervention containers will be handed in and will be counted and recorded by a non-affiliated researcher, student or member of the Nutrition Unit. In cases where more than 8 weeks has elapsed between the participant's screening visit and first study visit, Haemoglobin levels will be checked using the Haemocue (according to standardised procedure, involving a small finger prick) in accordance with the revised Hugh Sinclair Unit procedure. Anthropometrics will then be recorded. The volunteer will then rest for ten minutes before having blood pressure measured. Fasted blood samples will then be taken

by a research nurse or a trained phlebotomist. Afterwards, volunteer will be given breakfast and asked to fill in self-reported mood questionnaires (DASS-21, Hamilton scales for depression and anxiety, Spielberger State Anxiety Inventory as well as Bond Lader Visual analogue scales). Volunteer will then be thanked and briefed on next steps and upcoming visits will be scheduled or confirmed.

### Detailed methodological information: Blood samples

Similarly to the acute trial, 12 EDTA tubes and 2 serum tubes, which is a total of 60 ml of whole blood approximately, will be obtained. Blood samples will be processed accordingly in order to obtain plasma or serum, which will then be stored and used at a later stage to test the levels of neurotransmitters, and other mood related biomarkers and their metabolites (listed below):

- Plasma Dopamine and its metabolites, 3-O-methyl-Dopa and Homovanillic Acid
- Plasma Serotonin (5-HT) and its metabolite 5-hydroxindole acetic acid (5-HIAA)
- Plasma Monoamine Oxidase (MAO A and B)
- Plasma Adrenaline & Noradrenaline
- Plasma Cortisol
- Serum S100B proteins
- Plasma Gamma-Amino-Butyric Acid, Succinic Acid, α-ketoglutaric acid & glutamine

### Interim Visit

Towards the end of the washout period, i.e. weeks 3 to 6, volunteers will be asked to visit the Hugh Sinclair Unit of Human Nutrition to be provided with the next treatment drink (active or control accordingly) and also to report any concerns, ask questions and confirm they are happy to continue. Again, a non-affiliated member of the Nutrition Unit will be responsible for the administration of the intervention drinks. The next-and final-study visit will also be scheduled or confirmed.

### *Intervention Drinks*

The active intervention drink will be a hop-rich beer (provided by an external company) which is brewed using the method of dry-hopping. This means that dry hops are added to the beer after the boiling process. The aim of dry hopping is to extract and retain as much of the hop extracts in the finished product as possible, ensuring not only richer aromas but also higher hop concentrations. A widely available lager beer, matched for alcohol content (5% ABV) will be used as a control, since, based on previous research in our lab, some of the most common, widely available lager and pils style beers contain very low amounts of hop phenolics compared to ales and, of course, dry-hopped beers. The phenolic profile of the intervention beer, both the active and the control, will be determined using HPLC at the end of the clinical part of the study.

### *Sample Size and Statistical Analysis*

A sample size of at least 33 participants was determined based on a similar published study by Kyrou and colleagues [124], who measured self-reported mood following hops extract intake in young adults. This was in agreement with a sample size that was estimated using an online calculator to achieve a statistical power of 80% with a significance level of $p \leq 0.05$ assuming no dropouts.

Primary and secondary outcomes will be analysed per protocol using SPSS v25 (IBM, Armonk, NY, USA). LMM with an unstructured covariance matrix for repeat measurements will be carried out for each endpoint. Intervention group and Visit will be fixed factors in the model. Bonferroni corrected pairwise comparisons will be examined regardless of the significance of the overall F test statistic.

## Conclusion

This clinical trial protocol was designed to investigate the effects of hop flavonoids on mood in young adults that are likely to have common mood disorders, such as depression and anxiety. We aim to create a link between self-rated mood and biochemical/neurochemical parameters that are related to mood. We anticipate to see decreases in self-rated stress, anxiety and depression scores and changes in biochemical

parameters, possibly indicating increased GABA-ergic activity and a potential decrease in serum S100b proteins, following ingestion of hop phenolics in the form of a hop-rich beer. Furthermore, we will assess whether any observed changes in the biochemical parameters coincide with any improvements in self-rated mood as an attempt to identify potential biomarkers of mood in common mood disorders such as depression and anxiety in otherwise healthy young adults. Such findings can have an implication in the current ways of managing and treating common mood disorders and will create scope for future research in mental health.

## Author's Contribution

M.G. contributed to the following: project conception and design; protocol development and operationalisation; ethics application; clinical trial registration.

GENERAL DISCUSSION

# Answers, Questions and Future Directions

There is increasing interest in dietary polyphenols as nutritional supplements for the improvement of neurocognitive performance, based on previous research suggesting that various sources of polyphenolic compounds have such properties, as discussed and reviewed in previous chapters. Although there is evidence that cocoa as well as various berry derived polyphenols have the ability to enhance cognitive function, which has been clinically tested and reviewed to a great extent [49, 148] as an attempt to better understand the bigger picture and clinical relevance of those findings, there are still unanswered questions and literature discrepancies. Even though there is encouraging evidence regarding the efficacy of various sources of polyphenolic compounds, the mechanisms through which they induce those effects remain unclear.

There is a body of research supporting that the neurocognitive benefits seen after the consumption of polyphenol-rich treatments are associated with beneficial effects on vascular function, since better cognitive performance is associated with greater cardiovascular health [149]. In addition, age related cognitive dysfunction occurs with cardiovascular disease and other comorbidities, including reduced cerebral blood flow [74]. Consistent with these claims, we showed that 24 weeks of cocoa flavanol supplementation can improve vascular function, seen as increases in FMD response and reduced heart rate, as well as reduced plasma concentration of NEFA after a 12-week wash-out, all of which are predictors of vascular health [150]. These findings are of great clinical importance when it comes to establishing ways of improving arterial function and potentially the treatment and/or prevention of cardiovascular disease, which, according to the World Health Organisation, is one of the main causes of death worldwide [151]. These results also add to previous clinical findings supporting the hypothesis that flavonoid supplementation can enhance endothelial function [150].

However, one of the objectives of this thesis was to question whether said vascular effects are predictors of beneficial neurocognitive effects and whether they can be attributed to the polyphenolic constituents of cocoa. Although we failed to substantiate

this foundation, we showed that increases in cognitive performance seen after chronic cocoa flavanol supplementation might be attributed to secondary bioactive constituents found in our treatments, such as theobromine and caffeine, two naturally occurring cocoa methylxanthines. This creates plenty of scope for future research, and an urgency to differentiate the effects of flavanols from those of theobromine. Although the clinical effects of theobromine in humans are insufficiently investigated, there is some evidence supporting that methylxanthines might be the main psychoactive constituents of cocoa [51], even though they occur in much lower concentrations than flavanols. Moreover, the interaction of methylxanthines with cocoa flavanols can, according to previous research, result in a greater enhancement of FMD, mainly due to an increase in flavanols absorption, but in this case no vascular effects of methylxanthines alone are detected [52]. This, along with findings from animal studies indicating that theobromine improves cognitive function and intra-neuronal amyloid-β protein levels, the accumulation of which in the brain is a typical characteristic of Alzheimer's disease [152], sets directions for future research. The questions that arise here are: Do methylxanthines, such as theobromine, have the ability to increase flavanol absorption and is that the indirect mechanism whereby methylxanthines benefit neurocognitive health? Although our data cannot support that-in fact the overall neurocognitive benefits do not line up with the overall vascular benefits, therefore our findings could not be attributed to the methylxanthines on this occasion-it is still our inclination that this theory has a lot of promise and requires further investigation in the future.

In agreement with previous findings suggesting that flavonoids can improve glucose metabolism and insulin response [19, 81, 82, 106, 107, 119], we showed that grapeseed flavonoids can reduce the postprandial blood glucose peak time, by approximately ten minutes compared to placebo, which supports the hypothesis that flavonoids can reduce glucose uptake. We further observed reductions in fasting glucose concentrations following a 24-week supplementation of both cocoa flavanol and matched placebo that sustained after the cessation of the treatment, which could, again, be attributed to the methylxanthines found in both treatments. However, that would go against research findings showing an unfavourable effect of theobromine on fasting glucose and insulin [153]. Based on our observations, it is speculated that effects on glucose metabolism and insulin response are more likely to be noticeable on a postprandial level, and less so after

chronic polyphenol supplementation. Although these findings could have important implications on the prevention and treatment of type 2 diabetes, and the associated risks of cardiovascular dysfunction, we highlight that further research is required to establish whether such glucoregulatory effects coincide with improvements in neurocognitive function and whether this is an underlying mechanism though which polyphenols elicit beneficial neurocognitive responses.

With regard to cognitive performance, although we did not demonstrate any significant improvements in any of the cognitive behavioural outcomes, we attempted to shed light on how polyphenol-rich interventions may have the ability to attenuate mental fatigue that occurs during highly demanding or stressful situations. Consistent with Massee and colleagues [22] we showed that acute grapeseed flavonoid supplementation can maintain cognitive performance during a stress-inducing cognitive test battery. Although Massee and colleagues only managed to show those effects on an acute and not on a sub-chronic level, we observed similarly unchanged cognitive outcomes following 24 weeks of cocoa flavanol supplementation. Albeit non-significant, this lack of cognitive decline could be suggestive of protective effects of flavonoids against mental fatigue, especially considering the increased levels of sedation observed in our data.

We also saw patterns of retroactive interference, predominantly in the acute trial, where study participants struggled to distinguish between different testing sessions across the day and the week, which had a negative impact on learning and memory. That could be because participants performed the cognitive test battery on more than one occasions (twice a day, two days a week), which led to confusion and a subsequent decline in cognitive performance during certain tasks, mainly memory tasks. This pattern was not seen in our chronic trial, were participants completed the same cognitive test battery only once per testing visit. We therefore believe that this profound drop in memory function in the acute trial was likely due to fatigue and/or retroactive interference. Although that had, seemingly, no impact on 'global cognition' it still implied that some tasks were very challenging and others too easy for this population sample. This limitation needs addressing in future research. We suggest that long and multi-domain cognitive test batteries may be overwhelmingly complicated especially when completed twice across the day, and we are inclined to lean towards domain specific approaches, when it comes to cognitive behavioural testing, as discussed in Chapter 1. However, as yet, there is no

consensus with regard to what cognitive tests are best, more sensitive or more suitable for nutrition intervention studies, and the methods used in the literature are vastly inconsistent. The different patterns of cognitive performance that we observed during different cognitive tests of different difficulty and cognitive demand levels, for instance extremely high performance on inhibition and sustained attention type of tests, as well as verbal fluency tests, might be an indication of 'ceiling effects'. In that case, any beneficial effects that dietary flavonoids may have had, would be undetectable. On the contrary, poorer performance both at baseline and post-intervention observed during highly demanding tests, such as corsi blocks, that require extreme coordination of various executive functions as well as high-level spatial working memory, might be where flavonoids can have more prominent effects. Gratton and colleagues, showed that flavonoids can increase cognitive performance only during highly demanding cognitive tasks and they further suggest that future research should use difficulty-graded cognitive challenges for testing the effects of dietary flavonoids on human cognitive function [44], which we are inclined to agree with, based on the vastly different behavioural patterns amongst different neurocognitive tests in our data.

In addition, the overall lack of significant improvements in cognitive function in our research could also be explained by the fact that our population sample was high functioning. Therefore, small changes in cognitive performance were basically undetectable. Future research should aim to address this limitation by testing the effects of polyphenol-rich interventions in subjects that are in high risk of dementia or have been diagnosed with some level of cognitive decline. Not dissimilar to the study of Desideri and colleagues [19], who reported beneficial effects of chronic cocoa supplementation on executive performance, at a verbal fluency task, in a sample of MCI individuals. This approach not only supports the notion that cognitively impaired individuals may benefit more from polyphenol interventions but also that a single task, or single domain approach might be more effective in the assessment of those effects.

As an attempt to follow up the above mentioned limitations regarding the different methods of assessing cognitive performance across the literature, we aimed to establish a basis to support the 'domain focused' approach, by conducting a systematic review of clinical trials. We focused on executive function, a broad domain that has been investigated by many in nutrition research, including ourselves, but rarely as an independent outcome.

To our knowledge, this is the first systematic review in the field that focuses on executive function, whilst highlighting the critical importance of executive function in cognitive health. Our results were partially in agreement with the systematic review of Barrera-Reyes and colleagues [49], who report that the most commonly evaluated domains in nutrition intervention trials with cocoa-based treatments were indeed executive function and memory. Furthermore, we are in agreement with the fact that there is immense variability across clinical trials investigating the effects of cocoa flavanols on cognitive function, which makes the analysis and interpretation of the results extremely challenging. This also puts the whole area of research into perspective and highlights the need for consistency in future research.

Moreover, we aimed to bring the effects of dietary polyphenols on mood into focus, an area of great clinical importance as it relates to mental health and potentially mental disorders. We showed that both chronic cocoa and acute grapeseed flavonoid supplementation led to changes in self-rated mood, seen as increases in sedation. Although these self-reported measures of mood are subjective and should be interpreted with caution, we saw a noteworthy pattern in sedation in both intervention strategies. This is in agreement with the hypothesis that some polyphenolic compounds have the ability to interact with the GABA-ergic system in the brain, thus exerting sedative effects [96], however this suggested neurochemical mechanism requires further investigation in humans. We speculate that said increases in sedation, both acute and chronic, may be beneficial in the sense of preventing any stress induced cognitive decline, however we failed to verify our hypothesis that cortisol, a glucocorticoid that plays an important role in the regulation of stress, has any involvement in that process. Although previous research has shown that polyphenols can have beneficial effects on cortisol response [72, 73], in our experience both salivary and serum cortisol levels appear to have a circadian pattern that is challenging to manipulate. Therefore, we are inclined to believe that any anxiolytic effects of polyphenol intake on mood may, more likely, be driven by their ability to allosterically modulate GABA receptors, than by influences on cortisol response, however further investigation on humans is required to confirm this speculation.

In addition, we endeavoured to assess the above mentioned conjectures regarding the effects of polyphenol-rich interventions on mood by developing a clinical trial protocol that would aim to determine the levels of serum GABA and its metabolites, as well as other

neurotransmitters, and, possibly establish a biochemical marker of mood. Several biochemical markers of mood have been mentioned and researched in the literature [154], however there is no conclusive evidence as to whether those biomarkers can be used clinically, for diagnostic and research purposes, especially when it comes to mild mood disorders, such as mild anxiety, that are difficult to test and diagnose. We hypothesised that using a more potent polyphenolic source as a treatment for common mood disorders, that has been previously shown to interact with the GABA-ergic system, in a sample of the population that are likely to have such disorders might yield stronger, more noticeable benefits on mood. Using a different source of polyphenols this time, i.e. hop polyphenols, which are known to be potent phytochemicals with oestrogen-like properties and have been previously shown to exert sedative and anxiolytic effects, may lead to more prominent effects and will likely answer some of the questions that emerged from our previous clinical trials (Chapters 2 and 3) with regards to effects on mood and sedation.

This research can have important implications in the area of mental health and the alternative ways of treatment of common mental disorders as it could potentially support claims that polyphenolic compounds, such as the ones found in Gingko biloba extracts, can be effective as adjunctive treatments for depression, enhancing the efficacy of SSRI's, in elderly patients with depression [155]. It may also generate further questions and answers regarding depression and other mood disorders in the ageing brain, since depression is a common comorbidity in dementia [156]. Additionally, it could reveal certain neurochemical mechanisms of action through which the polyphenolic compounds of interest benefit the nervous system, which could be via stimulating serotonergic neurotransmission [157], effect previously attributed to naringenin, a flavanone that is naturally occurring in grapefruit [130, 158]. This can cycle back to cognitive performance, shedding light on the potential modulating effects of serotonin receptor agonists on cognition, memory and learning, a mechanism that is still poorly understood [159].

In summary, this thesis has contributed to the field by providing evidence supporting the beneficial effects of polyphenol-rich nutritional interventions on mood, vascular function, glucose metabolism and, to an extent, cognitive function. Most importantly, it has created plenty of scope for future research, both in the area of mood and mood disorders and the area of cognitive performance and decline. Although I only partially succeeded in proving my primary hypothesis: Flavonoid-rich interventions can

enhance cognitive function and mood, I have produced some evidence to suggest the biological activity of dietary flavonoids and the potential to link them to brain function. My team and I observed beneficial effects of flavonoids on mood and attempted to explain the lack of beneficial effects on cognitive performance. Through the limitations of my research, and the speculations, as explained throughout this thesis, as well at the scientific findings and limitations of other researchers, I attempted to expand both my knowledge as well as create new knowledge in the field of dietary flavonoids and nutritional neuropsychology. As a closure note on what I have learnt through this journey and what I am inclined to believe, bearing in mind that we cannot produce any conclusive statements at this stage, I strongly believe that additional attention should be given to the potential psychoactive effects of polyphenolic compounds from a neurochemical point of view, since, as discussed above and in earlier chapters, the behavioural outcomes are subjective, often hard to interpret and the methods used to assess them are very inconsistent. This could also help expand on current theories supporting interactions of dietary flavonoids and their metabolites with neurotransmitter receptors and the whole CNS. Furthermore, even though cocoa and berry polyphenols have been thoroughly investigated for their cognitive and physiological effects, there is still a gap between the cognitive outcomes and the underlying biological and biochemical mechanisms. Current and future research should aim to link the cognitive behavioural effects with physiological effects, and modern brain imaging methods have been playing an influential role in the field. In addition, it is my opinion that prenylated flavonoids in hops, a polyphenolic source that is underresearched, has a lot of promise, and future research should explore the psychoactive properties of hop polyphenolics in humans, as there is a scarcity of published clinical research on these compounds.

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