Characterising the Response to Blueberry Dietary Interventions Aimed at Improving Cognition and Vascular Function

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Abstract

Inter-individual variations exist in response to dietary factors and to the pathophysiologic development of endpoints related to vascular diseases and cognitive impairment. Therefore, the evaluation and characterisation of responses to a dietary intervention targeting vascular and cognitive health is of importance. A series of investigations were set out. Firstly, previous evidence of randomised controlled trials (RCTs) supplementing fruit and targeting vascular and/or cognitive improvement was sought and evaluated by systematic reviews incorporating meta-analyses. Collectively, the reviews have shown that the consumption of berries with dosage ranging from 22 to 45 g powder, 150 to 300 g frozen berry and 100 to 500 ml juice resulted in a 3.68 mmHg reduction on systolic blood pressure and 1.68 mmHg reduction on DBP. A human dietary intervention with 37 participants was performed comparing two forms of blueberry; either whole fresh blueberry (160 g), freeze-dried blueberry powder (20 g) or placebo control (microcrystalline cellulose) in a 1-week single-blinded cross-over RCT in a relatively young outwardly healthy population. There was no significant effect of either blueberry intervention to improve either vascular function or cognition. No significant putative discriminating urinary metabolites between interventions were found using supervised multivariate analysis. The response to the intervention was calculated for each endpoint using percentage change (+ / -%) compared to the baseline. Extensive inter-individual variation was found in vascular health parameters (- 141 % - + 525 %) and cognitive domains (- 114 % - + 96 %) post-interventions, but there was no consistent response following the two interventions between and within participants. Although several discriminatory metabolites were found between responder (RS) and non-responder (NRS) groups it was not possible to identify predictors of response using receiver-operator-curve analysis. To conclude, we did not find a predictive urinary metabolite as a potential biomarker for differentiating between RS and NRS and no consistent individual responses following both blueberry and blueberry powder interventions were found. This is the first blueberry intervention applying quartile division to characterise response in vascular and cognitive endpoints following a specific dietary intervention. The overall approach for defining a metabolic signature of response could be used in the future for tailoring personalised nutritional advice.

Publication

Peer reviewed publication arising from this course of investigation:

Wang, Y. Gallegos, J.L. Haskell-Ramsay, C. et al. Effects of chronic consumption of specific fruit (berries, citrus and cherries) on CVD risk factors: a systematic review and meta-analysis of randomised controlled trials. Eur J Nutr (2020). https://doi.org/10.1007/s00394-020-02299-w

Wang, Y. Haskell-Ramsay, C. Gallegos, J.L. et al. Effects of chronic consumption of specific fruit (berries, citrus and cherries) on cognition and mood: a systematic review and meta-analysis of randomised controlled trials. (Under review*)*

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Declaration

I declare that the work contained in this thesis has not been submitted for any other award and that it is all my own work. I also confirm that this work fully acknowledges opinions, ideas and contributions from the work of others.

Any ethical clearance for the research presented in this thesis has been approved. Approval has been sought and granted by the Northumbria University's Faculty of Health and Life Sciences Ethics Committee (reference: 10113) on 30th October, 2018.

I declare that the Word Count of this Thesis is 44,967 words

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Signature:

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Chapter 1. Introduction

1.1 Background

Cognitive decline and impaired vascular function are common and important characteristics that differ in extent between individuals [1, 2]. Cognitive decline with ageing has been shown to increase the risk of neurodegenerative diseases and impaired vascular function is also involved in the pathogenesis of cardiovascular diseases (CVD) [1, 2]. The modulation of cognition and vascular function by normal genetic variation is well established while individual variation is mainly influenced by lifestyle behaviours, with an unbalanced diet being one of them [1, 3, 4]. Therefore, to identify individuals that show improved responses following a specific dietary intervention can help to improve an individual's dietary regime to maximise health benefits.

Recent developed technologies allow the use of high-throughput '-omic' analysis in nutritional research [5], and metabolomic analysis can help to identify variations in metabotypes across individuals that show different responses to the biological endpoints. Overall, this PhD programme investigated dietary whole blueberry and freeze-dried blueberry powder interventions aimed at improving a range of endpoints related to vascular function and cognition and characterised the inter-individual variation in each endpoint to categorise participants as responders (RS) or non-responders (NRS) to the intervention. Finally, we aimed to identify metabolic predictors of response to the intervention using metabolomics.

1.1.1 Vascular function and vascular assessments

Cardiovascular disease (CVD) is currently one of the major causes of death worldwide. World Health Organisation (WHO) has reported that 31 % of global death (17.9 million) in 2016 were attributable to CVD [6]. Therefore, vascular homeostasis and function are important for health and well-being. Improvement of blood pressure, the plasma lipid profile including cholesterol and triglycerides have been shown to associate with risk reduction for CVD [7]. Therefore, maintaining normal blood pressure level and blood lipid profile were important for maintaining normal vascular function. Endothelial function is also an important indicator of vascular health. Endothelial dysfunction is a pathological state of the vascular endothelium (the inner layer of blood vessels) and

is a major manifestation underlying atherosclerosis, CVD or other vascular diseases [8]. The key to healthy ageing in terms of vascular change is also to maintain homeostasis of endothelial function [9]. The circulating endothelial-dependent relaxing factor, nitric oxide (NO), possesses a wide range of biological properties that regulate vascular homeostasis, modulate vascular dilator tone, balance local cell growth and protect the vessels from platelets aggregation, indicating an essential role in maintaining normal vascular function [10].

There are different non-invasive methods assessing vascular health in human interventions that contribute to inter-individual variation in response to the assessment tools [11]. The most prevalent measurements utilised in previous studies assessing vascular health include pulse wave velocity (PWV) for assessing arterial stiffness and flow-mediated dilation (FMD) for assessing endothelial function [12-15]. Other methods assessing vascular health including the reactive hyperaemia index (RHI), augmentation index (Aix), the lase doppler flowmetry and iontophoresis have been applied in a wide range of studies [16-19]. PWV reflects the speed of the blood pressure wave to move down the blood vessel, it is a good indicator of arterial dispensability and stiffness [20]. Vascular (dys)function has also been assessed by PWV in previous interventions [12-15]. Flow mediated-dilation (FMD) has been largely used in studies assessing endothelial (dys)function as the gold standard tool [13, 21-23]. However, there were also inconclusive reports using FMD, and on other methods (e.g. the reactive hyperaemia index/RHI, augmentation index/Aix, the lase Doppler flowmetry and iontophoresis etc.) to assess vascular (dys)function [16-19]. The methods measuring vascular health have been compared previously and PWV presented with better repeatability and reproducibility than FMD, so FMD was not used in the current study [11]. It should be noted that the choice of vascular assessment tools could explain a level of variation in different interventions, as reviewed in Chapter 2.

1.1.2 Cognition and cognitive assessments

Cognitive function is comprised of cerebral activities that involve various cognitive domains, such as attention and memory, to help our brain process and react to every-day tasks [24]. Cognitive function is commonly assessed through the domains of attention, executive functions, memory functions, language, visual perception and

processing, social and emotional processing [25]. The cognitive domains categorised in studies often vary depending on the cognitive ability that researchers intend to assess, for example, researchers have argued that the information processing speed and attention underlie the domain of executive functions [26].

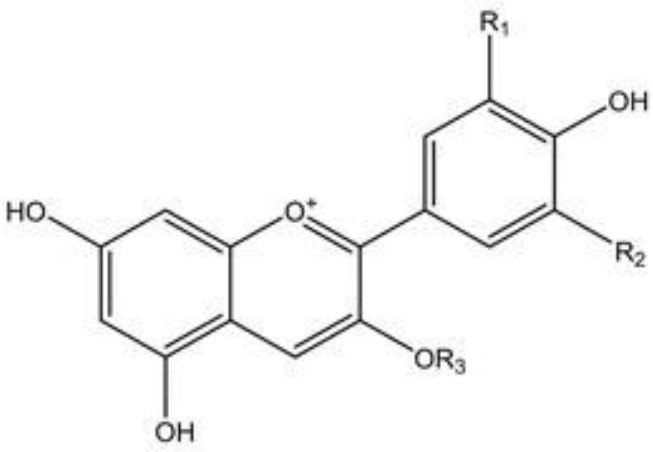
Neurodegenerative diseases like dementia and Alzheimer's disease are shown to be associated with cognitive impairment [27]. The measurement of cognitive impairment is important since the rising prevalence of dementia [27] and the benefits of earlier detection and prevention [28]. Cognitive tasks used for the cognitive assessment normally depended on the task requirements [25]. For example, for the assessment of executive function, tasks such as Stroop Colour and Word Test [29, 30], Serial 3s and/or Serial 7s Subtractions [29, 31], Corsi Block-Tapping Task [29], Digit Span (backward) [32, 33] are usually used. For memory assessment, tasks such as List Learning Tasks and Word Recall (e.g. California Verbal Learning) [34, 35], Paired Associate Verbal Learning Test [31, 32], and Picture Memory are often used [29]. Self-rated Scales have also been used to assess mood in a range of studies [29-31]. However, the impact of blueberry interventions on specific cognitive domains could not be quantitatively compared with a number of other trials because of the large variability in the assessment tools and scoring interpretations. This concern also presented in other dietary trials assessing cognition [36]. For example, the number of correct answers or the time taken to complete the task have been interpreted per study to assess processing speed, as per the meta-analysis in this area [37]. For example, here in the meta-analysis in Chapter 3, we only categorised the time taken to complete the task as assessing processing/psychomotor speed and the accuracy (or the number of the correct answers) assessing attention or working memory, depending on the specific tasks, in order to achieve quantitative comparison across studies. Consistently reported cognitive performance tools are recommended for future studies to better interpret study results.

1.1.3 Blueberries

Blueberries have become very popular in the United Kingdom. In 2018 the United Kingdom imported 48,000 tonnes of blueberry (the genus Vaccinium), together with the national production the average annual consumption reached an estimated 0.7 kg

per capita [38]. Blueberries contain significant amounts of nutrients, such as vitamin K, vitamin C, folate, K, Mn, Cu and an array of polyphenols which have been shown to exert neuro- and vascular-protective in *vivo* and in *vitro* [39, 40] and for this reason are supplemented in the current interventions. Anthocyanins are the major class of polyphenols in blueberries (approximately 92 mg/100 g) and include monoarabinosides, monoglucosides and monogalactosides of cyanidin (Cy), petunidin (Pt), peoni- din (Pn), delphinidin (Dp) and malvidin (Mv) and their glucosides [41] **(Figure. 1.1).**

Figure. 1.1 The major classes of anthocyanidins [42]



Anthocyanin	R_1	R_2	R_3
Cyanidin	OH	H	H
Delphinidin	OH	OH	H
Malvidin	OCH_3	OCH_3	H
Pelargonidin	H	H	H
Peonidin	OCH_3	H	H
Petunidin	OCH_3	OH	H
Cyanidin-3-glucoside	OH	H	Glc
Delphinidin-3-glucoside	OH	OH	Glc
Malvidin-3-glucoside	OCH_3	OCH_3	Glc
Pelargonidin-3-glucoside	H	H	Glc
Peonidin-3-glucoside	OCH_3	H	Glc
Petunidin-3-glucoside	OCH_3	OH	Glc

The anthocyanin profile in berries is a factor explaining variability in the biological responses observed in dietary interventions with berries. For instance, blueberries contain primarily delphinidin, malvidin, and petunidin whereas raspberries and blackberries contain primarily cyanidin, pelargonidin and malvidin [43]. Although consumption of anthocyanins can be in the range of 200 mg/d [44], the bioavailability appears low as they are believed to be poorly absorbed and rapidly excreted [45]. The rate of polyphenol absorption from blueberries could be influenced by dose administered [46], and the matrix of the food source [47], and several studies have suggested that the rate of anthocyanin absorption is influenced by their chemical structure. Targeted analysis of bioactives reveals plasma concentrations of 0 to 4 micromolar from an approximately 50 mg dose with excretion ranging from 0.3 % to 43 % [45]. Studies have pointed to an approximately 1 - 2 h peak max in plasma [48-50], with a peak max in urine from 2 h [50, 51], but metabolites can persist in urine for

5 d after consumption [51]. The bioavailability of blueberry metabolites will vary between individuals and is dependent on complex absorption, distribution, metabolism and excretion (ADME) mechanisms [52]. Variations can be due to phase I and II metabolism of blueberry anthocyanins and the enzymes could play a role at the microbial, age, smoking, sex, and genetic levels to induce variability in anthocyanin metabolism [53, 54]. Indeed, the anthocyanin metabolites such as glucuronides, sulphates and glycoside derivates [48-50] formed during phase 2 metabolism, are present in large diversity and intensity [51]. Bioavailability has also been compared between whole blueberries and blueberry juice. An acute metabolite profiling study examined the difference in the plasma and urinary metabolome 2 h following ingestion of either blueberry juice or whole blueberries, and found higher intensity of phenolic metabolites (including those conjugated to sulphate, glycoside or glucuronide), and fatty acyl derivatives in urine following whole blueberry intake compared to blueberry juice intake, however further time points are required for a full biokinetic profile [55]. Nevertheless, the differences were relatively minor. Furthermore, Kuntz *et al*. compared the bioavailability of selected anthocyanins from grape and blueberry juice with a smoothie and found no difference in plasma pharmacokinetics and recovery of the major anthocyanin species, but found significantly higher concentrations of the phenolic acid 3,4-dihydrobenzoic acid after ingestion of the juice [56]. These data may help to explain bioavailability variations in the blueberry products. Also, a systematic review has shown that the intake of fruit juice offered similar protection against cancer and cardiovascular disease to the intake of whole fruit [57], and thus a similar proportion of bioactive phytochemicals must remain in the processed products.

1.1.4 The effect of blueberry intake on vascular function and cognition

The normal functioning of our brain demands energy for various neuronal activities (e.g. synthesis and actions of neurotransmitters, nerve impulse propagation, metabolism of homocysteine etc.), all these functions require sufficient and constant supply of micronutrients to the brain [58, 59], which indicates that dietary factors have an important influence on cognitive function. There is also commonly accepted evidence from recent years that normal vascular function is also associated with heart-healthy eating behaviours [60]. One in *vivo* study observed that only cyanidin and delphinidin could induce the inhibitory effect on the redox-sensitive p38 MAPK and c-

jun-N-terminal kinase pathways that have been shown to improve platelet function, an indicator for reduced blood pressure [61]. This provides a potential mechanism of action for benefits to vascular function by blueberry ingestion.

Single nutrients, such as vitamins, minerals, phytochemicals and polyphenols (e.g. cocoa flavonoids) from plant - based foods have shown beneficial effects in improving cognition, and vascular health [62-66]. However, from nutritional perspective, employing single whole foods instead of single components as a supplementation in interventions is more reasonable. Unlike natural bioactive compounds, synthetic single nutrient supplements are likely to be metabolised through different pathways. For example, concern has been raised over the use of supplemental folic acid instead of the consumption of leafy green vegetables in that synthetic folic acid is metabolised in the liver instead of the gut similar to natural folates [67]. Therefore, the protective effects on vascular function and cognition of whole fruit intervention or fruit intervention in different forms (e.g. powder, juice) instead of single nutrients are worth exploring.

As reviewed in Chapters 2 & 3, only one study has supplemented whole frozen blueberry [68]. There have been interventions supplementing freeze-dried blueberry powder (22 - 45 g, 4 - 8 weeks), blueberry juice (480 ml, 8 weeks) that have reported either no effects or improved effects on blood pressure (BP) [69-74], PWV [69, 73], blood lipids [71, 73, 75] and nitric oxide (NO) [69, 73, 76]. For cognition and mood assessment measuring memory, executive function, attention, psychomotor speed or mood scales, long term interventions (100 mg extract powder - 200 g whole blueberry equivalent, 6 - 24 weeks) have found inconclusive results on cognitive performance [29, 68, 77].

Chapters 2 & 3 & 7 have systematically reviewed and discussed dietary blueberry interventions targeting high-risk populations that have demonstrated positive findings on the improvement to vascular health and/or cognitive health. In conclusion, low-risk populations (< 60 years old and healthy) receiving either blueberry powder or whole blueberry (100 mg extract - 560 g whole blueberry equivalent) or concentrate (30 ml) supplementations both acutely (1 - 24 h) and chronically (6 - 24 weeks) have shown less beneficial effects compared to high-risk populations towards vascular- and

cognitive-protective benefit following blueberry consumptions independent of the various dosage and duration implemented.

1.1.5 The effect of processing on blueberries

Food processing, food temperature and pH might also modify blueberry anthocyanin content bio-accessibility and bioavailability, depending on the technology and processing conditions [78]. Processing, such as smashing or thermal conditions, can damage the cell structure of blueberry releasing cytoplasmic content that can make bioactive compounds like anthocyanins more accessible for absorption [79]. Moreover, the presence of other constituents formed from the technological process or added from food matrix could modify the bioavailability of the anthocyanins due to their liability to bind, solubilize, or stabilize anthocyanins [78]. In previous interventions, the blueberry powder was usually consumed with a meal [69, 72] and the interaction with the food matrix could be one of the main factors determining bioactive stability and accessibility.

In terms of the nutritional value, blueberry powder normally contains a lower sugar content compared to the whole blueberry and the glucose load in the fruit matrix has been shown to delay anthocyanin absorption, which could result from the competitive action of glucose and anthocyanin on the sodium-dependent glucose cotransporter SGLT-1 [80]. The blueberry skin is rich in anthocyanins and usually kept during the freeze-drying processing of the blueberry to powder [81]. It has also been shown that fibre content is contained during the processing from blueberry to powder [82]. The technological processing to blueberry powder could also cause increased bio-accessibility and absorption of bioactives in relation to the whole blueberry [78]. The metabolic difference and treatment effects shown following whole blueberry and processed blueberry interventions are reviewed and discussed throughout Chapters 5 - 7.

1.1.6 Inter-individual variations in vascular and cognitive response to dietary intervention

It is agreed that effective dietary strategies can help to reduce the global burden of nutrition-related non-communicable diseases (NCDs) [83]. Most dietary interventions and the corresponding guidelines focus on population averages [84, 85]. However, a range of recent studies have addressed the high inter-individual variations in response to dietary interventions that demand the development of characterising response to the clinical endpoints to achieve optimal nutrition [86, 87].

The characterisation of responses and variation to dietary interventions is important to maximise health and optimise personalised nutrition. When exploring the inter-individual variations in response to the clinical endpoints, standard deviation (SD) is a measure of variation at the endpoint and most studies assess the group effect andreport SD as a reflection of group response variation [16]. There is also a number of studies that have calculated the coefficient of variation (CV) as it is also a measurement of group response variation [88, 89]. In the current study the response level was calculated taking relative changes to baseline into account for each individual, to further illustrate intra-individual changes in response to the intervention.

For the endpoints assessing blood pressure and arterial stiffness as shown in Chapter 2, blueberry powder studies presented SD of 8.00 - 11.00 mmHg in the response of SBP [69-72], SD of 1.20 - 9.77 mmHg in the response of DBP [69-72] and SD of 0.90 - 2.14 m/s in the response of PWV [69, 70]. It has been shown that lowering SBP by 5 mmHg or more could decrease CVD risk by 3% to 38% [90, 91] and the minimal clinically important difference (MCID) for PWV is a reduction of 1 m/s [92]. Therefore, it's important to explore the range of response following the same intervention at the individual level even though the group effect may not be statistically significant. The inter-individual variation to intervention endpoints are not limited to fruit supplementations. For example, a ketogenic dietary intervention reported a 103 % and 68 % variation (CV) among participants in response to blood triglyceride and glucose concentration respectively [88].

For the endpoints assessing cognition and mood, the inter-individual differences in cerebral blood flow dynamics, neural correlates, heritability, physical, social environment, and personality all constitute complex associations between individual response and specific cognitive domain phenotype. The review in Chapter 3 demonstrated a larger variation across studies assessing memory (SD of 2.2) relative to executive function (SD of 0.08 - 0.16) following either blueberry powder or blueberry concentrate intervention [77, 93, 94]. Apart from fruit interventions, a large variation in the correct response to working memory (SD of 181.35) and mood (SD of 12.71) assessments have also been shown among healthy individuals following a Mediterranean dietary intervention [95]. There are also multiple factors affecting variations in the cognitive response among individuals. It has also been shown that age-related changes in cognition and the susceptibility of different cognitive domains to ageing are not unitary across all individuals and the susceptibility of cognitive domains to ageing are not uniform neither [96]. Certain aspects of memory and attention have shown attenuated performance with age while others suggested a significant decline [96]. Although from a big picture perspective, cognitive decline seems to be one of the deficits seen in ageing [97], many older adults can out-perform young adults or at least maintain the same level as the young on some cognitive tasks, for instance, to keep good comprehension by effectively using context to interpret message in speech and language [98]. In addition to ageing, which has been shown to lead to cognitive decline [96], brain structure differences estimated for individuals have also been shown to associate with inter-individual variations in cognitive functioning [99]. For instance, the variation in response for the choice reaction time (the time taken to indicate a choice in cognitive tasks) assessing psychomotor speed across individuals has been shown to correlate with brain fractional anisotropy of the optic radiation [100].

Nevertheless, currently the in-depth characterisation of inter-individual variation in response to vascular and/or cognitive endpoint following dietary interventions is scarce. Potential influencers of inter-individual variation in vascular and cognitive response to dietary blueberry interventions include bioavailability and metabolism of blueberry polyphenols, SNPs (single nucleotide polymorphisms), and gut microbiota between individuals [101]. The health status of participants has also been shown to affect the response to dietary interventions in biomarkers of vascular and cognitive

functions. For example, individuals with Metabolic syndrome (MetS) have a higher degree of endothelial dysfunction (assessed by RHI) compared to individuals with multiple CVD risk factors such as elevated blood pressure, waist circumference, lipid profile and fasting blood glucose level without MetS [102]. Ageing has also shown to be associated with less elastic blood vessels which contributed to the augmented vasoconstrictor and reduced vasodilator responsiveness [103]. More studies similar to ours that aim to explore the inter-individual variation in response to vascular and/or cognitive endpoints following dietary interventions and the factors found to influence the response have been compared and contrasted in Chapter 6.

1.1.7 Metabolite profiling for investigating inter-individual variation

Metabolomics is a technique that investigates small-molecule metabolite profiles of biological fluids that reflect biochemical processes [5]. Metabolomics explores a whole range of endogenous or exogenous metabolite profiles found in a biological system, combined with high-throughput analytical chemistry and multivariate data analysis [104]. Identification of metabolomic biomarkers has been commonly used in research investigating metabolic pathways, thus the approach could pave the way for characterising individuals in intervention studies combined with physiological and biochemical assessments [105]. Metabolomic profiling has also been extensively used in nutritional research including randomised clinical trials and observational studies [104].

For example, metabolomic profiles have been characterised and differentiated between multiple dietary patterns including a low-fat diet, a low glycaemic index diet, a very-low-carbohydrate diet, and a healthy diet concordant with WHO eating guidelines [106]. Metabolomic profiling has also been applied to investigate metabolomic changes associated with single food intake, such as polyphenol-rich foods [107]. Urinary metabolomic profiling following dietary intakes of polyphenol-rich foods including coffee, tea, red wine, citrus fruit, apple and pear, and chocolate has reported five polyphenol metabolites with a high predicting ability of polyphenol-rich food intake [107]. Using untargeted metabolomic profiling, higher intakes of fruit and whole grains haven also been shown to link with higher levels of the plasma metabolite hippurate that acted as biomarker for reduced odds of having MetS (OR: 0.795[0.082];

P = 0.026) [108]. Metabolomic profiling has also been applied to investigate the nutrient-metabolism interactions, for example the phospholipid metabolism and pro-inflammatory response following vitamin E supplementation [109]. Bioavailability of bioactive has also been investigated using metabolomic profiling following dietary interventions. An investigation of anthocyanin bioavailability following bilberry consumption has suggested that about 20 % of anthocyanins could be absorbed in the small intestine during 8 h after the ingestion and were detected in both urine and plasma circulations (Cmax 777 ± 192 nmol/l) [110]. Metabolomic analysis utilising Liquid Chromatography-Mass Spectrometer (LC-MS), as adopted in the current study, would also provide analysis of a wide range of small molecules including the hydrophilic polyphenols, the main phytochemicals in blueberry [111].

Inter-individual (between-individual) and intra-individual variations need to be taken into account for categorising response following a dietary intervention and usually inter-individual variation rather than intra-individual variation could account for most of the variability in metabolite profile that showed association with response [112]. For example, a prospective longitudinal study used Cox regression and assessed the association of metabolite profiles in patients with different cognitive status for identifying discriminating metabolites that represent the between - individual variations in the cognitive response [113]. After adjustment for lifestyle and physiological factors, increased intensity of metabolite N-acetyl-1-methylhistidine was significantly associated with greater 6 - year change in Delayed Word Recall scores (β = -0.66 words; $P = 3.65 \times 10^{-4}$); 2 metabolites (one unnamed and a long-chain omega-6 polyunsaturated fatty acid found in vegetable oils were significantly associated with less decline on the Digit Symbol Substitution Test (β = 1.25 digit-symbol pairs, $P = 9.47 \times 10^{-5}$); another 2 unnamed compounds and three sex steroid hormones were associated with an increased risk of dementia ($P < 3.9 \times 10^{-4}$) [113]. Based on existing evidence, metabolic profiling of individuals following blueberry interventions were used to explore the biological pathways between blueberry metabolism and the responses to vascular and cognitive endpoints in the current thesis.

1.2 Novelty of the work

The impact of fruit interventions (different types and processed forms) and their effect on improving cognitive/vascular function with a focus on individual responses is underexplored in comparison to studies assessing only group effect following an intervention. Therefore, the current thesis aims to characterise individual response as responder (RS) and non-responder (NRS) group at the both vascular and cognitive endpoints following the blueberry interventions. Apart from inter-individual variation during metabolism, substantial heterogeneity in the categories and forms of fruit chosen and durations in the interventions might also contribute to inconsistent results of dietary fruit's health benefit [16, 114]. So far, the benefits of blueberry consumption in cognitive and endothelial decline are inconclusive, and there are insufficient clear mechanisms linking blueberry consumption to human cognition or vascular health [41, 115]. Therefore, the current thesis also aims to investigate the protective benefit of chronic dietary blueberry interventions in different forms for both vascular and cognitive function using systematic reviews and meta-analysis.

There are emerging needs to investigate metabolomic responses to an intervention and to further develop personalised approaches such as the validation of biochemical markers and therefore apply them as indicators of diagnosis and treatment of cognitive/vascular function decline. Therefore, another aim of the current thesis is to explore the metabolic pathways underlying different vascular and cognitive responses following the blueberry interventions. In future, similar approaches can be used to fully understand the variability in response within and between individuals and this can further lead to the development of more personalised nutritional plans.

1.3 Chapter synopsis

In the present thesis, Chapters 2 & 3 conducted systematic review and meta-analysis of RCTs assessing vascular and cognitive functions that spanned over 1 week to help inform the choice of fruit intervention to a specific category and/or processed form. Chapter 4 describes the notion of using a blueberry intervention, the population, and the design of a crossover RCT, including the participants recruitment, sample size calculation, experimental procedure on each study visit, clinical and biochemical

measures used for collecting experimental data and human samples. Chapter 4 also described the mathematical method basing on previous research evidence to quantify the RS and NRS groups. Statistical methods used for analysing study results from the clinical endpoints and the metabolomic assay are also described.

Chapter 5 reports the study results including participant demographics at baseline, sample size, and the treatment effects shown on vascular and cognitive functions following the two interventions compared to the control. The metabolomic differences originating from whole blueberry and blueberry powder interventions were investigated and discussed. Chapter 6 reports inter-individual response to each biological endpoint and consistency across participants. The chapter then goes on to characterise metabolomic between RS and NRS groups. Predictor metabolites that can act as biomarkers for predicting RS are also explored. Effects of blueberry interventions assessing the vascular and cognitive endpoints are discussed and compared to previous interventions in Chapter 7. Chapter 7 also summarises the main findings from each chapter and discusses the gap in knowledge that the current study has filled, as well as implications for exploring individual responses to a dietary intervention to benefit health.

1.4 Objectives

- **i.** To systematically review the effects of chronic dietary fruit interventions (≥ 1 week) in different categories (e.g. berry, cherry, citrus) and processed forms (e.g. whole, juice, powder) on vascular and cognitive functions, and apply a meta-analysis where available to help inform the fruit intervention.
- **ii.** To analyse and compare the effects of chronic dietary interventions supplementing whole blueberry and freeze-dried blueberry powder on cognitive and vascular functions in healthy adults.
- **iii.** To characterise inter-individual responses to vascular and cognitive endpoints to the interventions and assess consistency of response within and between participants
- **iv.** To identify any predictive metabolic biomarkers that may predict response in vascular and/or cognitive endpoints to blueberry interventions and to further

understand the mechanisms underlying different individual responses to the blueberry interventions aimed at improving cognition and/or vascular function.

1.5 Hypothesis

i. A specific category and processed form of fruit interventions with largest number of studies reporting positive findings on vascular and/or cognitive functions can be identified and supported by the meta-analyses evidence where available.

ii. There will be statistical significance of the improvement to at least one of the primary vascular and/or cognitive endpoints following the whole blueberry and blueberry powder interventions compared to the control in healthy adults and no difference in endpoints between whole blueberry and freeze-dried blueberry powder intervention arms.

iii. Individual response at the vascular and cognitive endpoints following the whole blueberry and freeze-dried blueberry interventions can be characterised and the consistency of the individual response can be characterised across the primary endpoints following the two blueberry interventions.

iv. A predictive metabolic biomarker can be identified following the whole blueberry and/or freeze-dried blueberry powder interventions for predicting response in vascular and/or cognitive endpoints among a group of healthy individuals.

Chapter 2. Systematic review and meta-analysis of fruit interventions assessing vascular function

The section 2.1 is arising from publication: Wang, Y. Gallegos, J.L. Haskell-Ramsay, C. et al. Effects of chronic consumption of specific fruit (berry, citrus and cherries) on CVD risk factors: a systematic review and meta-analysis of randomised controlled trials. Eur J Nutr (2020). https://doi.org/10.1007/s00394-020-02299-w

2.1 Effects of fruit consumption on vascular function: a systematic review and meta-analysis of randomised controlled trials.

2.1.1 Introduction

Current WHO recommendations for fruit and vegetable consumption are for a minimum 400 g/d [116]. A recent meta-analysis indicated that an intake of 800 g/day of fruit was associated with a 27 % reduction in relative risk of vascular diseases [117]. It is well recognised that vascular health can be affected by several dietary factors [118]. Interventional studies also provided evidence supporting the consumption of a range of fruit and fruit juice to reduce vascular disease risk factors. For example, consumption of fruit containing relatively high levels of anthocyanins and procyanidins, such as berry, has been shown to improve vascular disease risk factors, namely endothelial dysfunction, dyslipidaemia, platelet aggregation, and hypertension [119, 120], whereas flavanone-rich fruit, such as orange, were effective in improving hypercholesterolaemia [119].

Fruit juice and powder may be effective methods to increase overall fruit consumption, which may explain the emerging intervention studies investigating health benefits with fruit powder and juice supplementations. In regards to nutritional value, freeze-dried fruit powder that is devoid of water retains concentrated bio-accessible antioxidants, fibre and other components [121]. Research has suggested that the juicing process can lead to a lower content of fibre and certain bioactives such as polyphenols, vitamins, and minerals [122, 123], while other research suggests that processing can increase the bioavailability of bioactives [124]. A recent single-dose bioavailability study showed only minor differences between whole fruit and the juice [55].

An initial search for fruit interventions assessing vascular function was conducted and most studies supplemented berry, citrus, and cherry fruit, therefore, this systematic review focused on only berry, citrus and cherry fruit interventions. The initial search covered both acute (< 24 h post-prandial study) and chronic interventions and the reviews in Chapters 2 & 3 focused on only chronic interventions. Acute studies are discussed in Chapters 4 - 7. For chronic studies, most were of at least 1 week duration so 1 week was used as a cut off, as described in the eligibility criteria below. The systematic review and meta-analysis were reduced to comply with the word limit but the full details can be found in the publication.

2.1.2 Objectives

Given the potential for differing nutritional value of varying types and forms of fruit, it is likely that their health benefits will also differ. However, little is known about the comparative effect of processed fruit on cognitive and vascular function. A review of this type is important to clarify the evidence base for the type and form of fruit that is most vascular-protective and to further inform our interventional design. The objective of this study was to systematically review and meta-analyse available human interventions to evaluate the potential effect of consumption of whole, freeze-dried, powdered fruit and fruit juice on CV risk factors and vascular function in randomised controlled trials (RCTs) to inform the type and processed form of a fruit-based interventional design.

2.1.3 Methods

We searched for studies investigating the effect of berry, citrus and cherry supplementations on vascular function. Berry, citrus and cherry are different types of fruit. Berry so defined is a fleshy fruit that usually contains no stone or pit and includes grapes, blueberries, strawberries, pomegranates etc; citrus is ‘sour’ fruit and includes most commonly oranges, grapefruits, tangerines, lemons, and limes; cherry is a fleshy drupe and categorised as stone fruit [125]. The following specific inclusion criteria were applied: 1) study design: RCTs; 2) subjects: adult subjects ≥ 18 years of age; 3) interventions: interventional RCTs providing or promoting berry, or cherry or citrus fruit

or their juice or freeze-dried, or powdered fruit consumption; 4) intervention length: at least 1 week; 5) control: control groups without components of citrus fruit, cherry, or berry, likely placebo group; 6) outcomes: The primary outcomes were the whole body measurements: systolic and diastolic blood pressure (SBP and DBP) and the endothelial (dys)function assessed by flow-mediated dilation (FMD) and arterial stiffness assessed by pulse wave velocity (PWV); the secondary outcomes were the blood biomarkers including circulating fatty acids Triglycerides (TAGs) and total cholesterol (TC), low-density lipoprotein cholesterol (LDL - C) and high-density lipoprotein (HDL - C); vascular inflammatory biomarkers such as high-sensitivity C-reactive protein (hsCRP), Nitric Oxide (NO), Intercellular Adhesion Molecules (ICAMs) and Vascular Adhesion Molecules (VCAMs) were also explored (details described below); 6) only English-language and peer-reviewed articles were included. No restriction of publication year was applied.

2.1.4 Data sources

The present systematic review and meta-analysis was conducted in accordance with Cochrane [126] and Centre for Reviews and Dissemination guidelines [127] and was reported according to PRISMA guidelines [128]. The protocol has been registered with PROSPERO, the International Prospective Register of Systematic Reviews (Registration number CRD42018091896). Two researchers (YW, JLG) assessed articles independently for inclusion eligibility. The searches using PubMed, Web of Science, Scopus and psycARTICLES were conducted from inception until January 2020. No restriction of publication year was applied, and the search result covered studies published between 1960 and 2020.

The search of the investigated themes in this review was as following: (Fruit OR citrus OR orange OR berry OR berries OR grape OR blueberry OR blueberries OR blackberry OR blackberries OR raspberry OR raspberries OR cranberry OR cranberries OR cherry OR cherries) AND ("endothelial function" OR "vascular function" OR "vascular risk factors" OR hypertension OR "blood pressure" OR BP OR "pulse wave velocity" OR PWV OR "flow-mediated dilation" OR FMD OR lipid OR

cholesterol OR LDL OR HDL OR triglyceride OR biomarkers OR inflammatory OR Nitric Oxide OR NO OR ICAM OR VCAM OR CRP AND (trial OR intervention).

2.1.5 Study selections

YW and JLG selected articles independently for eligibility. Articles were moved to the next screening phase or discarded when full disagreement was reached. Any disagreements that were not resolved were handled by JKL serving as an arbitrator. All records were exported to EndNote X8 reference management software. The selection of eligible studies was based on two steps. Firstly, the title and abstract of each study were screened for relevance; full texts were then reviewed for those without certainty for inclusion. Reference lists of included papers and relevant systematic reviews were also screened by hand-searching for additional articles.

2.1.6 Data abstraction

Data were extracted by YW and JLG independent of each other, their selections for accuracy were reviewed in a meeting. Corresponding authors were contacted via e-mail to request information if there were missing data or for clarification. Data from endpoints and the baseline were obtained. A pre-defined data extraction form in Microsoft Excel 2016 was used to input studies data, which included information on 1) author and published year; 2) study design; 3) population characteristics (ethnicity, mean age, sex, mean body mass index (BMI), health status and sample size at baseline); 4) treatment details (intervention type, length, dosage and frequency); 5) control group settings; 6) retention rate; 7) measured outcomes for both experimental group and placebo group at baseline and the longest post-intervention time point to avoid the bias of selectively choosing data.

2.1.7 Risk of bias assessments

Study quality for RCTs was assessed by Jadad Score (0 to 5), which took into account whether a trial was randomised and blinded with appropriate procedure, and whether dropouts were well recorded; a score ≥ 3 indicates a high-quality trial [129].

Publication bias was assessed by Funnel plot and Egger's test, 'trim and fill' method was implemented to identify and correct for funnel plot asymmetry arising from publication bias [130].

2.1.8 Data synthesis

R studio version 3.5.2 and the package "meta" [131] were used to pool and meta-analyse data from collected studies. Subgroup analysis with at least 10 studies supplementing berry was implemented to estimate the specific effects and the heterogeneity for each interventional subgroup. Sensitivity analysis was performed to investigate the impact of studies adjusting for participants' physical level [132, 133] and also the impact of juice quality on the meta-analysis results.

All pooled results were presented as weighted mean difference with 2-sided *P* values. 95% confidence intervals (CIs) and prediction intervals were both presented in the results. The FMD value was expressed in percentage unit and the PWV value was expressed in m/s; the conversion of cm/s to m/s for PWV value was applied when necessary for pooled mean differences in meta-analysis. For blood lipids, the conversion factor 1 mmol/L = 38.67 mg/dL was used for total, HDL, and LDL cholesterol level and 1 mmol/L = 88.57 mg/dL for triglycerides level [134] where applicable.

The Hartung-Knapp-Sidik-Jonkman method for random-effects meta-analysis [135] was applied. Heterogeneity was estimated by Cochrane *Q* statistics and the consistency of study results was assessed by I^2 statistics as an extension of Cochrane *Q* statistics and an $I^2 > 50$ % is considered for a high heterogeneity level [136]. The effect sizes based on the weighted mean difference (WMD) between treatment groups were used when measurement units of assessed outcomes were comparable across studies. The standardized mean difference (SMD) was used when studies have used different measurement units and the conversion had failed.

2.1.9 Literature search

In accordance with PRISMA guidelines [128], **Figure. 2.1** describes the selection process of included studies. The search for literatures assessing vascular function and cognition was carried out together but only the final record for vascular function was reported here. The initial search produced 13861 articles from the four databases, this record was reduced to 8613 articles after duplicates were removed. After screening of the titles and abstracts for eligibility, the final selection identified 45 trials assessing vascular function. From trials included, 38 trials assessing vascular function were entered into the meta-analysis.

2.1.10 Study characteristics

For studies assessing vascular disease risk factors **(Table. 2.1)**, forty-five studies were included in this systematic review, of which 18 were cross-over randomized controlled trials (RCT) and 27 were parallel RCTs [16]. The sample size of both experimental and control group in the interventions ranged from 5 to 63. The total sample size for the intervention group was 1130; the total sample size for the control group was 1109. Participants' characteristics at baseline also vary across studies; most trials recruited healthy subjects (n = 13), while there were 7 studies with participants manifesting increased vascular disease risks (elevated lipid profiles and hypertension) and 3 with diagnosed CVD or coronary heart disease (CHD); 18 with metabolic syndrome (inclusive of overweight); 1 with mild-to-moderate dementia, 1 with chronic obstructive pulmonary disease, 1 with type 2 diabetes and 1 with end-stage renal disease [16]**.** However, there were not enough studies with different participants' characteristics to do sub-group analysis to explore the effect of participants' characteristics at baseline.

For studies assessing vascular function, there were 32 studies (71 % of the interventions) that supplemented fruit juice and 13 studies supplemented whole fruit and fruit in other freeze-dried forms [16]. Treatments were all delivered in arms of experimental and control groups. The mean chronic treatment duration was 57 days with a standard deviation (SD) of 43 days (ranged from 7 days to 180 days).

Figure. 2.1 Flow diagram of study selection for the review

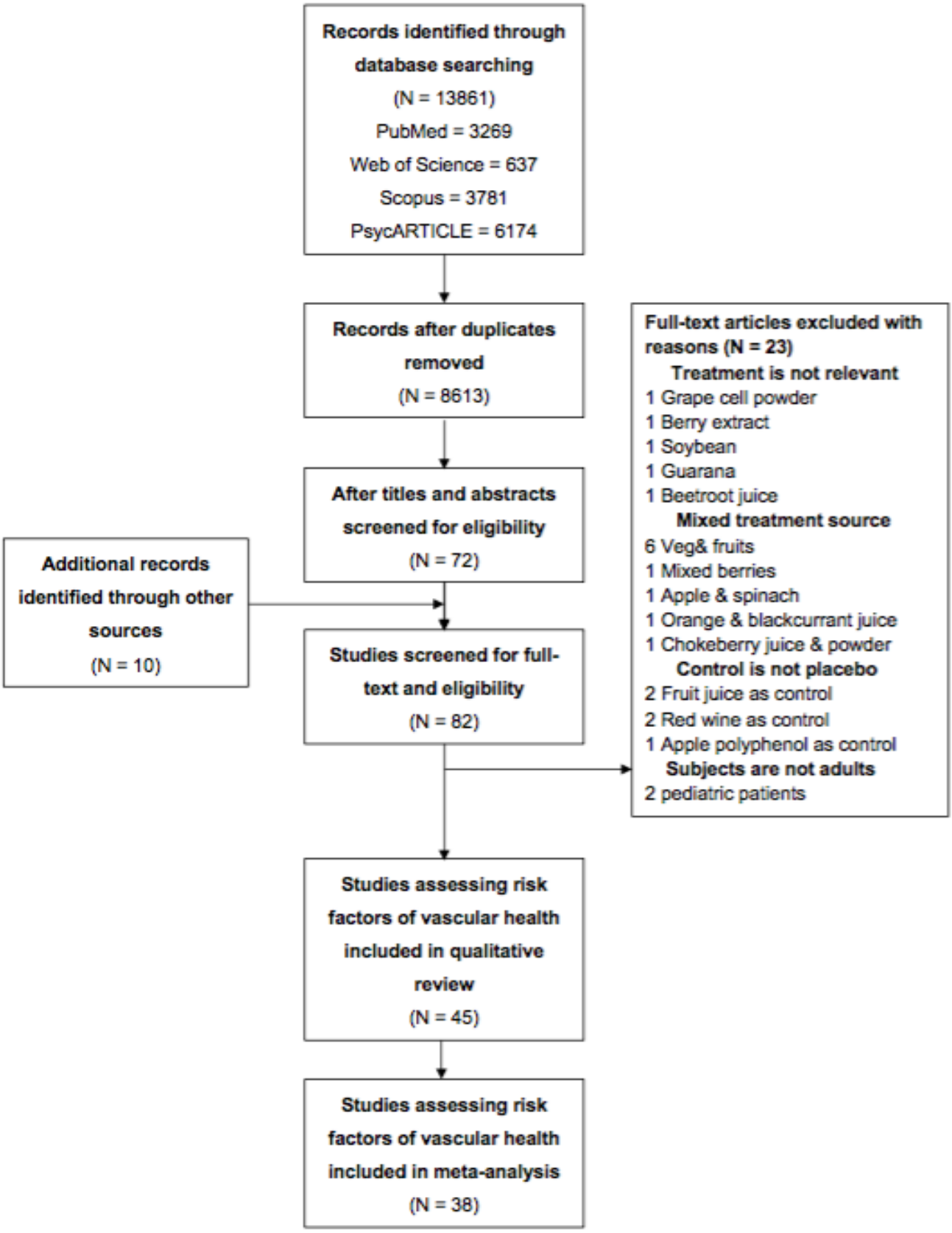


Table. 2.1 Summary of fruit interventions assessing vascular function

Reference	Study design	Sample (sample at baseline/N, age/years, % male, mean BMI (kg/ m2), health status, ethnicity (%))	Intervention type, dose and duration	Control group	Reten-tion rate	Outcomes	Total Jadad score (0 to 5)
Bardagjy, A. S. et al. (2018) US	Double blinded cross-over RCT	N =23(20), age 48.6 ± 15.4, 20 %, BMI 37.0 ± 9.9, obese but otherwise healthy adults, N/A	60 g grape powder was equivalent to 330 g or 2.2 cups of fresh grapes and contained 297 mg total polyphenols (as gallic acid equivalents) for 4 weeks	PBO was matched to GP in calories, macronutrients, taste, and appearance but provided zero polyphenols/serving.	87 %	SBP, DBP, TC, HDL, LDL, TG, CRP, ET-1, IL-6: oxLDL, sICAM, sVCAM, TNFc	3
Barona,et al. 2012 (Colombia)	Double blinded cross-over RCT	N=25, age 51.3 ± 9.6, male and BMI N/A, metabolic syndrome, N/A	46 g/d grape powder equals to 2 serving of fresh grapes for 30 days	Placebo	96 %	SBP, DBP, FMD, TG, HDL, Glucose, BMI, Nox, sICAM-1, sVCAM-1, E-selectin	2
Boldaji, et al. 2019 (Iran)	Cross-over RCT	N=40(38), age 47.8 ±13.3, 61 %, BMI 23.9 ± 4.8, End-Stage Renal Disease patients on dialysis 3 times a week for at least 3 months, N/A	100ml pomegranate juice, 3 times a week after subjects' dialysis session for 8 weeks	No intervention as control	97.60 %	SBP, DBP, TC, HDL, LDL, TG, IL-6	2
Buscemi, et al. 2012 (Italy)	Single-blinded cross-over RCT	N=19, age 48±13, 53 %, BMI 32.1±4.9, subjects with increased CVD risk, N/A	500 ml/d red orange juice for 1 week	placebo drink (12 healthy non-diabetic subjects acting as control group)	91 %	FMD, GTN (glyceryl-trinitrate), hs-CRP, IL-6, TNF-α, NO, PCs (protein carbonyl)	2
Constans, et al. 2015 (France)	Single-blinded cross-over RCT	N=25, age 53.8±2, 100 %, BMI 26±1, mild hypercholesterolemic men (LDL-C between 130 and 190 mg/L), N/A	200 ml (3 times/d) blond orange juice for 4 weeks	control beverage	96 %	glucose, TC, LDL, HDL,TC/HDL, TG, ApoA-1, ApoB, Lpa, hsCRP, Brachial FMD, sICAM-1, sVCAM-1, sE-selectin	3
Desai, T. et al. (2018) UK	Single-blinded cross-	N=11, age 30 ± 10, N/A, BMI 24.43 ± 3.23, healthy, N/A	30 ml Montmorency tart cherry juice for 20 days	placebo	100 %	SBP, DBP, TC, LDL, TG, IL-7	3

Table. 2.1 (Continued)

	over RCT						
Dohadwala, et al. 2010 (US)	Cross-over RCT	N=63, age 43±12, 69 %, BMI 28±3.8, stage 1 hypertension, 42 % black	490 ml/d concord grape juice containing 965 mg anthocyanins for 8 weeks	Placebo drink	77 %	SBP,DBP, TG TC, LDL, HDL	3
Dohadwala, et al. 2011 (US)	Double blinded cross-over	N=44, age 62±10, 68 %, BMI 29.5±4.5, coronary heart disease, Black 45.5 %	480 ml/d double-strength cranberry juice for 4 weeks	calorie, taste, and appearance-matched placebo beverage containing no polyphenols	91 %	SBP,DBP,FMD, Baseline diameter, Dilation to nitroglycerin, Baseline flow, FMD, Hyperaemic flow, InPAT ratio, Carotid-radial PWV, Carotid-femoral PWV	4
Habauzit, et al. 2015 (France)	Double blinded cross-over RCT	N=52, age 58±4, 0 %, BMI 25.7±2.3, Postmenopausal woman, 100 % Caucasian	340 ml/d (2 times of 170 ml) concentrate blond grapefruit juice for 6 months	Isocaloric Control drink	92 %	SBP, DBP, Pulse pressure, FMD dilation, Baseline brachial diameter ,PAT ratio, Pulse pressure, NO, Endothelin 1	4
Holland, et al. 2018 (UK)	Open label cross-over RCT	N=45(41), age 52.2 ± 13.6, 49 %, BMI 29.0 ±5.1, healthy, N/A	500 ml/d (2 times of 250 ml) blood orange juice providing 50 mg anthocyanins for 4 weeks	500 ml blonde OJ without anthocyanins	91 %	SBP, DBP, ba PWV, cf PWV, NO, CRP, TG, HDL-C, LDL-C	2
Lamport, et al. 2016 (UK)	Double blinded cross-over RCT	N=25(19), age 43.2 ± 0.6, 0 %, BMI 24.6 ± 0.5, healthy, N/A	355 ml/d concord grape juice for 12 weeks and assessed at 6, 12 weeks	energy-, taste-, and appearance-matched placebo	77 %	SBP, DBP	4
Martin, 2018 (US)	Cross-over RCT	N=13(10), age 38.1 ± 12.5, N/A, BMI 32,2 ± 4.6, overweight and obese, N/A	240 ml/d tart chery juice for 4 weeks	placebo juice	77 %	IL-6, IL-10, TNF-, MCP-1, hsCRP	4
Millar, 2018 (US)	Double blinded cross-over RCT	N=20, age 53.5 ± 10.1, N/A, BMI 33.0 ± 4.77, metabolic syndrome, N/A	60 g/d grape powder (contributing 195 mg total polyphenols) for 4 weeks	placebo powder	100 %	Total cholesterol, HDL-C and HDL particles, TG	3

Table. 2.1 (Continued)

Morand, et al. 2011 (French)	Cross-over RCT	N=24, age 56±1, 100 %, BMI 27.4 ± 0.3, overweight healthy, N/A	500 ml/d orange juice for 4 weeks	control drink + placebo capsules (starch)	100 %	SBP, DBP, Pulse pressure, Glucose, Insulin, Triglycerides, Total cholesterol, LDL ,HDL,CRP,IL-6,vWF,sICAM-1,sVCAM-1,NOx	5
Riso et al. 2013 (Italy)	Cross-over RCT	N=18, age 47.8 ± 9.7, 100 %, BMI 24.8 ± 2.6, subjects with CVD risk factors, N/A	Freeze-dried wild blueberry powder 25 g/d in water, equals to 250 ml/d for 6 weeks	placebo drink consisted of 250 mL water, 7.5 g fructose, 7 g glucose, 0.5 g citric acid and 0.03 g blueberry flavour	89 %	RHI,FRHI,AI,AI@75,Diastolic pressure, SBP, Total NO,sVCAM-1	2
Ruel et al. 2013 (Canada)	Double blinded cross-over RCT	N=35, age 45 ± 10, 100 %, BMI 28.3 ± 2.4, overweight, N/A	500 ml/d of 27 % cranberry juice for 4 weeks	placebo juice	100 %	Heart rate, Systolic BP, Diastolic BP , MAP, Resting AIx ,Δ AIx salbutamol, Δ AIx GTN,Global endothelial function, NOx ,Uric acid, Oxidized LDL , sICAM-1 , sVCAM-1 , sE-selectin	3
Siasos, et al. 2014 (Greece)	Double blinded cross-over RCT	N=26, age 26.34 ± 4.93, 38 %, BMI 23.21± 4.10, healthy, N/A	Weight equivalent 7 ml/kg/d of 100 % concord grape juice for 2 weeks and assessed at 1 week and 2 weeks	The grapefruit placebo juice matched the flavour, colour, calorie, and sugar profile of the CGJ but did not contain any polyphenols	100 %	FMD,PWV/carotid-femoral, Total cholesterol, LDL-C, TG, Serum glucose, SBP, DBP	4
Willems, et al. 2015 (UK)	double-blind cross-over RCT	N=13, age 38 ± 8, 62 %, BMI 23±2, healthy, N/A	6g/day (138.6 mg anthocyanins) blackcurrant powder in water for 1 week	blackcurrant juice 3-4 mg anthocyanins per dose	77 %	SBP, DBP, mean arterial BP, heart rate, Stroke volume, cardiac output, peripheral resistance	2
Basu, A. et al. 2014 (UK)	Parallel RCT	N=60, high dose intervention: high dose control: low dose intervention: low dose control: 15: 15 : 15 : 15, age 49 ± 10, 9 %, BMI 36 ± 6.5, obese adults with elevated serum lipids, N/A	High-dose 50 g/d freeze dried strawberry (10 % weight of fresh strawberries) and low-dose	high-dose calorie- and fibre-matched control 44g/d; low-dose calorie- and	100 %	BMI, SBP, DBP, glucose, insulin, Total cholesterol,LDL-C,HDL-C,LDL:HDL,VLDL-C,TGs,hs-CRP,sVCAM-1,sICAM-1	2

Table. 2.1 (Continued)

			25 g/d freeze-dried strawberry for 12 weeks	fibre-matched control 24g/d			
Basu, et al. 2010 (US)	Parallel RCT	N=30, intervention: control 15:12, age 47.0 ± 3.0, 7 %, BMI 37.5 ± 2.15, metabolic syndrome, N/A	Freeze-dried strawberry beverage (50 g freeze-dried strawberries ~ 3 cups fresh strawberries) for 8 weeks	4 cups of water/d	90 %	BMI, SBP, DBP, glucose, Total cholesterol,HDL-C,LDL-C,VLDL-C,TGs,ICAM-1,VCAM-1	2
Basu, et al. 2011 (US)	Parallel RCT	N=31, intervention: control 15:16, age 52.0 ± 8.0, 0 %, BMI 40.0 ± 7.7, metabolic syndrome, N/A	240 ml/d containing 458 mg total polyphenols of cranberry juice for 8 weeks	Placebo drink	97 %	SBP, DBP, Total cholesterol, HDL-C,LDL-C,TGs	3
Basu, et al. 2010 (US)	Single-blinded parallel RCT	N=66, intervention: control 25:33, age 50.3 ± 3.0 SE, 8 %, BMI 37.8 ± 2.3, metabolic syndrome, N/A	480 ml freeze-dried blueberry beverage (50 g freeze-dried blueberry) for 8 weeks	480 mL water and vanilla extract	73 %: 72 %	SBP, DBP, TG, Total , LDL-, HDL-, ox LDL- cholesterol	2
Cerda 2006a (Spain)	Parallel RCT	N=30, intervention: control 15:15, age 60 ± 10.9, N/A, BMI 31.4±4.8, chronic obstructive pulmonary disease, N/A	400 ml/d pomegranate juice (2660 mg/d anthocyanins) for 5 weeks	Placebo drink	100 %	Total cholesterol, HDL-C,LDL-C,TGs	3
Curtis, P. J. et al. (2019). UK	Double blinded parallel RCT	N=144 (115), 1 cup : 1/2 cup: control 37:39:39, age 63± 7, N/A, BMI 31.2 ± 3.0, metabolic syndrome, N/A	26 g blueberry powder equivalent to 1 cup (150 g) and 1/2 cup (75 g) blueberry for 6 months	dextrose, maltodextrin, and fructose, which were produced as a purple powder, with blueberry aromatics generated from natural (no anthocyanin) and artificial colour and flavourings	80 %	SBP, DBP, TC, HDL, LDL, TG	3
Chew, B. et al. (2019) US	Double blinded parallel RCT	N=79 (78), intervention: control 40 : 38, age 43.1 ± 1.1, N/A, BMI 30.8 ± 0.4, non-smoking overweight, N/A	450 ml/d cranberry extract beverage (CEB) for 8 weeks	The placebo beverage was designed to look, smell, and taste such as the CEB, but did not contain cranberry constituents.	98 %	CRP, IL-6, IL-10, IL-23, TNF-α; IFN-γ	3

Table. 2.1 (Continued)

Chai, S. C. et al. (2019).US	Parallel RCT	N=37, intervention: control 20:17, age intervention: control 28.5 ± 3.7: 27.3 ± 4.2, 40 %:53 %, BMI 70.0± 3.7: 69.5 + 3.9, older adults, N/A	68 ml/d of Montmorency tart cherry concentrate was diluted with 412 mL of water for 12 weeks	Control drink was prepared by mixing unsweetened black cherry flavoured Kool-Aid (Kraft Foods, Chicago, IL, USA) with water.	100 %	TNF-α, CRP, ET-1, NO, OxLDL	3
Duthie 2006b (Scotland)	Parallel RCT	N=20, intervention: control 11:9; age 18-40 years old 0 %, BMI N/A, healthy, N/A	750 ml/d cranberry juice for 2 weeks	Placebo drink (6.72 mg/d)	100 %	Total cholesterol, HDL-C,LDL-C,TGs	2
Dow, et al. 2013 (US)	Parallel RCT	N=74, intervention: control 37:32, age 41.8 ± 10.7, 30 %, BMI 32.1 ± 4.1, obese or with additional metabolic syndrome (42 %), non-Hispanic white race (62.3)	Low bioactive diet plus one and half of fresh grapefruit daily for 6 weeks	A low bioactive diet devoid of citrus	93 %	sVCAM-1, hsCRP	1
Flammer, et al. 2013 (US)	Parallel RCT	N=84, intervention: control 32:37, age 49.5 ± 16.2, 45%, BMI 27.7±5.9: 27.2 ± 5.5, peripheral endothelial dysfunction and CVD risk factors, N/A	460 ml/d (2 times of 230 ml/d) of (54%) cranberry juice (double-strength Ocean Spray® light cranberry juice cocktail) for 4 months	placebo juice beverage, an isocaloric formulation mimicking the flavour and colour of the cranberry beverage	82 %	RHI, SBP, DBP, pulse pressure, heart rate, AI augmentation via EndoPAT,hsCRP,VCAM,ICAM ,I1-6,TNF-alpha,oxLDL,Cholesterol,HDL, TG	3
Gonzalez-Ortiz 2011b (US)	Parallel RCT	N=20, intervention: control 10:10, age 25-55 years old, N/A, BMI 30.0-39.9, obesity, N/A	120 ml/d pomegranate juice for 1 month	Placebo drink	100 %	Total cholesterol, HDL-C,LDL-C,TGs	3
Hollis 2010a (US)	Parallel RCT	N=51, intervention: control 25:26, age 18-55, N/A, BMI 25.0-29.9, overweight, N/A	480 ml/d of concord grape juice for 12 weeks	Placebo drink	100 %	Total cholesterol, HDL-C,LDL-C,TGs	3
Jeong, et al. 2014(Korea)	Double blinded parallel group RCT	N=73, intervention: control 39:38, age 58.0 ± 9.2: 60.1 ±9.5, 47 %, BMI 26.3±4.3: 25.1±4.0, metabolic syndrome, N/A	750mg/d (4 capsules) powdered black raspberry for 12 weeks	Placebo group-cellulose, isomaltose, and corn powder.	92 %	Resting brachial artery diameter, Reactive hyperaemia brachial artery diameter,IL-6,NF-a,C-reactive protein,Adiponectin,sICAM-1,sVCAM	3

Table. 2.1 (Continued)

Jeong, et al. 2016 (Korea)	Double blinded parallel group RCT	N=51 intervention: control 26:25, age 56.4±9.2: 60.7±10.4, N/A, BMI 25.9±4.6, 24.7±3.9, metabolic syndrome, N/A	750mg/d (4 capsules) powdered black raspberry for 12 weeks	Placebo	100 %	SBP, DBP, heart rate, radial augmentation	3
Johnson, et al. 2015 (US)	Double blinded parallel group RCT	N=49 intervention: control 20:20, age 59.7±4.58: 57.3±4.77, 1 %, BMI 30.1±5.94: 32.7±6.80. pre- and stage 1 hypertension, N/A	22 g/d of freeze-dried blueberry powder for 8 weeks and assessed at 4 and 8 weeks	22 g macronutrient-matched control powder consisted of maltodextrin, fructose, artificial and natural blueberry flavouring, artificial purple and red colour, citric acid, and silica dioxide	83 %	SBP, DBP, Mean arterial pressure, Carotid-femoral pulse wave velocity, Brachial-ankle pulse wave velocity, Heart rate	4
Kent, et al. 2017 (Australia)	Parallel RCT	N=49, intervention: control 24:25, age 78.9±5.2 : 80.6± 6.6, 51 %, BMI 25.7±3.4: 26.6 ±3.5, mild- to moderate dementia, N/A	200 ml/d of cherry juice for 12 weeks	Flavonoids-devoid apple juice	86 %	Letter verbal fluency (executive function),SBP, DBP, heart rate, IL 6,hsCRP	4
Khan, et al. 2014 (UK)	Parallel RCT	N=66, high dose intervention: low dose intervention: control 21:22:21, age 51+11, 67 %, BMI 29.2±6.9, healthy, N/A	250 ml/d of high blackcurrant juice drink (81.5 mg/100 ml total polyphenols); low blackcurrant juice drink (27.3 mg/100 ml total polyphenols), for 6 weeks	Flavoured water	97 %	SBP,DBP,FMD,GTN-mediated vasodilation	3
Kim, 2018 (US)	Double blinded parallel RCT	N=43(37), intervention: control 19:18, age 46.6±11.5: 42.0±14.4, 31.6 %: 27.8 %, BMI 33.5±6.7, metabolic syndrome, N/A	325 ml/d pf acai berry beverage (containing 1139 mg L−1 gallic acid equivalents of total polyphenols) for 12 weeks	325 ml placebo beverage	86 %	Total cholesterol, TGs, hs-CRP, IL-6, TNF-a	2

Table. 2.1 (Continued)

Lynn, et al. 2012 (UK)	Parallel RCT	N=51(48), intervention: control 24:24, age 39±1.24 : 36.1± 0.92, 33 %, BMI 24.99± 1.26 24.99± 1.06, healthy, N/A	330 ml/d of pomegranate juice for 4 weeks	Lemonade drink-devoid of bioactive plant compounds, antioxidants or vitamins, and contained only a trace amount of sodium, similar energy and carbohydrate	100 %	PWV, SBP, DBP, MAP, Heart rate	3
Lynn, et al. 2014 (UK)	Parallel open-label RCT	N=47, intervention: control 25:21, age 38.3±6.16: 37.2±5.78, 38 %, BMI 24.6±3.63: 23.5±3, healthy, N/A	250 ml/d of cherry juice concentrate (30 ml diluted with 220 ml of water; Cherry Active®) for 6 weeks	Lemonade drink	98 %	PWV, hsCRP, SBP, DBP, Total cholesterol, HDL-C	2
Lazavi, et al. 2018 (Iran)	Parallel RCT	N=46, intervention: control 23:23, age 56.86± 8.47, 33.3 %: 38 %, BMI 29.22±3.98: 27.78 ± 3.45, patients with type 2 diabetes, N/A	200 ml/d pomegranate juice for 8 weeks	No intervention	100 %	SBP, DBP, TC, HDL, LDL, TG, ApoB, ApoA	5
Leal, et al. 2019 (Brazil)	Parallel RCT	N=20, intervention: control 10:10, age 64.9±4.0: 72.9± 5.6, 38.5 %, BMI 24.7±1.0: 26.6± 1.1, hypertensive elderly, N/A	200 ml/d of grape juice for 12 weeks	No intervention	91 %	SBP, DBP	1
McAnulty, et al. 2014 (US)	Parallel RCT	N=25, intervention: control 13:12, age 46.15:±11.92:39.92±13.38, N/A, BMI 27.8± 5.46: 24.23± 3.44, sedentary males and females, N/A	38 g/d blueberry powder, equivalent to 250 g rehydrated berries for 6 weeks	placebo powder contained a blend of maltodextrin, fructose, BB flavouring, colouring, citric acid, and a flow agent (silica)	100 %	SBP, DBP, Aix (Augmentation Index),ASP (aortic systolic pressure), cPWV	2
Novotny et al. 2015 (US)	Parallel RCT	N=60, intervention: control 30:30, age 49.8±11.1: 50.0±11.6, 48 %, BMI 27.8± 3.8: 28.9± 4.5, healthy, N/A	480 ml/d of cranberry juice for 8 weeks	flavour/colour/energy –matched placebo beverage	93 %	Total cholesterol, LDL cholesterol, HDL cholesterol, TGs, apo A-I,apo A-II, apoB, sICAM, sVCAM, Diastolic BP, Systolic BP,CRP	4

Table. 2.1 (Continued)

Sumner, et al. 2005 (US)	Parallel RCT	N=45, intervention: control 26:19, age 69±11, 89 %, BMI 28±6, CHD and myocardial ischemia patients, 86.67 % white	240 ml/d of pomegranate juice for 3 months	Placebo drink	93 %	SBP, DBP, Total cholesterol, HDL-C,LDL-C,TGs	4
Stull, et al. 2015 (US)	Double -blind parallel group RCT	N=46, intervention: control 23:23, age 55±2: 59±2, 36 %, BMI 35.2 ± 0.8: 36.0 ± 1.1, metabolic syndrome, N/A	45 g/d of freeze-dried blueberry powder, equals to 2 cups of fresh whole blueberry/ consumed with 24-oz yogurt and skim milk-based smoothie, for 6 weeks	identical smoothie without the blueberry powder	87 %	Glucose, Insulin, Triglycerides, Cholesterol,LDL,HDL,24h-SBP,24h-DBP,RHI	4

Among the fruit juice category, most studies evaluated the effect of cranberry juice, grape juice, pomegranate juice, cherry juice, orange juice (n = 7, 5, 5, 5, 4 respectively). The mean dosage applied for these types of juice was 480 ml, 353 ml, 238 ml, 173.6 ml and 425 ml respectively. The remaining interventions included blueberry juice (n = 1), grapefruit juice (n = 1), barberry juice (n = 1), blackcurrant juice (n = 1), strawberry juice (n = 1) and acai berry juice (n = 1) . Among whole fruit and fruit in other freeze-dried forms of interventions, 4 studies supplemented freeze-dried blueberry powder. Portion conversion of powder to whole fruit was provided in each study; typically the mean dosage of blueberry powder supplementations was 32.75 g (equivalent to approximately 1.5 cups of fresh blueberry). Three trials supplemented freeze-dried grape powder. The mean dosage of grape powder supplemented was 55.33 g, which is equivalent to approximately 2.5 cups of fresh grape. The remaining 5 studies supplemented other berry (powdered raspberry, powdered blackcurrant, freeze-dried strawberry) and citrus fruit (1.5 portion of grapefruit following a low bioactive diet).

2.1.11 Study quality

For studies assessing vascular disease risk factors, the average retention rate for all included trials was 92.64 %, of which 30 out of 45 RCTs obtained no less than 3 points of total Jadad score. Trials generally provided adequate description of methods and procedures, although only 40 % and 33.33 % of RCTs implemented true randomization and true blinding respectively with an adequate description of methods (e.g. computerised statistical randomisation). Jadad scores were provided in **Appendix. 1.1.**

2.1.12 Meta-analysis

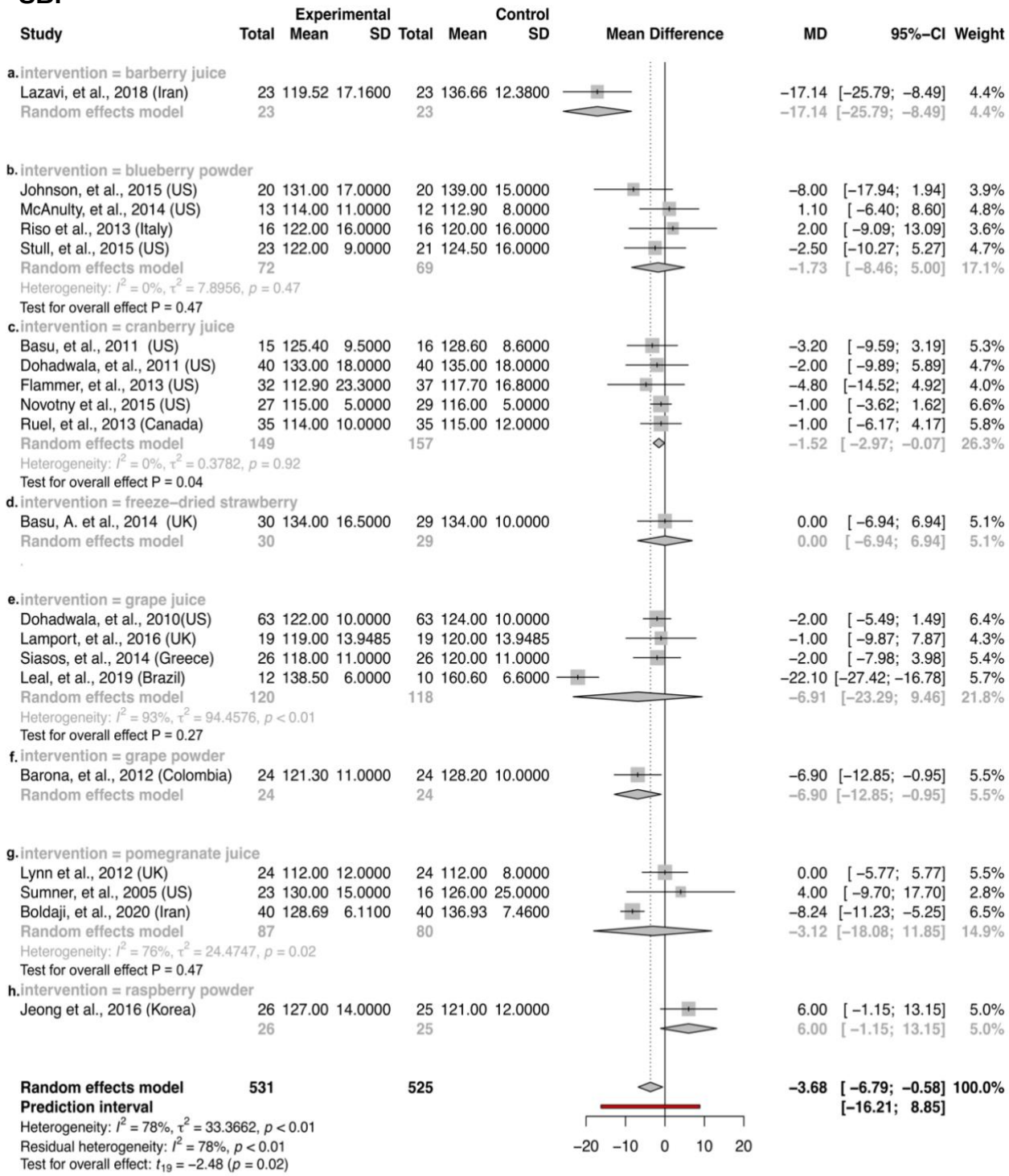
Thirty-eight trials were included in the meta-analysis. The meta-analysis of 38 studies assessing FMD, PWV, SBP, DBP, levels of TAG, TC, HDL-C and LDL-C and levels of vascular inflammatory biomarkers ICAMs, VCAMs, hsCRP and NO were displayed in forest plots [16]. The interventions used in these studies supplemented blueberry powder, grape juice, grape powder, cranberry juice, orange juice, whole grapefruit,

pomegranate juice, raspberry powder, freeze-dried strawberry, acai berry juice, and barberry juice.

Our principal findings from a meta-analysis of interventions supplementing with berry (including 531 treatment and 502 placebo participants) including barberry juice, blueberry powder, cranberry juice, freeze-dried strawberry, grape juice, grape powder, pomegranate juice and raspberry powder suggested significantly reduced SBP by 3.68 mmHg [95 % CI - 6.79 to - 0.58; *P* = 0.02] **(Figure. 2.2)** and DBP by 1.78 mmHg [95 % CI - 3.43 to - 0.12; *P* = 0.04] **(Figure. 2.3)** respectively. There was no significant improvement of DBP by the citrus juice interventions compared to the control.

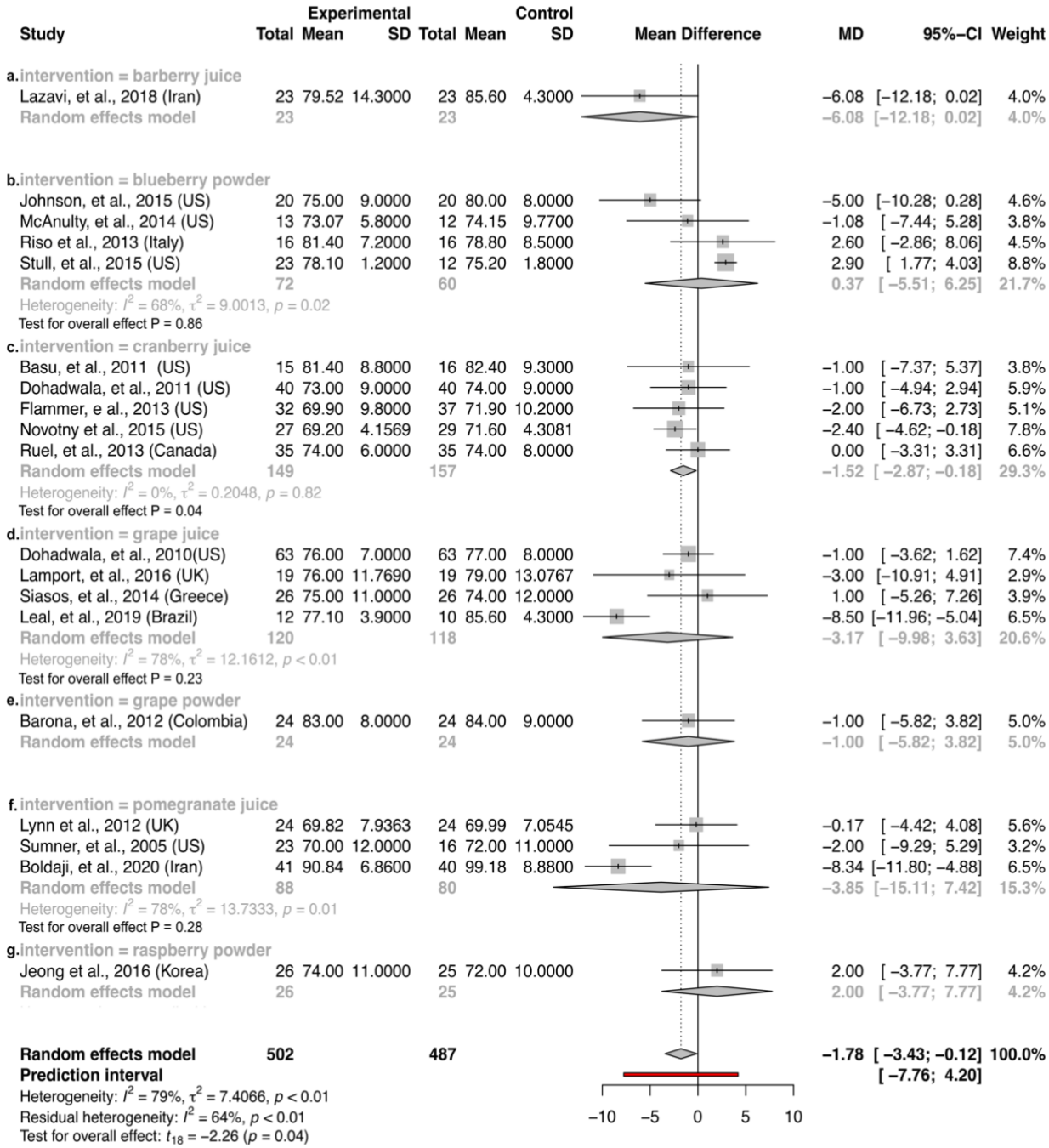
Subgroup analysis showed that specific interventions using cranberry juice, with mean dosage of 432 ml and length of 8 weeks including 149 treatment participants has significantly decreased SBP and DBP by 1.52 mmHg (95 % CI - 2.97 to - 0.07; *P* = 0.05) **(Figure. 2.3)** and 1.52 mmHg (95 % CI -2.87 to -0.18, *P* = 0.04) respectively. Two cherry juice interventions including 36 treatment participants with dosage of 30 ml for 20 days and 330 ml for 6 weeks separately also led to a significant reduction in SBP by 3.11 mmHg (95 % CI - 4.06 to - 2.15; *P* = 0.02)**.** The berry group including blueberry juice, cranberry juice, grape powder, pomegranate juice and raspberry powder was also shown to significantly increase sVCAM-1 levels by 14.57 ng/mL (95 % CI 4.22 to 24.93, *P* = 0.02) in the treatment group relative to the control. The I^2 test suggested significant substantial heterogeneities for berry group investigating the effects on SBP (I^2 = 78 *%, P* < 0.01) (**Figure. 2.3**) and DBP (I^2 = 78 *%, P* < 0.01) **(Figure. 2.4)**. Significant substantial heterogeneities for the citrus juice group investigating the effects on DBP (I^2 = 83 %, *P* < 0.01) were also presented **(Figure. 2.4)**.

Figure. 2.2 The effect of berry interventions including (a) barberry juice, (b) blueberry powder, (c) cranberry juice, (d) freeze-dried strawberry, (e) grape juice, (f) grape powder, (g) pomegranate juice and (h) raspberry powder assessing SBP



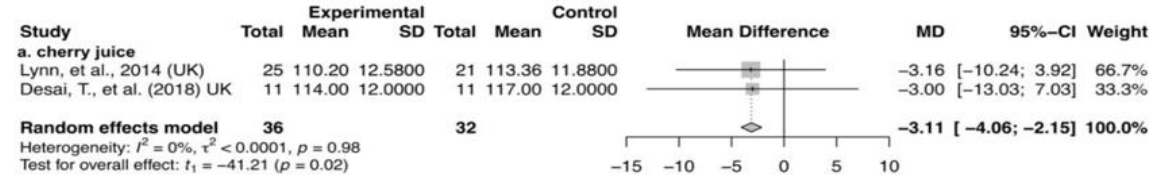
*Notes
a. 200ml, 56 days
b. 22g, 38g, 25g, 45g, equivalent to 1~ 2 cups of blueberry, 56 days, 42 days, 42 days and 42 days respectively
c. 240ml, 480ml, 460ml, 480ml, 500ml, 56 days, 28 days, 120 days, 56 days, 28 days
d. 3 cups, 56 days
e. 490ml, 355ml, 240ml, 200ml, 56 days, 84 days, 14 days, 84 days
f. 46g, equivalent to 2 cups of grape, 30 days
g. 330ml, 240ml, 100ml, 28 days, 90 days, 56 days
h. 750mg, 84 days

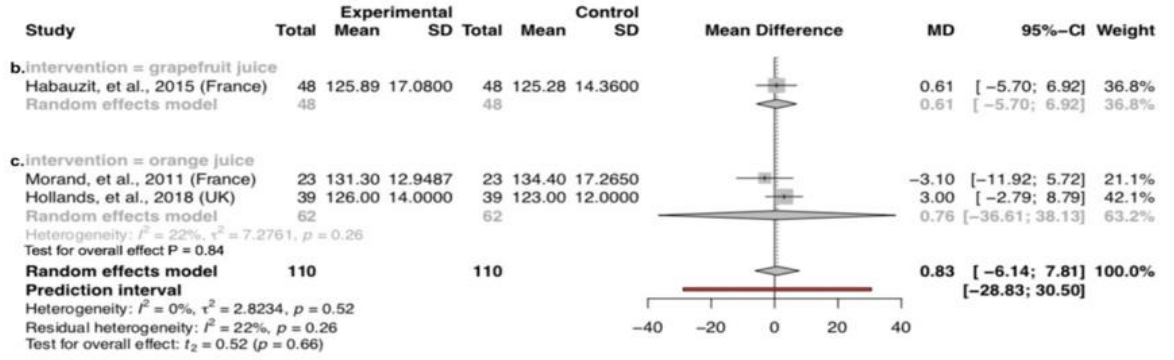
Figure. 2.3 The effect of berry interventions including (a) barberry juice, (b) blueberry powder, (c) cranberry juice, (d) grape juice, (e) grape powder, (f) pomegranate juice and (g) raspberry powder assessing DBP

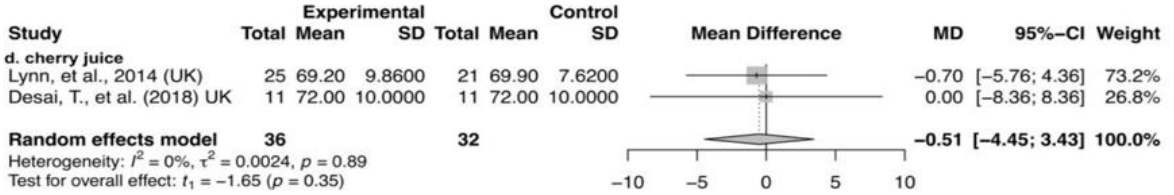


***Notes**
a. 200ml, 56 days
b. 22g, 38g, 25g, 45g, equivalent to 1~ 2 cups of blueberry, 56 days, 42 days, 42 days and 42 days respectively
c. 240ml, 480ml, 460ml, 480ml, 500ml, 56 days, 28 days, 120 days, 56 days, 28 days
d. 490ml, 355ml, 240ml, 200ml, 56 days, 84 days, 14 days, 84 days
e. 46g, equivalent to 2 cups of grape, 30 days
f. 330ml, 240ml, 100ml, 28 days, 90 days, 56 days
g. 750mg, 84 days

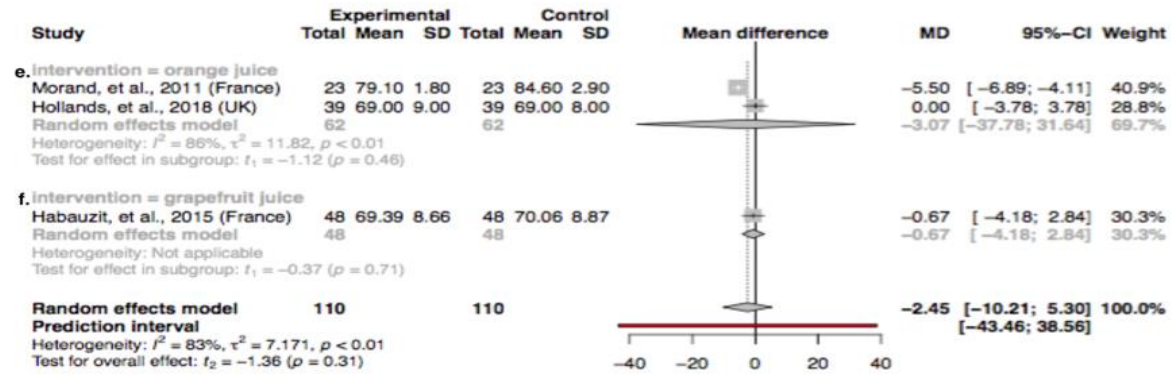
Figure. 2.4 The effect of (a) cherry juice, (b) grapefruit juice, and (c) orange juice interventions assessing SBP and (d) cherry juice, (e) grapefruit juice, and (f) orange juice interventions assessing DBP







*Note
a. 250ml and 30 ml, 42 days and 20 days
b. 340ml, 180 days
c. both are 500ml and for 28 days
d. 250ml and 30 ml, 42 days and 20 days



*Notes
e.500ml, 500ml, 28 days, 28 days
f. 340 ml, 6 months

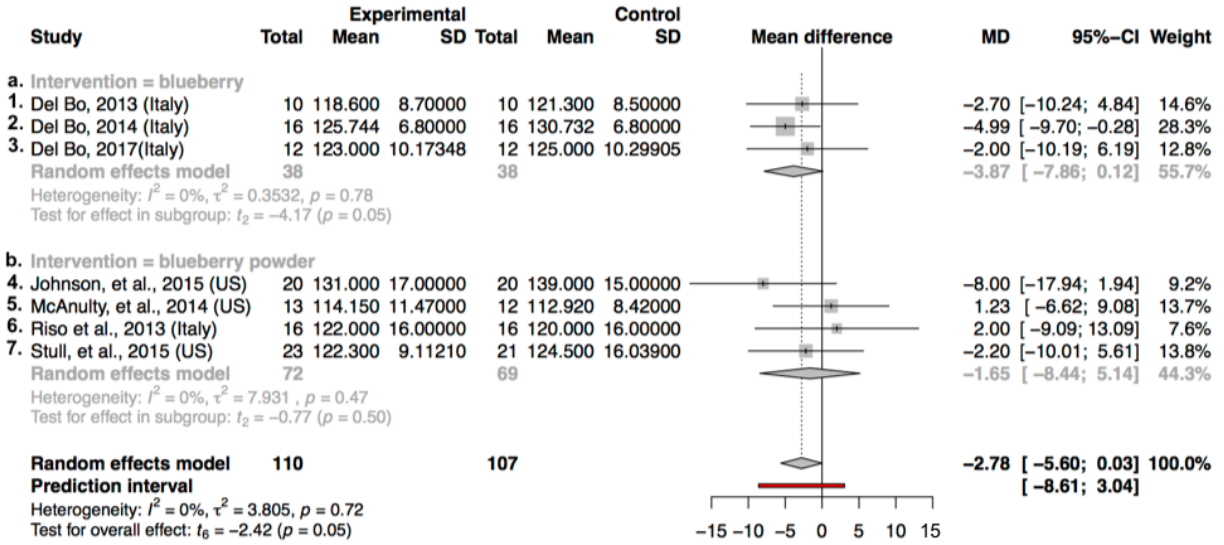
There were no significant effects of other included intervention groups on other vascular and inflammatory markers: FMD, PWV, TAG, TC, LDL-C, HDL-C, ICAMs, VCAMs**,** NO or hsCRP. The forest plots of these markers were provided in **Appendices. 1.2 - 1.3.** The I^2 test suggested no heterogeneity for berry interventions assessing the effect on FMD (I^2 = 0 *%, P* = 0.39) and non-significant moderate heterogeneities for berry interventions assessing the effect on PWV (I^2 = 58 *%, P* = 0.07). The I^2 test suggested significant substantial and moderate heterogeneities for the berry group (I^2 = 71 *%, P* < 0.01) and cherry juice (I^2 = 55 *%, P* = 0.14) investigating the effects on TC respectively. There are significant moderate heterogeneity for the berry group investigating the effects on HDL-C (I^2 = 56 *%, P* < 0.01)**;** non-significant moderate heterogeneities were shown for the berry group investigating the effects on TAG (I^2 = 36 *%, P* = 0.08)**,** LDL (I^2 = 37 *%, P* = 0.08). Funnel plots and the egger's test for the berry group showed an overall symmetric distribution of the interventions around the standard error for the investigated outcomes of TAG, TC, LDL-C (egger's tests *P* > 0.05)**.** Funnel plots and the egger's test for the berry group showed an overall symmetric distribution of the berry interventions around the standard error for the investigated outcomes of SBP; asymmetric distributions were shown for the berry group investigating the effect on DBP and TAG, trim and fill method was further implemented to adjust for the publication bias **(Appendix. 1.4).**

2.1.13 Principal findings

The berry group overall has shown improvements to both SBP and DBP by over 3 mmHg and 1 mmHg respectively, which revealed the potential of the berry group to play a beneficial role in the diet to maintain vascular health. The dosage of juice and freeze-dried powder in the included berry group ranged from 100 ml - 500 ml and 750 mg - 46 g respectively. One freeze-dried strawberry study supplemented 3 cups. The duration of the berry group interventions ranged from 14 days to 120 days. Specifically, high doses of 432 ml cranberry juice showed improvements to blood pressure in our meta-analysis. Small studies of cherry juice, categorised as stone fruit, using up to 330 ml showed improvement to systolic blood pressure but not diastolic blood pressure.

However, there are evidence from acute interventions (≤ 24 hours) showing that frozen blueberries [137, 138] have improved SBP in meta-analysis and blueberry interventions combing both acute and chronic interventions also improved SBP significantly, which showed vascular protective potential of blueberry supplements (Shown in **Figure. 2.5**). The largest number of interventions supplementing powder were also using blueberry powder in this review with high heterogeneity existed, which could be accounted by the variations of the study design. It would be of nutritional interest to test powered berry against whole fruit if more RCTs with consistent dosage and intervention length were conducted.

Figure. 2.5 Forest plot of (a) small sized acute studies supplementing frozen blueberry and (b) chronic studies supplementing blueberry powder for improved SBP



*Note:
1. 24h, 300g
2. 2h, 300g
3. 2h, 300g
4. 22g, 56 days
5. 38g, 42 days
6. 25g, 42 days
7. 45g, equivalent to 1~ 2 cups of blueberry, 42 days

These findings suggested that berry interventions, especially using juiced cranberry may effectively reduce SBP and DBP. However, the limitations within each cranberry juice intervention included small sample size [139], not refraining from other polyphenol-rich foods/beverage throughout [12, 140, 141], and different blood pressure between groups at baseline [142] that have made the meta-analyses results inconsequential.

2.1.14 Previous evidence of RCTs assessing vascular function

For interventions assessing vascular function, our review showed that blueberry and grape in both juiced and freeze-dried forms have been supplemented frequently, however, this quantitative analysis only displayed an improvement on the outcomes by the consumption of cranberry juice and cherry juice.

A previous systematic review investigated the impact of fruit polyphenols on blood lipids (n = 17), platelet function (n = 9), BP (n = 9) and endothelium-dependent vasodilation (vascular function) (n = 7) and suggested that polyphenols from fruit such as pomegranate, purple grape and blueberry are particularly effective at preventing hypertension compared to other vascular disease risk factors [119]. The berry group in particular was shown to possess vascular-protective properties; the underlying mechanisms highlighted include inhibitory effects on inflammatory gene expression, oxidative stress, carbohydrate digestive enzymes and foam cell formation as well as increased effect on nitric oxide synthase following anthocyanins, the major polyphenol in berry [120].

In another meta-analysis of 95 prospective studies of fruit and vegetable intake, Aune *et al.* found that fruit juice intake had little association with CVD and total cancer, while slight inverse associations were observed for CHD with Risk Ratio (RR) [95 % CI, 0.79 (0.63 - 0.98)], stroke with RR [95 % CI, 0.67 (0.60 - 0.76)] and all-cause mortality with RR [95 % CI, 0.87 (0.83 - 0.91)] every 100 g/d increment, however, the very low number of fruit juice studies (n = 2) made these findings preliminary and more studies are needed before any firm conclusions can be drawn [117]. Aune *et al.* also reported an inverse association between high vs. low berry consumption and all-cause mortality in the meta-analysis, whereas no similar associations were observed for CHD and CVD [117]. Knowledge about powdered fruit forms for the association with disease risk is currently very limited [143].

However, there is evidence showing that increasing the consumption of fruit juice by one serving per day was associated with a 7 % greater incidence of type 2 diabetes (95 % CI, 0.8 % to 14 %) [144] and there was also greater risk of weight gain with higher consumption of fruit juice, probably because of the high sugar content and excess calories provided [145]. Fruit juice contains quantities of sugar classified as ‘free’ sugars like sucrose, compared with whole fruit in which the sugars are classified as intrinsic. Increased dietary fructose following sucrose intake is reported to increase de novo lipogenesis (DNL) levels and VLDL, which has been shown to increase the risk of developing non-alcoholic fatty liver disease (NAFLD) [146]. Therefore, cautious

interpretations should be made for choosing fruit juice interventions or for promoting fruit juice consumption as healthy options to increase fruit and vegetable intake.

Our review has also shown elevated sVCAM-1 levels after berry interventions, however, some authors have suggested that the magnitude of the increase in sVCAM-1 may not be clinically relevant, as the other vascular inflammatory markers did not change between the treatment and the control group after the interventions [147], which is also in line with the results of other inflammatory markers in our review. Aside from this, Bardagjy *et al.* and Ruel *et al.* reported significantly higher sVCAM-1 levels in the treatment group at the baseline compared to the control, which may contributed to the elevated sVCAM level after the interventions in the berry-treated group [141, 148]. There is also limited study data under some types of fruit interventions (i.e. grape powder and pomegranate juice) investigating all risk factors to be meta-analysed. Although the consumption of a range of berry have been linked with alleviated disease risks, considering the results from our review and previous evidence, current evidence is insufficient and inconsistent to substantiate the consumption of specific berry or other fruit as a vascular-protective dietary strategy.

2.1.15 Implications for the intervention design

Several points were raised from these reviews to help inform our study design:

- **i.** Cranberry and cherry juice studies have shown improvements to SBP, however we have to emphasize that the analyses were limited to either small samples size, unbalanced baseline blood pressures, or neglected diet refrainment. Furthermore, improvements to other outcomes were not observed, which overall does not support the notion that the consumption of these specific fruit powder or other fruit juice will confer a vascular-protective benefit.
- **ii.** Future interventions on supplementing berry (i.e. blueberry, cranberry, grape) with a sufficient sample size are warranted, as these appeared to have the biggest potential to improve vascular function.
- **iii.** No fresh fruit was identified in the previous interventions, which could be due to the difficulty of storage and allocation during the intervention. Fresh whole fruit is generally how the fruit is consumed in the general population, which highlights a novel and necessary intervention for future studies.
- **iv.** Apart from fruit juice, most RCTs supplemented freeze-dried powder and this form could also be an effective method to increase overall fruit consumption. Increased intake of “free sugar” in fruit juice has been shown to increase the risk of weight gain, type 2 diabetes and non-alcoholic fatty liver disease (NAFLD) [144, 145, 149], which is a controversial option considering fruit juice for health promoting and also for our study design.
- **v.** Supplementing whole frozen blueberry in acute duration (300 g, < 24h) [18, 137] along with supplementing blueberry powder in chronic duration (equivalent to 150 - 300 g whole blueberry, 6 - 8 weeks) [69-72] to both young and older participants may significantly reduce SBP to benefit vascular function. A long term investigation for whole blueberry assessing vascular function is lacking and worth exploring.

Chapter 3. Systematic review and meta-analysis of fruit interventions assessing cognition

3.1 Effects of fruit consumption on cognition : a systematic review and meta-analysis of randomised controlled trials.

3.1.1 Introduction

It is well recognised that cognitive impairment can be affected by several dietary factors [118]. One dose–response meta-analysis with a total of 31,104 participants and 4,583 incident cases of cognitive impairment and dementia showed that an increment of 100 g per day of fruit and vegetable consumption was related to an approximately 13 % (Odds Ratio/OR = 0.87, 95 % CI 0.77 - 0.99) reduction in cognitive impairment and dementia risk [150]. A number of interventional studies showed positive results indicating that intake of a range of flavonoid-rich fruit (e.g. blueberry, orange juice) could improve both immediate and chronic cognitive performance or mood in older adults [151]. Currently there is correlation across cognitive domains (i.e. working memory and executive function) [152], and no correlations of the improvements for different cognitive domains following fruit interventions were found.

A number of potential underlying mechanisms have been identified including the interaction of fruit polyphenols, and antioxidants with intracellular neuronal and glial signalling pathways, energy metabolism, regulation of cerebral blood flow, and protection against neurotoxins and neuroinflammation [153-155]. However, findings are contradictory, for example a previous study using cherry juice has shown no cognitive benefit in middle-aged [156] or older adults [157] with and without dementia following acute (≤ 24 hours) supplementation. Whilst sample size was indicated as a factor in these null findings, the length of intervention as well as types and forms of fruit may also be important.

3.1.2 Objectives

Given the potential for differing nutritional value of varying types and forms of fruit, it is likely that their health benefits will also differ. However, little is known about the comparative effect of processed fruit on cognitive. A review of this type is important to clarify the evidence base for the type and form of fruit that is most cognitive-protective and to further inform our interventional design. The aim of this study was to

systematically review and meta-analyse available human interventions to evaluate the potential effect of consumption of whole, freeze-dried, powdered fruit and fruit juice on cognition and mood in RCTs to support findings from the previous chapter and inform the blueberry interventional design.

3.1.3 Methods

We searched for studies investigating the effect of berry, citrus and cherry supplementations on cognitive health. The inclusion criteria for interventions and participants was the same with the section 2.1.3. The primary outcomes were memory, executive function, psychomotor speed and mood (details described below).

3.1.4 Data sources

The protocol and databases used were the same with section 2.1.4. The search of the investigated themes in this review was as following: (Fruit OR citrus OR orange OR berry OR berries OR grape OR blueberry OR blueberry OR blackberry OR blackberries OR raspberry OR raspberries OR cranberry OR cranberries OR cherry OR cherries) AND cogniti* OR memory OR “executive function” OR “reaction time” OR “psychomotor speed” OR attention OR mood) AND (trial OR intervention).

3.1.5 Study selections

Procedure of study selections was the same with section 2.1.5.

3.1.6 Data abstraction

Procedure of data abstraction was the same with section 2.1.6.

3.1.7 Risk of bias assessments

Jadad Score (0 to 5) [129], funnel plot and Egger's test [130] were used to assess study quality and publication bias, as described in section 2.1.7.

3.1.8 Data synthesis

R studio version 3.5.2 and the package "meta" [131] were used to pool and meta-analyse data from collected studies. Sensitivity analysis was performed to investigate the impact of studies adjusting for participants' physical level [132, 133] and also the impact of juice quality on the meta-analysis results.

All pooled results were presented as weighted mean difference with 2-sided *P* values. 95 % confidence intervals (CIs) and prediction intervals were both presented in the results. Basing on the categorised cognitive tasks, meta-analyses investigating the effects of berry interventions on memory, executive function and psychomotor speed [68, 77, 158-160] and 2 cherry juice studies assessing executive function and psychomotor speed were carried out [132, 161]. Two grape powder studies assessing MMSE (Mini Mental State Examination), which measures cognitive impairment, were also included [30, 162]. Memory and executive function both encompass the essential cognitive processes in a person's life [163]. Psychomotor speed assesses the individual's ability to detect and respond to a stimulus and therefore reflects the relationship between cognition and physical movements [164]. The random-effects model, heterogeneity and effect size estimations for meta-analysis were described in section 2.1.8.

3.1.9 Literature search

In accordance with PRISMA guidelines [128], **Figure. 3.1** describes the selection process of included studies. The search for literatures assessing vascular function and cognition was carried out together but only the final record for cognitive function was reported here. The initial search produced 13861 articles from the four databases, this record was reduced to 8613 articles after duplicates were removed. After screening of the titles and abstracts for eligibility, the final selection identified 14 trials assessing cognition. From trials included, 10 trials assessing cognition were entered into the meta-analysis.

3.1.10 Study characteristics

For studies assessing cognition and mood **(Table. 3.1),** there were 2 cross-over RCTs [158, 165] and 12 parallel RCTs [30, 68, 77, 132, 159-162, 166-169], 13 of these studies recruited older participants (aged 60 years or older) [30, 68, 77, 132, 159-162, 165-169]. Baseline characteristics of participants varied across interventions, 8 studies included healthy participants at baseline [68, 77, 132, 158, 159, 162, 165, 169], 5 studies included older people manifesting cognitive decline [30, 160, 166-168] and the remaining 1 study recruited diagnosed mild to moderate dementia [161]**.**

Figure. 3.1 Flow diagram of study selection for the review

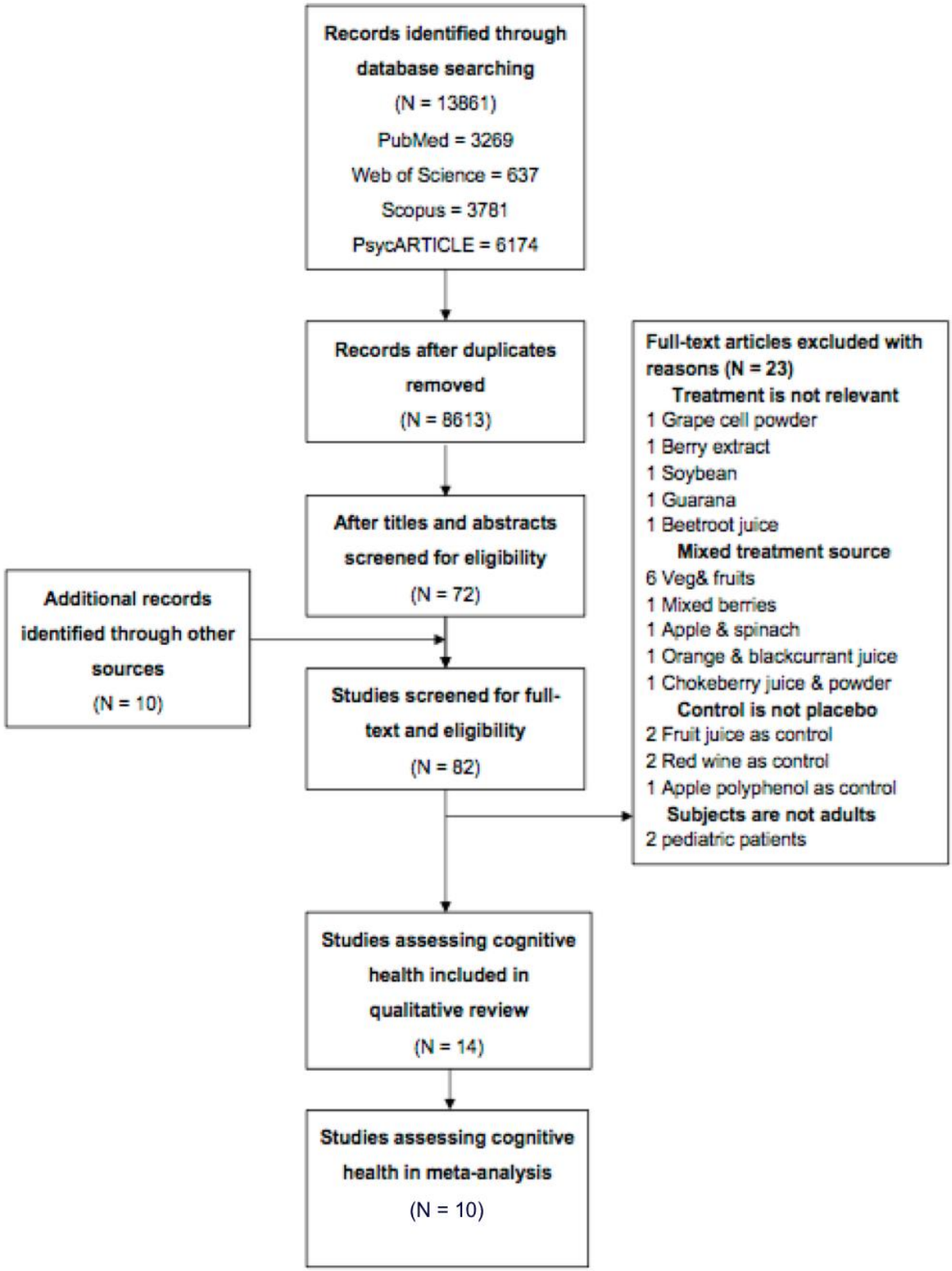


Table. 3.1 Summary of interventions assessing the effect of fruit supplementation on cognition and mood

Reference	Study design	Sample (sample at baseline/N, age/years, %male, mean BMI (kg/ m2), health status)	Intervention type, dose and duration	Assessments	Effects of intervention	Jadad score
(Bowtell *et al.* 2017)	Double-blind, parallel-group RCT	N=26 (12:14), intervention: control group 67.5 ± 3.0: 69.0 ± 3.3 years, intervention: control group 58.33 % : 42.86 % males, mean BMI intervention: control group 25.9 ± 3.3: 27.1 ± 4.0 healthy	30 ml blueberry (BB) concentrate (blueberry active) providing 387 mg anthocyanidins, 12 weeks, isoenergetic synthetic blackcurrant and apple cordial as control	Detection Task, Groton maze timed chase test and learning test, sequential letter 1-back and 2-back tasks, fMRI, serum sample	Improved executive function (Groton maze learning task accuracy). Also improved task-related brain activation (Brodman areas, presumes, anterior cingulate, insula and thalamus regions)	4
(Boespflug *et al*. 2018)	Double-blind, parallel-group RCT	N=21 (8:8), 80.4 (7.3) :75.5(4.8) years, mean BMI 26.2(3.6):26.4(2.4) with age-related memory decline	Blueberry powder: 12.5*2g equivalent to 148 g whole blueberry, 16 weeks, placebo powder: 12*2g	Sequential letter n-back tasks, fMRI	Trend for improving working memory at larger sample size (effect size d = 1.02). Also increased BOLD activation in the left pre-central gyrus, left middle frontal gyrus, and left inferior parietal lobe during tasks after BB treatment.	2
(Calapai *et al*. 2017)	Parallel RCT	N = 110 (**57**:52), 56-75 years, 48.2 % males, mean BMI 23.2±1.0, healthy	250 mg/d Cognigrape-V. vinifera fruit extracted powder and maltodextrin (30–40 %), 12 weeks, placebo was composed of maltodextrin	MMSE score, BDI, HARS, RBANS	Improved attention, language, immediate and delayed memory. Supplementation also produced a significant reduction in BDI (−15.8 %) and HARS (−24.9 %) scores with respect to baseline levels ($p < 0.0001$) and placebo ($p < 0.0001$ for BDI and $p < 0.05$ for HARS.	3
(Chai *et al.* 2019)	Parallel RCT	N = 20 (17: 17); 28.5 ± 3.7: (27.3 ± 4.2); 70.0 ± 3.7: 69.5 ± 3.9 years; 40 %:52.9 % males; BMI 28.5 ± 3.7: (27.3 ± 4.2); older adults with normal cognitive function	480 ml tart cherry juice (68 ml Montmorency tart cherry juice concentrate was diluted with 412 ml water) per day for 12 weeks; placebo consisted of mixing unsweetened black cherry flavoured Kool-Aid (Kraft Foods, United States) with water. Dextrose and fructose were added to match the carbohydrate content found in tart cherry	Memory ability, Memory contentment, Memory strategy, digit span, PAL first trial memory, PAL total errors adjusted, RTI movement time, RTI reaction time, RVIP A, RVIP mean latency, SWM strategy, SWM total error	Increased subjective memory in the domain of contentment with memory by 5 % and reduced movement time by 4 % in comparison with the control drink. Also reduced errors in episodic visual memory by 23 % compared to control drink as assessed by PAL task.	2

Table. 3.1 (Continued)

(Crews *et al.* 2005)	double-blind, parallel-group RCT	N = 50 (24:23); 69.28±6.45 years old, Healthy. Cognitive intact subjects	909 ml/d 27 % cranberry juice for 5 weeks, placebo drink	Immediate free recall, long term storage, short-term recall, long-term retrieval, consistent long-term retrieval, random long-term retrieval, cued recall, delayed free recall, delayed recognition, Faces I, Faces II, Digit symbol, Part A, Part B, Word page, colour page, colour-word page	A nonsignificant trend (P = 0.123) observed for twice as many subjects of subjective, self-report improved memory in cranberry group compared to placebo controls.	3
(Kean *et al.* 2015)	Double-blind, randomized, cross-over	N=37, 66.7 ±5.3 years, 35.13 % males, mean BMI 26.1±1.1, healthy older adults	High-flavanone (305 mg) 100 % orange juice, 500ml/d for 8 weeks, equicaloric low-flavanone (37 mg) orange-flavoured cordial (500 mL) as control	SBP, DBP, DSST, DSST dual, Go-No-Go RT, LF, LM, Serial Sevens, CERAD Immediate and Delayed, SWM, Immediate and Delayed VPA, PANAS Positive and Negative Affect Scale	Improved cognition (significant drink x visit interaction for global cognitive function and executive function). No effect on mood and BP.	5
(Kent *et al.* 2017)	Parallel RCT	N = 49 (24:25), 78.9 ± 5.2 : 80.6 ± 6.6 years, 51 % males, mean BMI 25.7 ± 3.4 : 26.6 ± 3.5, mild-to-moderate dementia	200 ml/d cherry juice, 12 weeks, flavonoid-devoid apple juice as control	RAVLT, SPOT, Boston naming test, TMT, Digit Span Backwards Task, Category and Letter Verbal Fluency, SB, DBP, serum sample	Improved cognition in memory and executive function and reduced SBP of 7.7 mmHg after juice treatment. No effect on Vitamin C and inflammatory markers.	4
(Krikorian, Nash, *et al.* 2010	Parallel double-blind RCT	N= 12 (5:7), 78.2±5.0 years, 66.67 % males, older adults with early memory decline but not dementia	100 % Concord grape juice, 6 and 9 ml/kg/d, 3 portions daily, 12 weeks, placebo beverage	CVLT, SPALT, GDS, glucose and insulin	Improved memory and insulin level after juice treatment. No effect on mood.	3
(Krikorian *et al.* 2012)	Parallel double-blind RCT	N= 21 (11:10), 76.9±6.1 years, 52.38 % males. mild cognitive impairment	100 % Concord grape juice 6.3−7.8 mL/ kg/d, 3 portions daily, 16 weeks, placebo beverage	CVLT, GDS, fMRI, SBP, DBP	Attenuated cognitive error (5.03 vs 7.16 interference errors on recognition memory task) and great activation in right superior parietal cortex and right middle frontal cortex regions after juice treatment. No effect on mood and BP.	2

Table. 3.1 (Continued)

(Lamport *et al.* 2016)	Double-blind, randomized cross-over design	N=25, 43.2±0.6 years old healthy females, mean BMI 24.6 ± 0.5	355 ml/d concord grape juice, 6wks and 12 weeks, energy-, taste-, and appearance-matched placebo	VVLT and VSLT IR & DR, RVIP, Grooved Pegboard, Tower of Hanoi, SBP, DBP, Subjective Mood, Driving performance	Better immediate spatial memory and aspects of driving performance after GJ intake. No difference in mood between groups	4
(Lee *et al.* 2017)	Parallel double-blind placebo controlled RCT	N=13 (5:5), 72.2± 4.7 years, 50 % males, mild cognitive decline	Grape formulation ---freeze-dried grape powder in 8 oz. water, 72 g/d---3 standard servings daily, 6 months, placebo formulation matched in appearance, flavour, smell, volume and content of fructose and glucose but free of polyphenols	MMSE, ADAS-Cog Hopkins Verbal Learning Test-Revised, Benton Visual Retention Test, Rey-Osterreith CFT, Boston Naming Test, LF, Category Fluency, Stroop, TMT Parts A and B, Wisconsin Card Sorting Test-64, WAIS-III Tasks, Wechsler Test of Adult Reading, Memory Functioning Questionnaire, Hamilton Mood Scales, Neuroimaging sVOI	Attenuated decline in brain metabolites at regions of right posterior cingulate cortex and left superior posterolateral temporal cortex and improved correlated attention after grape treatment. No effect on mood.	3
(Miller *et al.* 2017)	Parallel double blind placebo-controlled RCT	N = 42 (18:19). 67.8 ±4.6: 67.3 ±4.8 years, 28 %:37 % males, mean BMI 24.1±3.7: 24.0±2.5, age-related motor and cognitive decline	24 g/d freeze-dried blueberry powder. 90 days, placebo powder	CVLT, ANT, DS, TMT, TST, wMWM, GDS, POMS	Attenuated cognitive error and improved executive function after blueberry treatment. No effects on mood.	4
(Schrager *et al.* 2015)	Parallel RCT	N = 20 (13:7), 69.5±9.3: 68.4±7.7 years, 45 % males, mean BMI 26.4±3.9: 26.2±3.2, healthy	6-cup (48 ounce (1.4 kg))/week frozen blueberry, 6 weeks, placebo/carrot juice with low anthocyanins contents	Grip strength, SRT, adaptive gait tests, TMT-B	Reduced errors (76.9 % vs 57.1 % of participants in BB vs Control had reduced errors) and improved mobility after BB treatment.	3

Table. 3.1 (Continued)

(Whyte *et al*. 2018b)	Parallel double blind placebo-controlled RCT	N = 122-->112 (29:28:28:27); 70.8±3.88 years; 38.50 % males, healthy older adults	500mg, 1000mg blueberry powder or 111mg purified blueberry extract for 24 weeks, colour matched placebo	Rey's Auditory Verbal Learning Task (RAVLT), Picture Recognition Task, Corsi Block Task, Stroop Task, and Modified Attention Network Task (MANT), the Serial 3, Serial 7, and Sternberg task, the PANAS-NOW	No effect on cognition and mood after the blueberry powder intervention. Improved episodic memory performance in delayed word recognition and marginally significant improved visuo-spatial Corsi Block performance at 3, but not 6, months following blueberry extract intervention.	5

ADAS= the Alzheimer's Disease Assessment Scale, ADCS-ADL=the Alzheimer's Disease Cooperative Study-Activities of Daily Living, AG=Affect Grid, ANT=Attention Network Task, BDI=Beck Depression Inventory, CBFV=Cerebral Blood Flow Velocity, CERAD=Consortium to Establish a Registry for Alzheimer's Disease, CFT=Complex Finger Tapping, CPT=Continuous Performance Task, CRT=Choice Reaction Time, CS=Contrast Sensitivity, CVLT=California Verbal Learning Test, DBP=Diastolic Blood Pressure, DPR=Delayed Picture Recognition, DRS=the Dementia Rating Scale, DSST=Digit Symbol Substitution Test, DV=Digit Vigilance, DVR=Delayed Verbal Recall, DWR=Delayed Word Recognition, EEG= Electroencephalogram, fMRI=Functional Magnetic Resonance Imaging, FVT=Freiburg Vision Test, GDS=Geriatric Depression Scale, HARS=Hamilton Anxiety Rating Scale, IWR=Immediate Word Recall, IVR=Immediate Verbal Recall, LF=Letter Fluency, LM=Letter Memory, MMSE=Mini Mental State Examination, NWM=Numeric Working Memory, NIRS=Near-IR spectroscopy, NPI=Neuropsychiatric Inventory, PANAS=Positive and Negative Affect Scale, PP=Picture Presentation, POMS=Profile of Mood States questionnaire, RAVLT= Rey Auditory Verbal Learning Test, RBANS= Repeatable Battery for the Assessment of Neuropsychological Status, RT=Reaction Time, RVIP=Rapid Visual Information Processing, SBP=Systolic Blood Pressure, SFT=Simple Finger Tapping, SPOT=Self-ordered Pointing Task, SPALT=Spatial Paired Associate Learning Test, SRT=Simple Reaction Time, sVOI=Standardised Volume of Interest, SWM=Spatial Working Memory, TMT=Trail Making Task, TST=Task Switching Task, VAS=Visual Analogue Scales, vMWM=Virtual Morris Water Maze, VPA=Verbal Paired Association, VSLT=Visual Spatial Learning Test, VVLT=Visual Verbal Learning Test, WAIS=Wechsler Adult Intelligence Scale, WFC=Word Fragment Completion, WMS=Wechsler Memory Scale, WP=Word Presentation

For studies assessing cognitive health, there were 8 studies supplementing fruit juice, 5 studies supplemented fruit powder and only 1 study used whole frozen fruit. Among 14 studies, the mean intervention duration was 13 weeks, ranging from 6 weeks to 6 months. Among the juice supplementations, 3 studies were accumulated under the groupings of grape juice with mean dosage of 408 ml/d, 2 were cherry juice with mean dosage of 340 ml/d, 1 was cranberry juice with 32 ounces/d (around 942 ml), 1 was blueberry juice concentrate and 1 was orange juice study with 500 ml/d. Three studies supplemented blueberry powder with mean dosage of 16.3 g/d and 2 supplemented grape powder with mean dosage of 48.5 g/d; portion conversion of powder to whole fruit were provided in 3 studies [30, 160, 168]. For example, Lee et al. supplemented grape powder that was comparable to 3 servings of fresh grape daily (approx. 504 g/d fresh grape) [30]. Two studies supplementing blueberry powder were equivalent to providing 1 cup and 1.5 cups of fresh blueberry as measured in FDA recommended fruit portions respectively [160, 168]. Schrager et al. supplemented 200 g/d whole frozen blueberry [68].

3.1.11 Study quality

For studies assessing cognitive health, the average retention rate was 92 %; 11 out of 14 studies obtained no less than 3 points of the total Jadad score **(Appendix. 2.1).** Five studies implementing blinding, randomisation and control all provided adequate description of methods and procedures; 4 implemented true randomisation; 2 studies implemented true blinding, where the placebo were colour and taste matched to mask treatments, and the received treatment was not revealed until the statistical analysis was completed for double blinding.

3.1.12 Meta-analysis

As shown in **Table. 3.2**, for studies included in the meta-analysis, memory was assessed as either the number of correct responses or accuracy (%) in Immediate Word Recall, Delayed Word Recall or CVLT List Free Recall (California Verbal Learning Test); executive function was assessed as the total score, or the number of correct responses or arcsine transformation of the square root of the proportion of

correct answers in Digit Symbol Substitution Test, Digit Span, 2-Back Task or Rapid Visual Information Processing (RVIP); psychomotor speed was assessed as reaction time (RT, ms or s) of Trail Making Task, Reaction Time Test (RTI), Simple Reaction Time, 2-Back Task or RVIP. Studies incorporating grape juice, grape powder, blueberry juice, blueberry powder, cranberry juice and frozen blueberry constituting a berry group along with studies supplementing cherry juice, categorised as stone fruit, were able to provide sufficient data to run meta-analysis. There was insufficient data for mood to be entered into meta-analysis, but the mood assessment results for individual studies were reported in **Table. 3.2.**

All 14 included studies assessed cognition with 13 studies reporting improvement or trend for improvement in cognition; 4 studies assessed mood with 1 study supplementing grape juice finding a trend for mood improvement. The largest portion of studies assessing cognition supplemented frozen, juiced or powdered berry (11 out of 14 studies) with blueberry (n = 5) and grape (n = 5) being the most intensively studied interventions.

Table. 3.2 Cognitive domain classification under each intervention type

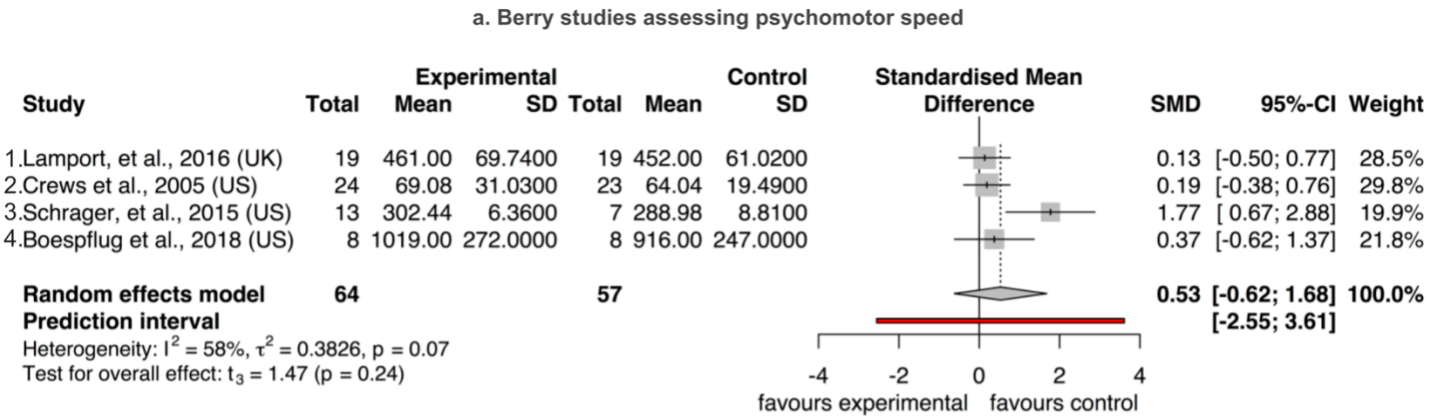
Cognitive domain	Intervention type	Study	Cognitive task entered into meta-analysis*	Unit of test scores*	Results of the chosen task
Memory	grape juice	Lamport, et al. 2016 (UK)	Visual verbal learning test-Immediate recall	accuracy/ %	No changes
	cranberry juice	Crews et al. 2005 (US)	Delayed free recall	correct numbers	No changes
	blueberry powder	Miller, et al. 2016	CVLT list A 1 free recall	correct numbers	No changes
Executive function	grape juice	Lamport, et al. 2016 (UK)	RVIP correct	correct responses	No changes
	cranberry juice	Crews et al. 2005 (US)	Digit symbol	raw score	No changes
	blueberry powder	Boespflug et al. 2018 (US)	2-Back task	arc	No changes
	blueberry juice	Bowtell et al. 2017 (UK)	2-Back task	arc	No changes
	cherry juice	Chai, S. C. et al. 2019 (US)	Digit span	score	No changes
		Kent, et al. 2017 (Australia)	Digit span backwards task (short-term memory)	score	**Final score between groups (*P* = 0.02)**
Psychomotor speed	grape juice	Lamport, et al. 2016 (UK)	RVIP reaction time	ms	No changes
	cranberry juice	Crews et al. 2005 (US)	Trial making task reaction time	total time	No changes
	frozen blueberry	Schrager, et al. 205 (US)	Simple reaction time	ms	No changes
	blueberry powder	Boespflug et al. 2018 (US)	2-Back task reaction time ms	ms	No changes
	cherry juice	Kent, et al. 2017 (Australia)	Trail making task reaction time	ms	No changes
		Chai, S. C. et al. (2019).US	RTI reaction time	ms	No changes
MMSE	grape powder	Calapai, et al. 2017 (Italy)	MMSE	score	**Change score between groups (*P* < 0.01)**
		Lee, et al. 2017 (US)	MMSE	score	No changes

*arc: arcsine transformation of the square root of the proportion of correct answers; CVLT: California Verbal Learning Test; RVIP: Rapid Visual Information Processing; RTI: Reaction Time Test; MMSE: Mini Mental State Examination

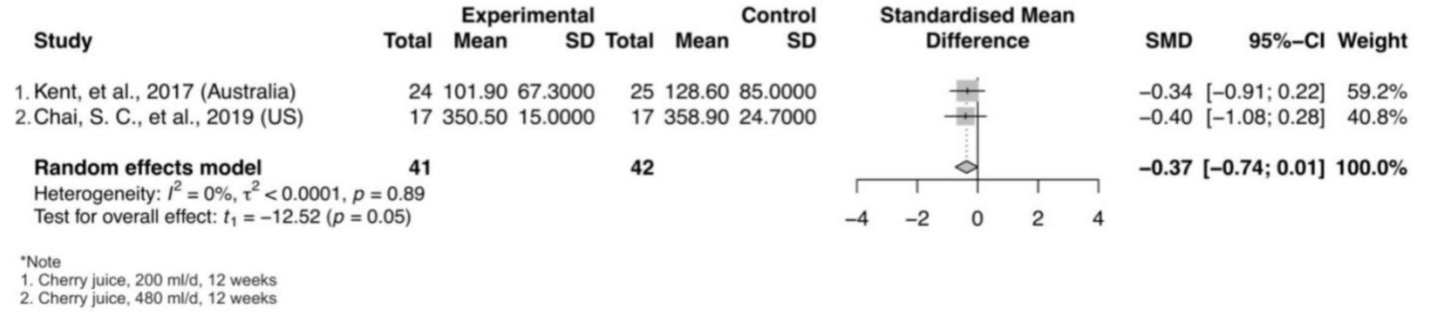
Notably, cherry juice induced a borderline significance in improvement of psychomotor speed after the intervention compared to control (SMD = -0.37, 95 % CI -0.74 to 0.01, P = 0.05, t_1 = -12.52**)** in **Figure. 3.2** The berry group induced no significant difference for psychomotor speed between intervention and control group (SMD = 0.53, 95 % CI - 0.62 to 1.37, P = 0.24, t_3 = 1.47). For the assessment of executive function, the berry group induced no significant difference between intervention and control group (SMD = 0.05, 95 % CI - 0.29 to 0.39, P = 0.79, t_5 = 0.45) and cherry juice induced no significant difference between the two groups (SMD = - 0.65, 95 % CI - 15.63 to 14.34, P = 0.68, t_1 = - 0.55). For memory assessment, the berry group induced no significant difference between intervention and control groups (SMD = 0.04, 95 % CI - 0.89 to 0.97, P = 0.87, t_2 = 0.19). Apart from the analysis assessing cognitive domains, two grape powder studies were able to provide MMSE (Mini Mental State Examination) data, but no significant difference was shown between intervention and control groups (MD = 0.87, 95 % CI -6.40 to 8.15, P = 0.37, t_1 = 1.53). The remaining forest plots are found in **Appendix. 2.2.** Funnel plots and the egger's test for the berry group showed an overall symmetric distribution of the berry interventions around the standard error for the investigated outcomes of executive function, memory and psychomotor speed; symmetric distributions were shown for the cherry interventions investigating the effect on executive function and psychomotor speed and grape powder interventions investigating the effect on MMSE test (egger's tests P > 0.05) **(Appendix. 2.3).**

Significant heterogeneity among studies was observed in the cherry juice studies assessing executive function (I^2 = 95 %, P < 0.01) and the berry group assessing psychomotor speed (I^2 = 72 %, P = 0.03). Higher physical activity levels were shown to link with better cognitive functioning in younger and older adults [170]; 2 studies supplementing blueberry juice concentrate and cherry juice concentrate instead of 100 % juice, could also cause variations to the juice quality and bioavailability [77, 132]. There was no heterogeneity in executive function assessment after the sensitivity analysis excluding studies applying physical activity adjustments, the sensitivity analysis also suggested no effect of juice quality on the meta-analysis results.

Figure. 3.2 The effect of (a) berry interventions and (b) cherry juice interventions assessing psychomotor speed



*Note
1. Grape juice, 355 ml/d, 12 weeks
2. Cranberry juice, 942 ml/d, 6 weeks
3. Whole frozen blueberry, 200 g/d, 6 weeks
4. Blueberry powder, 25 g/d, 16 weeks



*Note
1. Cherry juice, 200 ml/d, 12 weeks
2. Cherry juice, 480 ml/d, 12 weeks

3.1.13 Principal findings

Two cherry juice studies including 41 participants receiving cherry juice with mean dosage of 340 ml/d for 12 weeks induced a borderline significant improvement in psychomotor speed. However, It is important to note that only 2 studies were included and one of those had a low Jadad score (< 3) [132], which limits the impact of this finding. The long term investigation for whole fruit intervention assessing cognition is lacking and worth exploring. The current analyses also do not support the notion that the consumption of fruit powder or other fruit juice will confer a cognitive-protective benefit.

3.1.14 Previous evidence of RCTs assessing cognition and mood

Our systematic review collected a range of frozen, freeze-dried, powdered and juiced fruit interventions, specifically the berry group (n = 11) reporting positive findings on the impact on cognition or mood; the largest portion of studies involved fruit juice (8 out of 14 studies) with grape juice (n = 3) being the most intensively studied supplementations, followed by cherry juice (n = 2). As demonstrated by the meta-analysis, 2 cherry juice studies including 41 participants receiving cherry juice with mean dosage of 340 ml/d for 12 weeks induced a borderline significant improvement in psychomotor speed. However, it is important to note that only 2 studies were included and one of those had a low Jadad score [132], which limited the impact of this finding. No improvement was observed on executive function and memory.

However, our meta-analyses suggested no differences between the intervention and control groups in any cognitive domains following berry or other fruit-based supplementations. Overall, the individual interventions showing improvements in our systematic review still require further substantiation given that the meta-analysis only suggests that cherry juice may have cognition-protective potential.

The systematic review suggested the potential for whole blueberry, blueberry juice concentrate, blueberry powder, grape powder, grape juice, cherry juice, orange juice and cranberry juice supplements to improve cognitive health. Supplementing grape juice also showed potential to improve mood. From the meta-analysis, we found a borderline significant improvement in psychomotor speed following chronic consumption of cherry juice, which has high content of the flavonoids catechin, epicatechin, procyanidins and anthocyanins [171, 172]. The participants of cherry juice studies included here were older healthy and have dementia. Lower psychomotor speed has been found to be associated with increased risk of all-type dementia (hazard ratio [HR] 3.41, $p < 0.0001$), Alzheimer's disease-type dementia (HR 3.18, $p < 0.0001$), Parkinson's disease (HR 2.98, $p = 0.04$) and depressive symptoms (HR 1.53, $p = 0.03$) [173], and is therefore related to chronic mobilities associated with dementia.

Although the meta-analysis indicates a lack of evidence supporting improvements in mood by specific fruit interventions, another meta-analysis with 10 observational studies involving 227,852 participants suggested an inverse association of fruit (RR 0.83, 95 % CI, [0.77, 0.91]; *P* = 0.006) intake with risk of depression [174]. A previous systematic review has also assessed the association between cognitive benefits and fruit consumption but only included limited evidence from juice interventions, where improvements to memory in mildly cognitive-impaired adults after 12 - 16 weeks of consumption were illustrated [151]. The reported grape and blueberry juice studies [166, 167, 175] were identified in our screening process; however, Krikorian *et al*. was not eligible for inclusion in our review due to the lack of randomisation details and an unavailable standard deviation value for the grape juice studies that overall hampered the process of entering the data into our meta-analysis [34, 35]. The small sample sizes in these studies should also be noted. Support for positive cognitive effects of blueberry juice is evident from studies in children but these were not included here due to the eligibility criteria [176, 177]. In another review, consumption of blueberries or freeze-dried blueberry powder ranging from 30 mg concentrate to 460 g frozen whole blueberries for chronic interventions (6 - 48 weeks) and 30 g freeze-dried blueberries for acute intervention (2 h) were reported in their review to improve some measures of cognitive performance, particularly episodic memory in primarily older adults (> 60 years) [114]. There was also a systematic review that reported studies supplementing blueberries to children, older adults (> 60 years old) and adults with MCI [178]. The results indicated that cognitive benefits may be found for delayed memory, executive function and psychomotor function in older healthy and MCI adults and the dosage ranged from 15 - 30 g freeze-dried blueberry powder, 30 ml concentrate - 621 ml juice and 300 g whole frozen blueberries with duration ranging from 1 - 6 h acutely and 6 - 24 chronically [178]. Same studies that meet the eligibility criteria in the current reviewed have also been included here.

High levels of flavonoid metabolites (e.g. anthocyanidin) from blueberries can transport through the blood brain barrier into regions such as the hippocampus to impact on memory and learning [179]. In the pathogenesis of neurological conditions such as Parkinson’s disease, flavonoids have been shown to counteract the damage induced by reactive oxygen species (ROS) and neuroinflammation, and to modulate synaptic signalling and cerebrovascular blood flow [180]. Therefore, several potential

mechanisms could be in action. Previous epidemiological studies assessing neurocognitive health provided discordant evidence on the impact of differing levels of fruit and their processed forms. One review collated data from cohort studies with a follow-up of 6 months or longer but found no inverse association between regular fruit consumption and risk of dementia and cognitive decline [181]. One epidemiological study reported significantly lower hazard ratio (95 % CI) for probable Alzheimer's disease when comparing subjects who drank fruit juice ≥ 3 times per week with subjects who drank < 1 time per week: 0.24 (0.09 - 0.61) vs. 0.84 (0.31 - 2.29) [182]; whereas another showed no association between either habitual juice or fruit consumption and cognitive changes in middle-aged adults [183]. As mentioned above, knowledge about powdered fruit forms for the association with disease risks including cognitive decline is currently very limited [143].

It should be noted that in addition to the lack of consideration of different intervention types in previous reviews, generally the cognitive tasks chosen in each intervention study were not uniform because of the polyfactorial nature of neurocognitive measures [181]. There were also instances where different studies have used the same or similar tests to assess different cognitive domains. For example, as a valid and sensitive measure of cognitive dysfunction and changes, digit symbol substitution test (DSST) has been applied to assess processing speed and executive function in clinical neuropsychology [184]. In our meta-analyses, the chosen cognitive tasks were isolated from other domain-specific tasks to most likely pool the data as we can, however this pooling analysis overlooked the positive results from other domain-specific tasks in the trials (e.g. TMT-B has been reported in executive function assessment too), thus the effect size derived from the meta-analysis in each outcome assessment was inevitably underestimated.

Currently, the majority of evidence in this area has included the association between intake of fruit combined with vegetable intake and cognitive function. The positive findings also mainly arise from studies with older subjects. Degeneration-induced cognitive impairment is a common and important phenotype in ageing and is likely to be accompanied by increased risk of dementia or Alzheimer's disease [185], it is necessary for future intervention and epidemiological studies to continue to examine

the benefits of fruit in their various delivery forms on cognitive health in an older population.

Blueberry is the only fruit that was delivered in whole frozen form among chronic (> 1 week) interventions assessing cognition, which showed a trend for cognition improvement [68]. So far, research has mainly focused on fruit juice interventions, nevertheless, it should be noted that the daily consumption of fruit juice should not exceed 150 ml glass as set out by Public Health England guidelines [186]. Validation of metabolites and biomarkers for cognitive impairment should be incorporated into future trials to help identify the potential mechanisms underlying any influence between fruit-based intake and cognitive health.

To our best knowledge, this is the first systematic review and meta-analysis to compare the impact of various forms of specific fruit in isolation from other food supplementation on cognition and mood. Firstly, in addition to the comprehensive search of the literature in the topic, we also applied the newly developed Hartung-Knapp-Sidik-Jonkman method for modelling random effects in meta-analysis, in addition to a comprehensive search of the literature in the topic. Secondly, the interventions included in this review have assessed either general cognitive performance or specific cognitive domains using well-established cognitive tasks.

There are limitations to our review. Although our systematic review results showed the positive results in interventions supplementing with blueberry or grape, the small sample size and inadequate studies (< 2 studies) that used the same cognitive task in the searched literature may partially explain the lack of support from our meta-analysis. Even though studies supplementing cherry juice have shown a significant effect, they were not accompanied by improvements to other cognitive domains. The improvements were limited to relatively small sample sizes within 2 studies, so the implications of the study effect should be treated with caution. Heterogeneities presented in our results, however, were explored by subgroup analyses of different intervention subgroups, due to the limited number of studies under each participants characteristic and country region, we were unable to further compare among different baseline characterised subjects (i.e. physical activity, gender) and regions (i.e. western and other countries) to take helpful information for our study design.

3.1.15 Implications for the intervention design

Several points were raised from these reviews to help inform our study design:

- **i.** Combining evidence from RCTs assessing vascular function in Chapter 2, cranberry and cherry juice studies have shown significant improvement to blood pressure or borderline significant improvement to psychomotor speed (P = 0.05). However, we have to emphasize that the analyses were limited to either small samples size, unbalanced baseline blood pressures, or neglected diet refrainment. Furthermore, improvements to other outcomes were not observed, which does not support the notion that the consumption of these specific fruit powder or other fruit juice will confer a cognitive-protective benefit.
- **ii.** Future interventions on supplementing berry (e.g. blueberry, 100 mg extract powder - 200 g whole blueberry equivalent, 6 - 24 weeks) with a sufficient sample size are warranted, as these appeared to have the biggest potential to improve cognition in both young and old adults.
- **iii.** No fresh fruit was identified in the previous interventions, which could be due to the difficulty of storage and allocation during the intervention. Fresh whole fruit is generally how the fruit is consumed in the general population, which highlights a novel and necessary intervention for future studies.
- **iv.** Apart from fruit juice, blueberry and grape powders is supplemented the most and could also be an effective method to increase overall fruit consumption. Combining evidence from RCTs assessing vascular function in Chapter 2, the long-term investigation for whole blueberry assessing cognition is also scarce and worth exploring

Chapter 4. Intervention design and general methodology

4.1 Introduction

Considering the review results presented in Chapters 2 & 3 along with other epidemiological and meta-analyses evidence [187, 188], the consumption of a range of berries have been linked with improved vascular and cognitive health, however, the meta-analyses were insufficient and inconsistent to substantiate the consumption of a specific type of berry or other fruit as a vascular- or cognitive-protective dietary strategy. Nevertheless, the review helped focus the intervention to berries.

This chapter discussed dose-response studies supporting the choice of blueberry, the dosage and intervention duration. Intervention design, participant recruitment, study protocol, sample size calculation, statistical analysis and methodologies for biological sampling, clinical chemistry and measurements are also described.

4.2 Design of a berry-oriented intervention

Blueberry is the only fruit that was delivered in the form of whole fruit among our reviewed chronic studies assessing cognition. Some acute post-dose (300 g, 24 hours) blueberry interventions assessing blood pressure were also previously reported (95 % CI of - 3.87 [- 7.86, 0.12], $P = 0.05$). As shown in Chapter 2, the meta-analysis including both acute and chronic blueberry supplemental studies also showed overall reduction in SBP (95 % CI of - 2.78 [- 5.60, 0.03], $P = 0.05$). The largest number of RCTs supplementing fruit powder in this review were using blueberry powder (n = 7) with presence of variations in the study design and characteristics of participants. The mean dosage of blueberry powder supplementations was 25.7 g (ranging from 22 to 45 g, equivalent to up to 150 g fresh blueberry depending on the freeze-drying ratio). An uncontrolled, single arm, single blind pilot study was performed to investigate the time-response effects of 11 g blueberry powder (equivalent to 100 g whole blueberry) on FMD from 1 - 4 weeks [189]. The study reported significantly improved FMD at 1 week and sustained improvement at 2 weeks, suggesting optimal chronical time point for blueberry powder intervention to improve vascular function [189]. Another 6 month RCT, reviewed in Chapter 2, investigated the dose-response effects of 13 g and 26 g blueberry powder (equivalent to 75 and 150 g of whole blueberry respectively) on

vascular function [73]. The 150 g of whole blueberry-equivalent powder was shown to improve blood lipids profile and vascular function as assessed by FMD and augmentation index compared to 75 g whole blueberry equivalent powder [73]. In terms of the processing impact on the bioavailability and vascular effects of blueberry polyphenols, one RCT has reported similar improvement to FMD post-acute consumption (1, 2, 4, 6 h) of baked blueberry-containing product compared to unprocessed blueberry, despite significant differences in the plasma metabolites levels [190], Another time-response RCT was performed to investigate the effect of consuming blueberry juice acutely (1, 2, 5 h) on BP, cognitive function and plasma brain-derived neurotrophic factor (BDNF) levels [191]. Improvement to cognitive function and trends for attenuating BP increase and BDNF concentrations were shown but no time course effects of blueberry juice consumption were suggested [191]. However, the sustained dietary blueberry effect (> 24 h) on vascular and/or cognition function and human metabolites have not been compared between processed forms of blueberry and whole blueberry consumptions.

The National Health Service recommendation for blueberry is 2 adult portion sizes or 160 g per day [192] and is achievable through diet. From above, 160 g/d of whole blueberries was considered for the treatment dosage in the current study. Blueberry is very popular among consumers in the United Kingdom [38]. In an exploration of the contribution of berry phytochemicals to the human metabolome, a blueberry intervention provided a 60 % higher abundance of anthocyanins and 49 % more total polyphenols compared to a strawberry intervention [193]. The study applied a systematic review approach hence focused on blueberry and strawberry as they are the main berries focused on in epidemiological studies and have the most available clinical trial data to date [193].

A recent paper emphasized the need for additional research assessing the effect of processed fruit on health, as fruit in processed forms provide consumer options while reducing costs and food waste in the meantime [194]. Apart from fruit juice, fruit powder could also be an effective method to increase overall fruit consumption; it is useful to deliver dietary options to the public regarding different fruit groups and delivery forms. Blueberry powder is another strategy to increase fruit intake but the freeze-drying process for blueberry powder may lead to different levels of bioactives

compared to the whole blueberry [195]. Therefore, it is of nutritional interest to test powdered berry against whole berry if an RCT with consistent dosage and intervention length is conducted. It's also of nutritional interest to investigate an alternative form of the fruit to establish if these forms have similar effects to whole fruit.

The average intervention duration as reviewed in Chapters 2 & 3 ranged from 1 week to 6 months. No meta-analysis was performed based on the intervention duration. In this proposed RCT, a 1 week duration was chosen for the interventional duration. High rates of attrition (49.3 %) and difficulties maintaining participant compliance (reported by 37.8 % of participants) were major threats to the viability of long-term interventions (up to 12 months) [196]. Therefore, compliance issue for participants adhering to long term intervention and the logistical issue of storing fresh blueberries over long term were considered also. The current study also aimed to assess metabolic differences within blueberry treatments and between responder (RS) and non-responder (NRS) groups manifesting opposite responding levels. Therefore, the time taken for observing metabolomic changes over an intervention should be considered too. A phytochemical dietary study followed by magnetic resonance/mass spectrometry (NMR/MS) and multivariate analysis reported distinct discriminations of metabolomic profiles between the 1st void morning urinary samples of subjects following 2-d low-phytochemical diet and urinary sample following 2-d normal diet or following 2-d standard phytochemical diet [197]. This study suggested that the plasma elimination half-life for major phytochemicals is between 1.1 and 28.1 hours so that 2 days were adequate to show distinctive metabolomic changes with a dietary intervention, with 1 - 2 days of washout period implemented [197, 198]. Another short-term dietary intervention study using liquid or gas chromatography coupled with mass spectrometry (LC-MS/MS and GC-MS), and NMR spectroscopy metabolomic profiling reported distinctive dietary metabolite markers in fasting plasma and 24-hour urinary samples from subjects following a 4-days mixed intervention of red wine and grape juice extracts [111]. Therefore, to observe metabolomic changes during a dietary intervention, a shorter term is sufficient rather than longer conventional interventions which intended to foresee changes in clinical phenotypes. Therefore a 1 week duration is justified.

A cross-over design can reduce the influence of confounding covariates such as intra-subjects' variability, each cross-over participant can act as his or her own control; furthermore, an optimal cross-over design is statistically efficient that requires fewer subjects than a non-cross-over design [199, 200]. Similarly, a 1 week of wash out period was chosen after each intervention arm. Apart from the 2 cross-over treatment arms, a control arm was required. For RCTs collected from the literature review in Chapters 2 & 3, colour-matched placebo powder containing no polyphenols or calorie-matched polyphenols-free drink were implemented for the fruit powder or frozen blueberry as control.

Encapsulated microcrystalline cellulose powder, a plant derived fibre which is calorie-free and contains no equivalent bioactive to blueberry, was chosen as a placebo arm in this study. As a wash-out period needed to be implemented for 3 treatment arms in a cross-over design, the overall length of the trial would take 6 weeks and there was also a need for completing the trial within the certain time range of the project. Due to the procedural nature of the researcher assigning treatments to the participants, only participants were concealed to the placebo treatment. Ultimately, a randomised, placebo-controlled, counter-balanced-cross-over design was employed. The study was approved by Northumbria University's Faculty of Health and Life Sciences Ethics Committee (reference: 10113; approved: 30th October 2018) and was registered under ClinicalTrials.gov (ID: NCT04015258).

4.3 Participants

The current study adhered to Consolidated Standards of Reporting Trials (CONSORT) guidance [201]. The reviews combined with meta-analyses in Chapters 2 & 3 were used to help inform the blueberry intervention design and the participant groups, where that data was available. Previous fruit interventions have included participants with different age groups and different health status, although the subgroup analyses were not able to explore further and most studies included participants at older age or with high health risks, the study results were inconsistent. The current study aimed to recruit adults with age range of 18 - 60 years old with normal (18.5 - 24.9 kg/m^2) to overweight (25.0 - 29.9 kg/m^2) BMI so that to include generally healthy participants with low-mild health risks developing vascular and/or cognitive function.

Forty participants were required based on data from two previous studies targeting vascular health and cognition following 300 g and 2 cups (approx. 300 g) fresh-frozen blueberry supplementations respectively [68, 137]. An effect size based on the group mean difference (d = - 4.70) (e.g. treatment *vs.* control: 124.40 mmHg *vs.* 129.10 mmHg, $P < 0.05$) assessing SBP, with a SD of 6.8 mmHg following 300 g frozen blueberry intervention [137] and d = - 1.83 (treatment *vs*. control: 4.26 *vs.* 6.09, $P = 0.042$) assessing number of errors of psychomotor/adaptive test, with a SD of 1.74 following approx. 300 g frozen blueberry intervention [68] respectively indicated that total of 35 and 17 subjects were required for detecting an effect difference in the blueberry treatments separately at a two-sided 0.05 significance level with statistical power of 0.8. The sample size estimation was not performed for all the primary endpoints in the current study, therefore, a retrospective post hoc power calculation was performed (provided in section 5.4). Considering a dropout rate of 10 %, 40 people were required to be drawn in the study to see a treatment effect. From our approach to identify response at the 25^{th} (Q1) and the 75^{th} (Q4) percentile of the calculated response level of the endpoints (described later in section 4.9.1) we would need approximately 10 RS and NRS from the 25^{th} and 75^{th} percentiles respectively in a sample cohort of 40 participants.

From the above, 40 participants were needed to be recruited through an opportunity sample within Newcastle upon Tyne and the surrounding areas. The inclusion criteria included requirement of no relevant pre-existing medical condition/illness such as heart disease, hypertension (≥ 140/90 mmHg) and neuropsychological disorders, and no current use of prescription medications (excluding contraception pills); no smoking and had body mass index (BMI) of 18.5 -30 kg/m^2 (inclusive), which were all assessed on the screening visit; no current condition of migraines (> 1 per month) and no visual impairment that cannot be corrected with glasses or contact lenses; being proficient in English and equivalent to IELTS band 6 or above; did not regularly consume blueberry/blueberry-contained products more than twice a week; no current participation in other clinical or nutritional interventional studies and had no habitually used supplements within the last month (defined as more than 3 consecutive days or 4 days in total). Participants were not recruited if at least one of the criteria did not apply to them. Each participant would receive a £ 20 shopping voucher upon their completion of the study for a compensation.

4.4 Treatment

Fresh blueberries (*Vaccinium corymbosum. L.)* throughout four seasons were purchased from a local retailer using the same source; freeze-dried blueberry powder was purchased from Lio-Licious freeze-dried fruit range and processed from fresh blueberry (*Vaccinium corymbosum. L.* as provided by the supplier); the microcrystalline cellulose was purchased from Blackburn Distributions. The powder to fresh blueberry net weight conversion was provided by the supplier. Total polyphenol analysis (TPC) was completed for blueberry, blueberry powder and placebo capsules using the Folin Ciocalteau reagent method [202] **(Table. 4.1).**

The present study supplied either 160 g of fresh whole blueberry (4 handful portion, 2 of NHS adult portion size) or 20 g of freeze-dried blueberry powder (measured with tablespoon provided; equivalent to 160 g of whole fresh blueberry), or placebo capsule with plant-derived fibre microcrystalline cellulose (participants have been told that this was encapsulated blueberry extract components) to participants on separate occasions for 1 week with 1 week of washout period. Each participant was given each treatment in random sequence. The random permutation was carried out by the online randomisation tool (http://randomization.com/), the blinding of the placebo treatment was conducted by the researcher (YW) who co-ordinated intervention.

All treatments were prepared 1 day prior to the study visit and were given to the participants with 7 days portion. Blueberries were asked to be stored in the fridge to keep fresh. Blueberry metabolites could be used as marker of compliance adhering to the interventions. Compliance was also checked on each study visit using 1-day food diary as described below. Energy and macronutrient intake were also calculated throughout the trial using 1-day food diary to evaluate any displacement effects of the different dietary intervention arms.

Table. 4.1 Nutrient composition of freeze-dried blueberry powder and placebo compared with whole blueberry

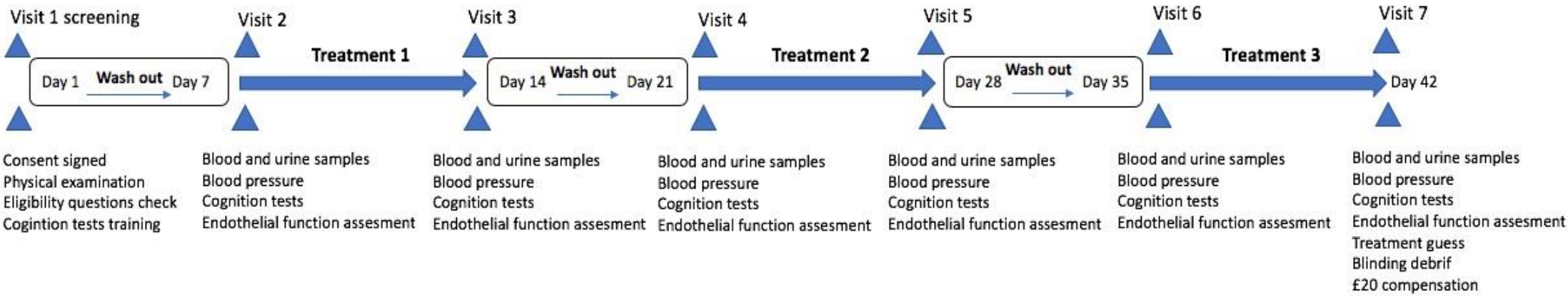
		Blueberry (per 160 g)[a]	Blueberry Powder (per 20 g)[b]	Placebo (per 1 g)[c]
Energy (kcal)		120.00	69.20	0.00
Fat (g)		0.32	1.30	0.00
From Saturates (g)		0.00	0.08	0.00
Total Carbohydrates (g)		23.20	13.90	1.00
From Sugars (g)		22.40	7.78	0.00
Protein (g)		0.96	1.40	0.00
Total Polyphenol Analysis (TPC)[d]	**Gallic Acid Equivalence (mg/d)[d]**	220.48	288.43	0.00

[a] US department of Agriculture National Nutrient Database for Standard Reference
[b] Lio-Licious freeze-dried blueberry powder information
[c] Blackburn Distribution information
[d] Analysed by the researcher (YW) at Northumbria University

4.5 Study protocol

Figure. 4.1 demonstrates the study timeline. The 1st visit was a screening appointment; upon arrival the participants were asked to give signed informed consent to participate. Once they have completed the consent form, they were measured for blood pressure for an eligibility check. This screening session usually lasted approximately 2 hours. They were also screened for general physical health (including measurement of demographic data (age, height, weight)) and criteria questions to confirm eligibility. All participants data were recorded in the pre-defined Case Report Form (CRF). Then the participants were trained on computerised cognitive tasks.

Figure. 4.1 Graphic demonstration of the study timeline



After the training, they completed a questionnaire based upon consumption of blueberry and related products. Once the participants met with the inclusion criteria, the participants were asked to completely avoid consumption of polyphenol-rich berry (e.g. blueberry, raspberry, and blackcurrant etc.), cherry, raisins and vegetables (e.g. eggplant, purple cabbage etc.), and any foods containing them, a list of food that they should refrain from taking were given to the participants. In addition to these, participants were also asked to stick to their usual diet. Before they left, the participants were given a 1-day food diary to record their dietary intake on the day prior the visit days (they should bring this diary to every appointment they attend). At the end of this screening session, they were provided with urine sample collection kits and instructions on their use. They were also asked to bring the completed samples for their second urination in the morning of the day on their next visit.

The 2nd, 4th and 6th visits were the baseline visits for each treatment. These visits took place 7 days after the last visit (ideally the same day of the week). Participants completed their urine collection on the morning of this visit and brought this sample with them to the laboratory (second urination of the morning). Their 1-day food diary questionnaire was also returned. The participants arrived at the laboratory having not consumed any food or drink other than water for 12 hours prior. Upon arrival at the lab, the researcher (YW) checked that they still complied with inclusion/exclusion criteria and were in good health and were well rested. Changes to medication and/or illness between visits were recorded if applicable. At this session they were required to bring the 20 ml spot urine in the morning (second urination of the day) with the given urine sample collection kit from the previous visit. Then 10 ml of their venous blood sample was taken by the BD Vacutainer™ Flashback Blood System into BD Vacutainer™ Heparin Plasma Tubes by a suitably qualified individual (YW). Then their systolic and diastolic blood pressure was measured (approx. 15 min). Following which participants underwent their computerised cognitive assessment (approx. 30 min). Finally, the non-invasive measurement of pulse wave velocity assessing arterial stiffness was performed (approx. 15 min). This was the completion of participant testing.

Participants were provided with a 1-day food diary and a urine sample kit upon their departure to record their food intake of the day prior to their next visit and were reminded to bring their 2nd urination to the next visit day. The participants were

instructed to take their treatment for the following 1 week, and adhere to the instructions and consume either (according to their treatment randomisation):

i. 160 g of fresh whole blueberry daily for 7 days; which were weighed prior to each study visit and provided to the participants. The blueberry was packed in 7 separate food grade sealed bags and participants were told to store the blueberries in a refridgerator and take one bag out for consumption daily. It was suggested that the blueberries were preferably consumed prior to lunch.

ii. Or 20 g of blueberry powder (for the next 7 days, which were measured in a sachet prior to each study visit and provided to the participants. The blueberry powder was to be measured with a tablespoon provided that had a 20 g marker line which was validated with Benchtop Electrical food scales. Participants were told to store the blueberry powder in a cool and dry place and take one tablespoon of the powder (measured with the marker line) for consumption daily. It was suggested to mix and consume the powder with water at room temperature, preferably prior to lunch.

iii. Alternatively, encapsulated placebo (the placebo content was revealed to the participants as blueberry extract) to be taken daily for next 7 days, which was provided to the participants. The 7 capsules were packed in food grade capsule containers and the participants were told to take 1 capsule with water for consumption daily, preferably prior to lunch.

Instructions on when and how to take the supplementations were carefully explained to each participant on their departure following each study visit. The procedure on the 3rd, 5th and the 7th visits was identical to the aforementioned procedure, in addition to that the participants were not be given treatments upon their departure so as to enter the 1-week washout period. On visit 7, all participants were asked to finish a treatment guess questionnaire in order to check their blinding status and compliance; the compensated voucher would be given to participants upon their completion on the last visit. The CRF and 1-day food diary templates are provided in **Appendices. 3.1 - 3.2.**

4.6 Biological sampling

i. **Plasma separation**: 10 ml of fasting venous blood was collected from participants by the BD Vacutainer™ Flashback Blood System into BD Vacutainer™ Heparin Plasma Tubes by the researcher (YW) and centrifuged for 10 minutes under 1200 relative centrifugal force (xg) to separate the plasma, which were stored in aliquots at -80 °C.

ii. **Urine collection**: 20ml of a spot urine sample (2nd urination of the day following the morning void) were collected and stored in aliquots at -80 °C

4.7 Primary and secondary endpoints

i. **Primary endpoints**: pulse wave velocity (PWV), systolic and diastolic blood pressure (SBP and DBP), accuracy of Immediate Word Recall and Delayed Word Recall Tasks, correct response of Serial 3s Subtraction, correct response of Serial 7s Subtraction, reaction time and accuracy of Digit Vigilance Task.

ii. **Secondary endpoints:** Plasma lipid profile including concentrations of plasma total and high-density lipoprotein (HDL-), low-density lipoprotein (LDL-) cholesterol and triglycerides, plasma nitrite concentrations. mental fatigue, alert, calm and content scores. Untargeted urinary metabolites. Measurements for these endpoints are described below.

4.8 Methodology

4.8.1 Anthropometric measurements

Participants' age, gender, height, and weight were recorded at the screening session. Height and weight were measured using digital scale (Seca Scales 703, Seca Ltd. Birmingham, UK) and BMI was calculated correspondingly.

4.8.2 Cognitive testing

A battery of computerised cognitive tests that would take approximately 30 minutes to complete was applied on each study day. Those tasks were provided by software

called the Computerised Mental Performance Assessment System (COMPASS, Northumbria University), which has shown stable sensitivity to a range of nutritional interventions [203, 204]. **Table. 1.2** summarises the domains in cognition trials that have demonstrated significant results in interventions. Attention and vigilance, long-term memory, working memory and mood were the ones with significant reports in fruit interventions and were entered into the tasks battery from COMPASS [30, 115, 161, 166, 167, 205-208]. **Table. 4.2** shows the selected cognitive tests and mood measures in the study.

4.8.3 Whole body measurements of vascular function

The following markers were measured in line with the assessment of vascular health:

i. **Ambulatory blood pressure (BP**): Systolic and diastolic blood pressures (SBP and DBP) were measured 3 times by a vital signs monitor (GE Carescape) with participants sitting in an upright position for 5 minutes. A first reading was taken but discarded. If the average reading was out of range, but the third was lower than the second reading, a fourth reading was taken. This reading alone was then used as the final measurement. Readings were taken > 1-minute intervals.

ii. **Pulse Wave Velocity (PWV):** PWV reflects the speed of the blood pressure wave to move down the blood vessel, it is a good indicator of arterial dispensability and stiffness [20]. It's a non-invasive whole-body measurement and has been applied in nutritional interventions before and is now commonly used [14, 209]. It was assessed by pulse wave time (s) dividing brachial-radial distance (m), PWV will be presented in the units of m/s. PWV of carotid artery and radial artery (crPWV) was assessed using SphygmoCor (ScanMed medical) and the cardiac rhythm was monitored using electrocardiogram (ECG) pad. The readings were repeated 3 times.

Table. 4.2 Cognitive and mood tasks completed at baseline and 1-week post-intervention for each treatment arm in the order of testing

Task	Description	Scoring	Domain
Bond-Lader visual analogue scales	The 16 Bond-Lader visual analogue scales were used; participants are asked to rate how much these descriptors match their current state by placing an 'x' on a line with the end points labelled 'not at all' (left hand end) and 'extremely' (right hand end); the results were combined to form 3 mood factors: "alert", "calm" and "content"	% along the line from left to right.	Mood
Word presentation	A series of words is displayed on the screen, one word at a time. In this case, 15 words were presented with a display time of 1 s and inter-stimulus interval of 1 s	-	
Immediate word recall	Participants are instructed to write down the words that were presented. In this case, 60 s were given to complete the task	Number of correct and number of errors	Episodic memory
Digit vigilance	A fixed number appears on the right of the screen and a series of changing numbers appear on the left of the screen at the rate of 150 per minute. Participants are required to make a response when the number on the left matches the number on the right. In this case the task lasted for 3 min	Accuracy (%), reaction time for the correct responses (ms) and false alarms (number)	Attention
Serial 3s subtraction*	A starting number between 800 and 999 appeared on the screen and participants were instructed to count backwards as quickly and as accurately as possible from this number in threes, using the linear number keys to make their response. Responses were cleared when the 'enter' key was pressed. Participants were only shown one number on screen and the rest of the numbers were generated by subtracting from the previous number in their head. In the case of incorrect responses, subsequent responses were scored positively if they were correct in relation to the new number.	Number total responses, correct responses, and number of errors	Working memory
Serial 7s subtraction*	This task is identical to the serial 3s subtraction task except that it involves the serial subtraction of 7s.	Number total responses, correct responses, and number of errors	Working memory
Mental fatigue Visual analogue scale*	The participant was asked to use the keyboard to click an 'X' at a point on the scale which best represents how they feel in response to the questions "How mentally fatigued do you feel right now", the 'X' can be repositioned if necessary.	% along the line from left to right.	Mental fatigue
Delayed word recall	Participants are instructed to write down the words that were presented to them at the beginning of the assessment. In this case, 60 s were given to complete the task	Number correct and number of errors	Episodic memory
Delayed word recognition	All target words that were shown during Word presentation plus an equal number of decoys are displayed on the screen one at a time. Participants indicate if they remember seeing the word earlier or not.	Accuracy (%) and reaction time for the correct responses (ms)	Episodic memory

*The task order Serial 3 – Serial 7 – Mental fatigue VAS was repeated 3 times during each testing

4.8.4 Clinical chemistry assessment

Plasma was used to measure concentrations of glucose, lipid status (total and HDL-LDL-cholesterol, triglycerides) and the nitric oxide (NO) intermediate nitrite (NO_2^-).

i. **Glucose:** The Randox Daytona GOD-PAP (Cat. No. GL8038) with measuring range of 0.200-35.5 mmol/l was used, a correlation of r=0.999 against another commercially available method was reported.

ii. **Lipids:** Plasma lipid status was measured using Randox Daytona GOD-PAP with measuring range of 0.22 - 21.7 mmol/l (Cat. No. CH200), and 0.1 - 13.4 mmol/l (Cat. No. TR210) for total cholesterol and triglyceride respectively. Randox Daytona Direct Clearance Method was used (Cat. No. CH1383) for measuring HDL-cholesterol with measuring range of 0.189 – 4.03 mmol/l. A correlation of r=0.999 against another commercially available methods was reported. LDL-cholesterol level was further calculated using Friedewald equation: LDL-cholesterol (mmol/l) = total cholesterol (mmol/l) – HDL-cholesterol (mmol/l) – (triglyceride/5) (mmol/l) [210].

iii. **Nitrite (NO_2^-):** The total amount of nitrite (NO_2^-) in deproteinised plasma was determined by using chemiluminescence method via the purge system of Sievers Instruments (model NOA 280i, Boulder, CO, USA) with repeatability of +/- 5 %. Plasma samples used for nitrite analysis was deproteinised by using ethanol to prevent foaming. Plasma samples were mixed with ethanol in a 2-fold dilution in microcentrifuge tubes and stood for 30 min. The microcentrifuge tube was centrifuged at 12500 rpm for 5 min. The supernatant then was removed for analysis. Standard solutions containing 10 nM, 50 nM, 100 nM, 1 µM, 5 µM, 10 µM, 50 µM and 100 µM of sodium nitrite ($NaNO_2$) were prepared with nitrate-free deionised water and analysed to construct a calibration curve.

4.9 Metabolomics

4.9.1 Sample extraction and preparation

Hydrophilic interaction chromatography (HILIC) was used on polar urine samples as if offers a broad coverage of compounds [211]. For urine sample preparation, urine (100 µl) was mixed with same volume of chilled LC-MS grade methanol (- 20°C) containing 0.125 % formic acid, mixed and allowed to chill in freezer (- 4°C) for 30 min. The sample was then centrifuged for 2 min at 4 °C at 10,000 rpm, then carefully transferred to a sample vial for HILIC analysis. QCs were prepared by pooling an aliquot of all samples together.

4.9.2 Mass spectrometer parameters

Urine samples were analysed on a Dionex 3000 Ultra High Pressure Liquid chromatography (UHPLC) system hyphenated to the Q-Extractive high resolution mass spectrometer system (ThermoScientific, Bremen, Germany). All solvents and ionization agent used were of analytical grade or higher unless stated. The chromatographic separation was performed on a Water Acquity Ethylene Bridge Hybrid (BEH) Amide analytical column (1 x 150 mm) with particle size of 1.7 micron at a flow rate of 100 µL/min, the column temperature was set to 45 ^{o}C. The binary buffer system was as follows: Buffer A was MilliQ water and Buffer B was ACN, both with 10 mM ammonium formate adjusted to pH 3.5 using formic acid. The LC profile was as follows: T:0 min: 95 % (B), T:2 min 60 % (B) ,T:5 min 40 % (B), T:7.5 min 40 %(B), T:7.6 min 95 % (B), T:10 min 90 % (B). The Heated spray ionization source (HESI) was set to the following parameters: Sheath gas flow rate of 50, the Aux gas flow rate was set to 13 and the sweep gas flow rate was 3. The Spray voltage of set to 3.5 kV with a Capillary temperature of 275 ^{o}C. The Aux gas heater temperature was adjusted to 425 ^{o}C. The MS1 mass acquisition range was as follows: 75 - 1000 m/z units at a mass resolution of 35,000 at approximately 7.6 scans per second, microscan: 1, lock mass: off. The AGC was set to 1e6 and the ion injection time was 100 mS^{-1}. A 3 µL injection was applied. The system was primed with a minimum of 10 sequential injections of a pooled QC to stabilise the HESI source and to check for chromatographic stability before starting the batch analysis. Samples were analysed

in random order, with pooled QC samples and blank injections. The data should be acquired in both Positive and Negative ionisation mode, however due to Covid-19 restrictions only positive mode was performed. Peak table generation and alignment was performed using compound discoverer 2.1 (ThermoScientific, Bremen, Germany) with an alignment window of 0.25 min, mass tolerance: 5 ppm and a signal intensity threshold of 200,000 counts with a signal to noise ratio of 5:1.

4.9.3 Data alignment and processing

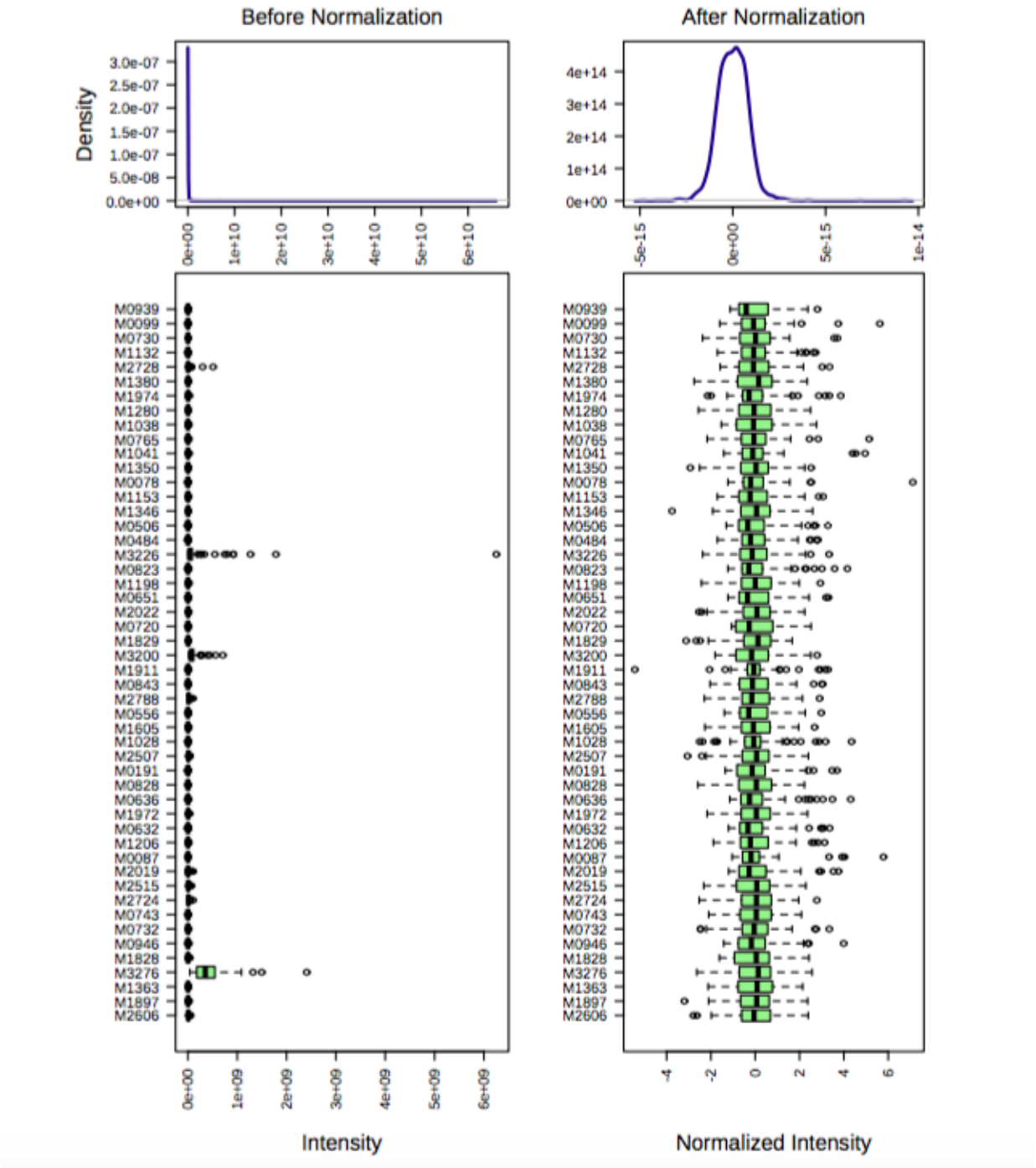
Post data acquisition processing and alignment was performed using Thermo Scientific™ Compound Discovery™ small molecule identification software (Thermo Fisher Scientific, Loughborough, UK). The software identifies compounds using automatic multiple database and spectral library search tools including mzCloud™, Chemspider™, KEGG, and BioCyc, and local database search tools such as the mzVault™ spectral library or mass lists. Positive mode data were categorised into different treatment groups for analysis. Pooled QC samples and sample blanks were also included and grouped accordingly in order to assess and evaluate system stability and track potential carry over effects throughout the entire batch analysis.

The resulting peak table was sequentially filtered using QC parsing with 30 % RSD to screen out features that showed instability or irreproducibility across the dataset, reducing the original feature list. The molecular features were categorised by putative molecular name, formula, accurate mass (mw), and retention time (min). The features were coded from M0001 to Mxxxx. This coding system remained throughout all chapters. Datasets were grouped by treatment, pre- and post- treatment, RS and NRS groups separately and uploaded to MetaboAnalyst 5.0 for univariate statistical analysis and multivariate chemometric analysis.

Non-parametric relative standard deviation (MAD/median) was used for filtering untargeted metabolomics datasets (i.e. spectral binning data, peak lists) with a large number of variables to screen out baseline noises [212]. Careful normalisation of MS peak intensities enable greater accuracy and precision in quantitative comparisons of metabolites abundance levels while also avoiding overfitting that would invalidate downstream statistical inference [213]. A log transformation helped to remove

heteroscedasticity from the data and correct for a skewed data distribution, autoscaling was able to remove the dependence of the rank of the metabolites on the average concentration and the magnitude of the fold changes and showed biologically sensible results after principal component analysis [214]. Log10 normalisation and autoscaling were implemented for intensity normalisation as shown in **Figure. 4.2.**

Figure 4.2 The effect of normalisation on signal intensities



* Box plots and kernel density plots before and after normalization. The boxplots show at most 50 features due to space limit. The density plots are based on all samples. Selected methods: Row-wise normalization: N/A; Data transformation: Log10 Normalization; Data scaling: Autoscaling.

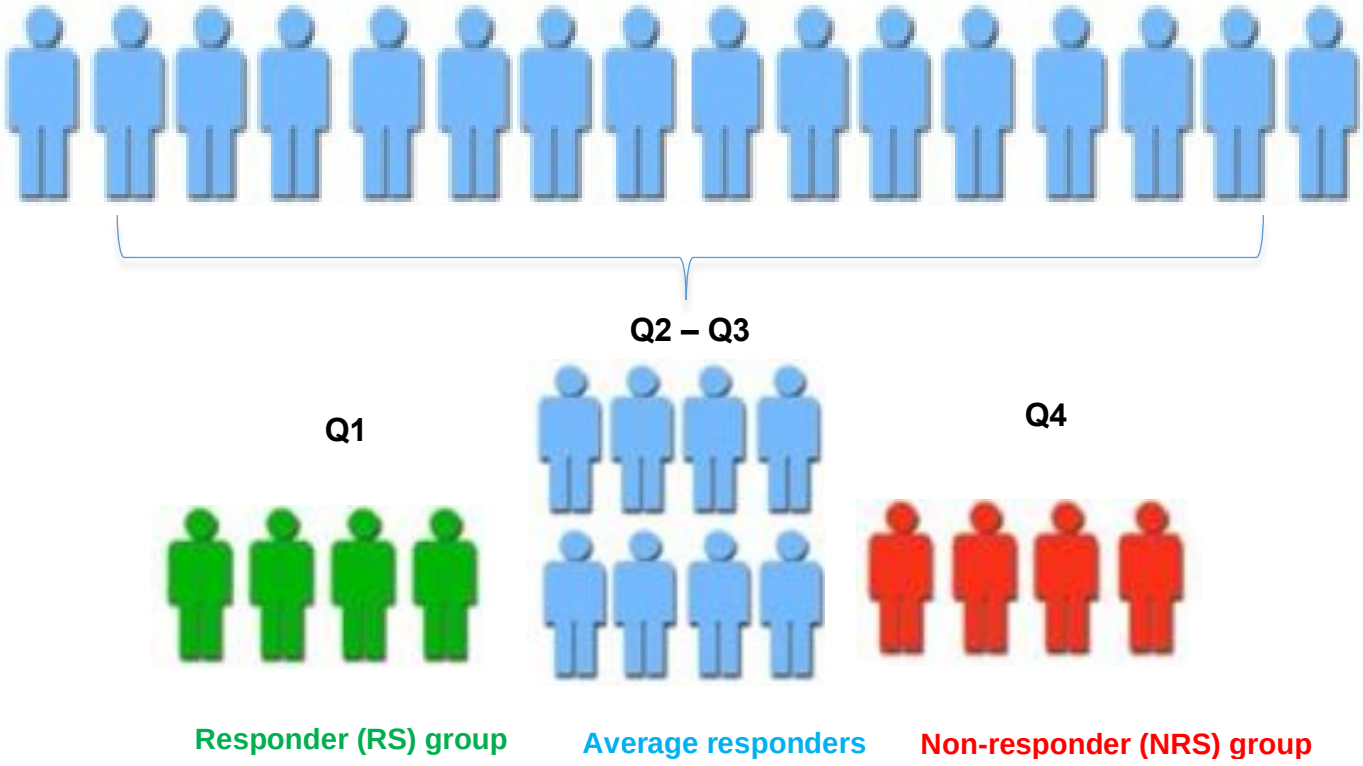
4.10 Data analysis

4.10.1 Characterisation of response in the biological endpoints

As a measure of variability in the descriptive statistics, the quartiles divided rank-ordered dataset into 3 points: the lower quartile, median and the upper quartile to form 4 groups (Q1, Q2, Q3 and Q4). Quartiles have been previously used to stratify responses to disease [215, 216]. For example, clinical studies have applied this mathematical calculation to characterise the best and worse responses for non-diabetic essential hypertensives following short-term thiazide treatment [217], physical fitness following training exercise [218] or coronary syndrome patients following the antiplatelet therapy etc. [219]. However, the quartile dividing variations following the supplementation of dietary blueberries and in the investigation of both vascular and cognitive health have not been incorporated previously. Basing on the effect direction, the Q1 or Q4 could be dividing either positive responders or the negative responders (so called non-responders) in this study, consequently the Q2 - Q3 represented the average responders.

The identification of RS to the treatments was performed by the calculation of response level first: response level = (post intervention score - baseline score)/mean group baseline score x 100 %. The calculated response levels were used to characterise subjects from highest response to lowest response level. Basing on the effect direction, the 25^{th} and the 75^{th} percentiles were applied to split participants with either improved or low responses as shown in **Figure. 4.3.**

Figure. 4.3 The characterisation of responder (RS) using quartiles division



4.10.2 Statistical analysis

The data for each assessed outcome from post intervention were analysed separately using linear mixed-effects models (MIXED) in SPSS statistics 26. This analysis is appropriate for repeated measured designs (e.g. cross-over design) that include a baseline covariate and fixed factors of random effects along with interactions among other confounding factors (e.g. treatment, testing day) that can be accounted for each subject [220].

The data for each assessed outcome were checked for homogeneity of variance (Levene's test) prior to the MIXED model. The post intervention measures were modelled including respective baseline values as covariate and the terms treatment, visit, treatment*visit, baseline as fixed factors. Participant was included as random factor. Repetition, treatment*repetition and treatment*visit*repetition interactions were further included in cognitive results analyses as there were repetitions for some tasks including Serial 3 and 7 subtraction tasks, digit vigilance task and mental fatigue in

one cognitive testing. Post hoc analysis used Least Significant Difference test (LSD) and adjusted *P* values for pairwise comparisons among treatments, visits and repetitions. One-Way ANOVA was used to analyse the difference of participants' dietary total energy, carbohydrate, fat and protein intakes between treatment groups pre- and post-intervention separately. Paired T-Test was used to analyse the difference in participants' dietary total energy, carbohydrate, fat and protein intakes within each treatment group,

A Chi-square test was used to assess the assciation of gender factor, BMI range with identified response. Other univariate and multivariate analysis were used in metabolomic analysis and described below.

4.10.3 Metabolomic analysis

For group-wise differences of features within and between interventions, unsupervised and supervised multivariate analysis was performed using MetaboAnalyst 5.0 [221]. For multivariate analysis, unsupervised method principal component analysis (PCA) was used to capture initial profile patterns and to screen out any outliers without using grouping information [109, 222]. The data was summarized into much fewer variables, called scores, which are weighted average of the original variables. The 2-D scores plot providing an overview of the various separation patterns between the most two significant PCs was used.

Then a supervised method, partial least squares-discriminant analysis (PLS-DA) model, was constructed to discriminate the profiles between samples taken pre- and post-blueberry interventions (within group), and also between treatment groups. MetaboAnalyst supports two types of test statistics for measuring class discrimination. To validate the model, cross validation (CV) of leave-one out CV (LOOCV) was used, presenting with model accuracy R2 and Q2 values as estimates of the predictive ability of the model. Typically a Q2 score > 0.4 and an R2 > 0.5 indicates a robust model, scores between 0.7 and 1.0 indicates a highly robust model [223]. A negative Q2 could be indicative of model overfitting [224]. To assess the significance of class discrimination, a permutation test was performed. In each permutation, a PLS-DA model was built between the data (X) and the permuted class labels (Y) using the

optimal number of components determined by cross validation for the model based on the original class assignment [225].

Heatmaps were used to illustrate the association between feature intensity and the intervention groups. Random forest (RF), developed by Breiman [226], is a machine learning method using a combination of tree-structured predictors (decision trees) and was used in the current study as a discriminatory metric. Each tree is constructed via a tree classification algorithm and casts a unit vote for the most popular class based on a bootstrap sampling (random sampling with replacement) of the data. RF measures the importance of a variable and includes Mean Decrease Accuracy (MDA) to measure how much it contributes to predictive accuracy. MDA is used to present how much accuracy the model loses by excluding each variable. The more the accuracy loses, the more important the variable is for the successful classification. RF has been shown to outperform other methods for imputing LC-MS untargeted metabolomics data [227]. The ion intensity following interventions were expressed in fold changes and ranked basing on the log2 transformed fold change values. These top features were futher ranked by MDA scores for each intervention group. Univariate analyses were also used to detect any significant features for group-wise difference within and between interventions.

4.10.4 Biomarker analysis

For responder (RS) and non-responder (NRS) participant groups, receiver operating characteristic (ROC) curve analysis was used to identify predictive biomarkers. The ROC curve analysis provides a predictive model to assess the diagnostic or predictive accuracy of a test with a continuous outcome by graphically displaying the cut-offs of the true-positive rate (sensitivity) and false-positive rate (1-specificity), and the area under the curve (AUC) for predictive accuracy [228]. Univariate analysis of single biomarkers and multivariate analysis of multiple biomarkers selected by the RF method was used. Single features that increased intensity in RS relative to NRS and/or ranked high in RF method was reported. After features were reported, pathways of discriminating features following blueberry interventions and between RS/NRS were identified where available in KEGG using KEGG ID generated from Thermo Scientific™ Compound Discovery databases.

Chapter 5. Effect of blueberry interventions on vascular and cognitive function

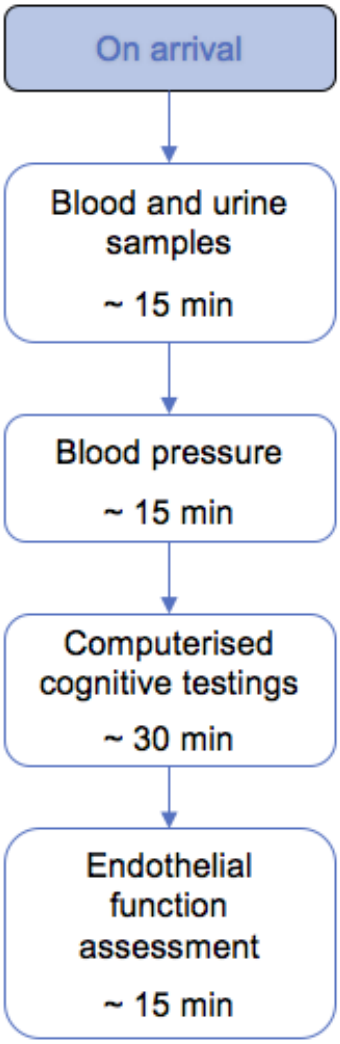
5.1 Introduction

The ultimate goal of the current study was to investigate the individual response to a blueberry intervention over multiple endpoints, in line with this, the interventional effect to improving vascular and cognitive responses following different blueberry supplementations was also evaluated. A one week duration of intervention was chosen based on previous evidence [16] and has also considered logistics for the participants; for example in the storage of fresh blueberry and number of study visits. This study applied a moderate dosage of whole blueberry and it was more applicable for participants in real world scenario consumption. The dosage of blueberry was equivalent to double the daily adult portion size as recommended by NHS [229], which constitutes one variety of the recommended 5 a day portions of fruit and vegetable intake in addition to the participants' usual dietary regime and it was expected to augment efficacy within a shorter term.

Assessment of vascular function and clinical chemistry included systolic and diastolic blood pressure (SBP and DBP), pulse wave velocity (PWV), plasma triglyceride, total-, HDL -, LDL - cholesterol levels, plasma glucose and nitrite levels. Assessment of cognition included domains of working memory, episodic memory, attention, as well as mood and mental fatigue. **Figure. 5.1** displays the procedure for participants to follow through on each testing day.

A linear MIXED model was used for statistical analysis as it included baseline as a fixed factor as well as adjusting for the covariate. This cross-over study consisted of 7 visits including a screening session, the baseline visit followed by the post-intervention visits. Due to the randomised treatment sequence, each post-intervention visit (3 in total) consisted of blueberry, blueberry powder and placebo group. Therefore, effect of visit order was included as a fixed factor in the statistical analysis. Participant was included as random factor. Participants completed 3 repetitions for working memory, digit vigilance and mental fatigue assessment during one test session so the effect of repetitions were also included as cofactor in the statistical analysis evaluating intervention effects on these domains.

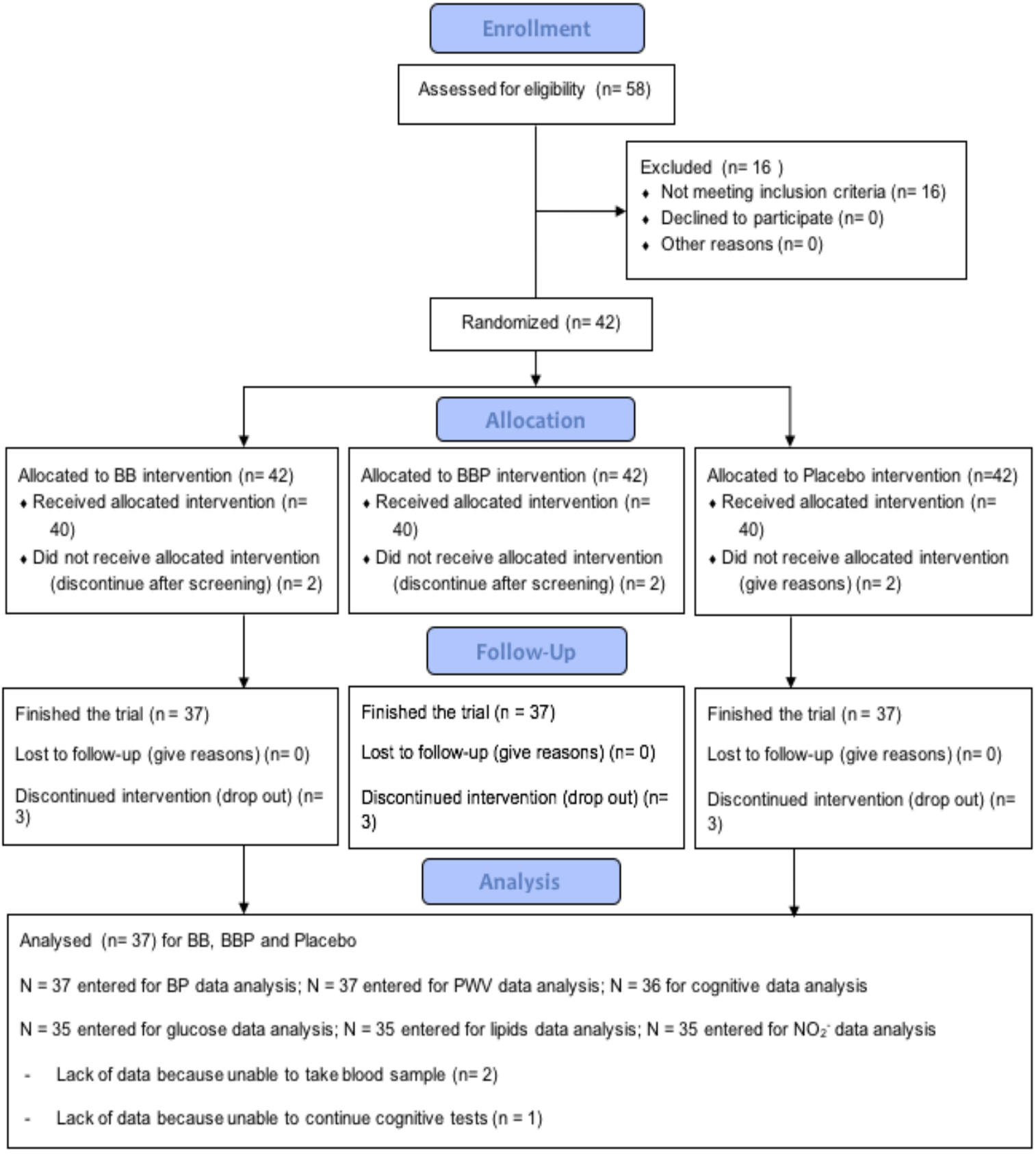
Figure. 5.1 Flowchart of the study day



5.2 Participants

Forty people received the interventions and 37 people finished the trial. Homogeneity of variance was checked in order to test the group variances prior to the statistical analysis. Depending on the indication of cognitive tests assessing attention and psychomotor speed, some data were excluded as outliers which could indicate that participants were not fully committed on the tests. The population for analysis **(see Figure. 5.2)** consisted of 37 adults (13 males/24 females).

Figure. 5.2 CONSORT flow diagram



* BB: blueberry; BBP: freeze-dried blueberry powder; BP: blood pressures; PWV: pulse wave velocity.

Participant characteristics at baseline can be found in **Table. 5.1.** Participants were asked to provide the food diary for the day prior to fasting for each study visit so that researcher can check whether they have consumed the blueberry intervention. Therefore, the compliance was checked on each study visit using the 1-day food diary. Two participants found out about the placebo during the trial and 1-day food diary confirmed 92 % of compliance. The data for these participants were retained for analysis.

Table. 5.1 Participant demographics

Variables	Value[a]
Age (years)	25.86 ± 6.81
BMI (kg/m^2)	23.15 ± 3.12
Gender	13 male, 24 female
Ethnicity	1 Black 2 Indian Asian 3 Chinese Asian 31 white European
Fruit and vegetables intake (portions/day)	2.13 ± 0.85
Berry intake (portions/day)[c]	0.06 ± 1.61

[a:] Data are expressed as mean ± SD; BMI: body mass index
[b:] Pre - Whole blueberry intervention *vs.* Pre - Freeze-dried blueberry powder intervention *vs.* Pre - placebo intervention
[c:] 1 portion size equals to 80 grams

The food diary was collected to calculate dietary energy intake throughout the trial and the mean energy and macronutrient intake values pre- and post-intervention groups are shown in **Table. 5.2**. Homogeneity of variances were tested prior to the analysis ($P > 0.05$). There was no statistical difference in the total energy, carbohydrate, fat and protein intakes of participants between and within intervention groups (**Table. 5.2)**.

Table. 5.2 Participant dietary intake pre- and post- interventions

	Pre				Post			
	Blueberry	**Blueberry powder**	**Placebo**	**Significance[a]**	**Blueberry**	**Blueberry powder**	**Placebo**	**Significance**
Energy (kcal)[b]	1580.697 (425.250)	1485.929 (406.151)	1487.353 (453.500)	*P* = 0.581	1583.227 (424.395)	1490.961 (409.881)	1489.849 (453.103)	*P* = 0.591
Total Carbohydrates (g)	170.112 (50.693)	161.009 (57.216)	163.453 (68.156)	*P* = 0.806	170.372 (51.247)	177.028 (86.868)	163.619 (68.344)	*P* = 0.735
Fat (g)	57.512 (22.635)	57.926 (22.238)	55.923 (24.775)	*P* = 0.932	58.650 (22.168)	59.163 (21.634)	57.388 (25.154)	*P* = 0.948
Protein (g)	79.629 (48.500)	65.235 (34.302)	71.323 (37.794)	*P* = 0.346	80.098 (48.638)	63.435 (24.886)	71.989 (37.826)	*P* = 0.206

[a]: Adjusted for between - group comparisons: least significant difference (LSD) $P \leq 0.0167$ for all pairwise comparisons;

[b]: Data are expressed as mean ± SD

Table. 5.3 summarises the frequency of berry intake for participants at baseline. Participants demonstrated low daily intake of berry (e.g. blueberry, raspberry, and blackcurrant) and cherry prior to receiving the blueberry interventions.

Table. 5.3 Frequency of berry and cherry intake at baseline

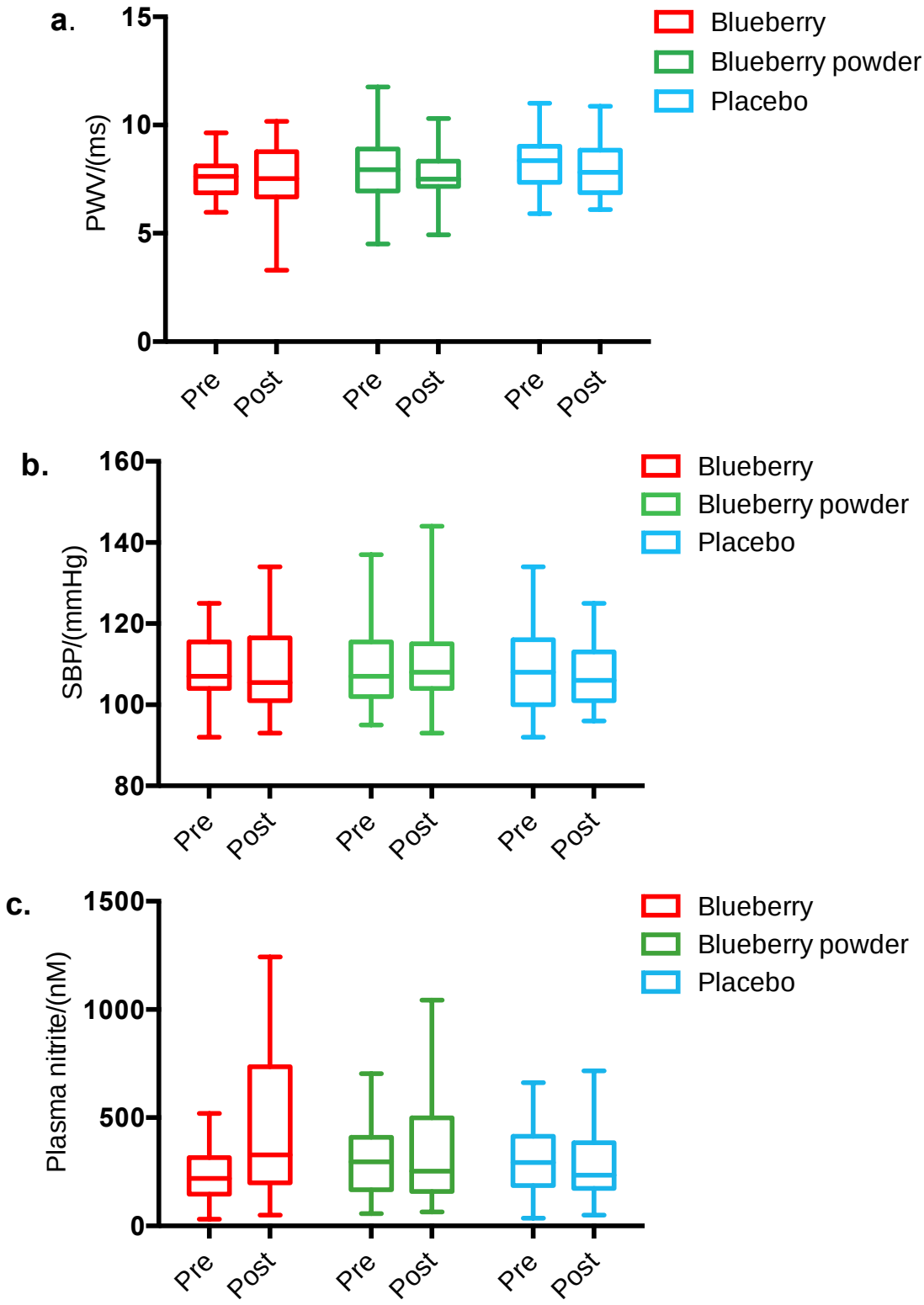
	Frequency							
Berry* and cherry intake frequency (N = 37)	Never	Once per month	2 - 3 times per month	once per week	twice per week	3 - 4 times per week	5 - 6 times per week	Every day
	10	8	16	2	1			
*blueberry, blackberry, blackcurrant, chokeberry, raspberry, strawberry, dark/purple grape included								

5.3 Results

5.3.1 Who body measurement of vascular function

No effect of treatment was found for SBP , DBP and PWV with covariance adjustment for baseline. LSD with adjusted *P* value was used for post hoc analysis for pairwise comparisons among treatments and visits. However, there was a significant treatment*visit interaction effect for PWV (F (4, 93.615) = 2.552, *P* = 0.044). **Figure. 5.3** displays the effect of interventions on selected endpoints including values of SBP and PWV**.** Pre- and post-intervention baseline-adjusted means of PWV and blood pressures are reported in **Table. 5.4**.

Figure. 5.3 The effect of blueberry interventions on selected vascular function endpoints including (a) PWV, (b) SBP, and (c) plasma nitrite levels*



*: The range of the error bars is the 95% confidence interval, the bottom and top of the box are the 25th and 75th percentiles, the line inside the box is the 50th percentile (median).

Table. 5.3 Change in PWV, blood pressure and plasma biomarker levels from pre- to post-intervention by the blueberry intervention groups[1]

	Blueberry intervention			Blueberry powder intervention			Placebo intervention			Effects[2]
	Pre	Post	△	Pre	Post	△	Pre	Post	△	
PWV, m/s	7.630 (0.153)	7.443 (0.239)	- 0.187	7.993 (0.258)	7.526 (0.234)	- 0.467	8.265 (0.209)	7.891 (0.244)	- 0.374	*P* = 0.344, Treatment x Visit*
BP, mmHg										
Systolic	108.727 (1.411)	108.704 (1.815)	- 0.023	110.278 (1.711)	111.395 (1.806)	1.117	109.314 (1.691)	109.732 (1.822)	0.418	*P* = 0.068
Diastolic	64.059 (1.440)	63.369 (1.398)	- 0.690	64.333 (1.482)	64.048 (1.388)	- 0.285	63.676 (1.395)	64.626 (1.406)	0.950	*P* = 0.536
Plasma biomarkers										
TAG	0.820 (0.053)	0.840 (0.075)	0.020	0.894 (0.059)	0.918 (0.071)	0.024	0.825 (0.061)	0.880 (0.074)	0.055	*P* = 0.836
Total cholesterol	4.302 (0.171)	4.557 (0.136)	0.255	4.567 (0.195)	4.533 (0.125)	- 0.034	4.509 (0.161)	4.324 (0.134)	- 0.185	*P* = 0.402
LDL- C	1.389 (0.147)	2.829 (0.136)	1.440	2.875 (0.138)	2.911 (0.128)	0.036	2.858 (0.128)	2.688 (0.136)	- 0.170	*P* = 0.318

Table. 5.4 (Continued)

HDL- C	2.749 (0.064)	1.439 (0.068)	- 1.310	1.514 (0.093)	1.443 (0.066)	- 0.071	1.487 (0.080)	1.516 (0.068)	0.029	$P = 0.485$
Glucose	5.755 (0.137)	5.952 (0.155)	0.197	5.818 (0.12)	5.788 (0.145)	- 0.030	5.854 (0.171)	5.625 (0.155)	- 0.229	$P = 0.334$
Nitrite/NO_2^-, nM	236.690 (21.086)	399.190 (47.030)	155.733	310.371 (31.311)	323.84 (45.19)	27.368	305.95 (32.708)	278.12 (45.249)	- 74.967	$P = 0.184$

△ Changed scores compared to the baseline: (post-intervention - pre-intervention)

[1]Values are baseline-adjusted means ± SEs unless otherwise indicated;

[2] Only P values for treatment effect was reported; Adjusted for pairwise comparison: least significant difference (LSD) $P \leq 0.0167$ for all pairwise comparison significance and effects observed. PWV, pulse wave velocity; BP, blood pressure; TAG: triglyceride; LDL- C: Low density lipoprotein cholesterol: HDL: high density lipoprotein cholesterol; nM, 10^{-9} mol/L;

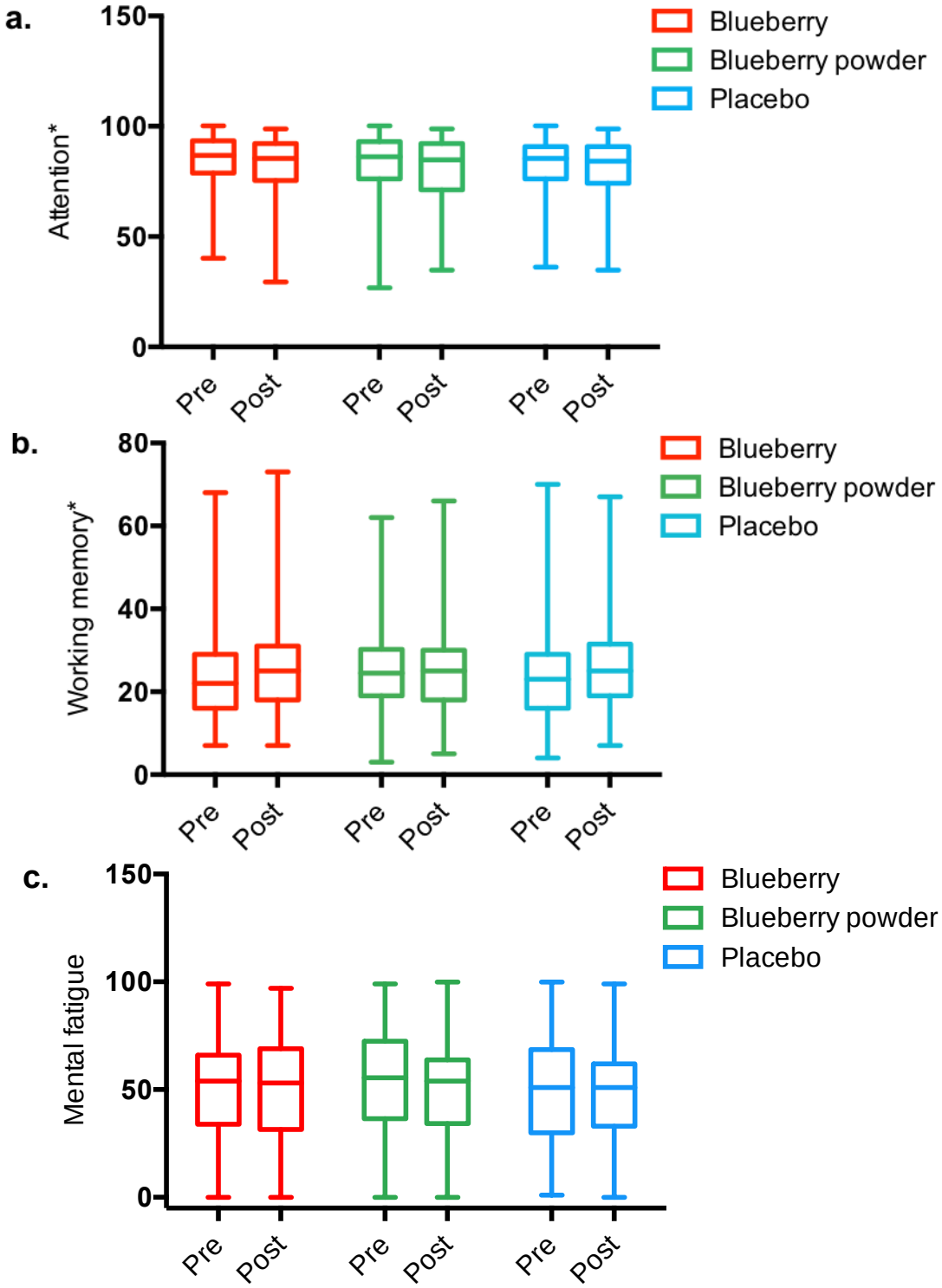
5.3.2 Plasma biomarkers

Table. 5.4 shows pre- and post-intervention baseline-adjusted means for plasma biomarkers for each intervention group. No effect of treatments, visiting days or the interaction effects of treatment*visit were shown for plasma triglycerides (TAG), total cholesterol, HDL - cholesterol, LDL - cholesterol, glucose and nitrite (NO_2^-) level with covariance adjustment for baseline. Both blueberry supplementation and blueberry powder supplementations reported improved NO_2^- level (+ 68.66 % and + 4.34 % separately) compared to the baselines whereas the placebo supplementation reported a decrease (- 9.10 %). However, the difference between the blueberry treatments and the placebo was not statistically significant. **Figure. 5.3** displays the effect of the interventions on plasma nitrite level.

5.3.3 Attention

Table. 5.5 shows pre- and post-intervention baseline-adjusted means for cognitive tasks. LSD with adjusted *P* value was used for post hoc analysis for pairwise comparisons among treatments, visits and repetitions. As shown in **Figure. 5.4**, although no difference of treatments was demonstrated by the interventions, effect of interventions on digit vigilance that assessed attention compared are displayed. There was a repetition effect on the correct response of the digit vigilance (F (2, 223.223) = 3.382, *P* = 0.036). There was significantly lower correct responses on the 3rd repetition compared to the 1st repetition (95% CI of - 2.877 [- 5.138, - 0.616], *P* = 0.013). There were also an effect of visit (F (2, 241.677) = 5.155, *P* = 0.006) and repetition (F (2, 241.357) = 8.357, *P* < 0.001) respectively on the reaction time (ms) of the correct responses to the test. The 3rd post- intervention visit showed significantly more slowed responses to the test compared to the 1st post-intervention visit (95% CI of 6.735 [2.314, 11.157], *P* = 0.003). The 2nd post-intervention visit also showed significantly more slowed responses to the test compared to the 1st visit (95% CI of 5.421 [1.201, 9.641], *P* = 0.012). The 3rd repetition showed significantly more slowed response to the test compared to the 1st repetition (95% CI of 8.643 [4.280, 13.006], *P* < 0.001) and the 2nd repetition also showed significantly more slowed responses to the test compared to the 1st repetition (95% CI of 6.587 [2.375, 10.798], *P* = 0.002).

Figure. 5.4 The effect of the blueberry interventions on selected cognitive domains including (a) attention, (b) working memory, and (c) mental fatigue scores*



* Attention assessed by the correct responses of example task: digit vigilance; working memory assessed by the correct number of example task: seral 7 subtraction; the range of the error bars is the 95% confidence interval, the bottom and top of the box are the 25th and 75th percentiles, the line inside the box is the 50th percentile (median).

Table. 5.5 Change in cognitive scores from pre- to post-intervention by the blueberry intervention groups[1]

	Blueberry intervention			Blueberry powder intervention			Placebo intervention			Effects[2]
	Pre	Post	Δ	Pre	Post	Δ	Pre	Post	Δ	
Working memory										
Total Serial 3s	42.505 (2.766)	45.535 (1.917)	3.030	42.380 (2.450)	44.961 (1.913)	2.581	43.448 (2.705)	44.620 (1.920)	2.115	*P* = 0.675
Correct Serial 3s	40.970 (3.065)	43.677 (2.611)	2.707	41.019 (2.692)	43.572 (2.610)	2.553	41.265 (3.006)	42.053 (2.620)	0.788	*P* = 0.388, Visit*
Error Serial 3s	2.168 (0.901)	2.224 (0.264)	0.056	1.837 (0.925)	2.301 (0.263)	0.464	2.49 (0.889)	2.048 (0.276)	- 0.120	*P* = 0.698
Total Serial 7s	26.143 (2.034)	28.191 (1.933)	2.048	26.815 (2.035)	28.001 (1.930)	1.186	27.133 (2.023)	28.848 (1.935)	2.705	*P* = 0.355, Treatment x Visit x Repetition*
Correct Serial 7s	24.753 (2.114)	27.637 (1.983)	2.884	25.571 (2.188)	27.694 (1.984)	2.123	24.949 (2.205)	26.602 (1.995)	1.849	*P* = 0.994
Error Serial 7s	2.423 (0.493)	2.338 (0.271)	- 0.085	2.469 (0.594)	2.271 (0.272)	- 0.198	2.626 (0.501)	2.536 (0.286)	0.113	*P* = 0.657
Episodic memory										
Delayed word recall - Correct	5.457 (0.317)	5.801 (0.350)	0.344	5.389 (0.250)	5.716 (0.345)	0.327	5.457 (0.295)	5.317 (0.355)	- 0.140	*P* = 0.445
Immediate word recall - Correct	7.286 (0.363)	7.736 (0.364)	0.450	6.972 (0.275)	7.113 (0.359)	0.141	7.171 (0.337)	7.366 (0.369)	0.195	*P* = 0.367

Table. 5.5 (Continued)

Word recognition - % Correct	78.286 (1.779)	81.001 (1.741)	2.715	80.37 (1.366)	82.298 (1.719)	1.928	82.381 (1.436)	80.569 (1.762)	- 1.812	*P* = 0.580
Word recognition - Correct RT	988.073 (40.362)	980.087 (35.539)	- 7.986	1026.525 (78.666)	1035.476 (36.115)	8.951	999.733 (31.007)	1003.691 (36.015)	3.958	*P* = 0.369
Attention									
Digit vigilance - % Correct	83.210 (1.949)	81.908 (2.081)	- 1.302	81.423 (2.862)	78.864 (2.076)	- 2.559	82.058 (2.006)	80.228 (2.090)	- 1.830	*P* = 0.414, Repetition*
Digit vigilance - Correct RT	501.190 (4.130)	505.842 (4.216)	4.652	502.692 (5.263)	506.862 (4.202)	4.170	505.27 (4.333)	509.254 (4.231)	3.984	*P* = 0.720, Visit*, Repetition*
Digit vigilance - False alarm	8.471 (0.851)	9.918 (1.004)	1.447	9.019 (1.078)	9.753 (1.000)	0.734	9.775 (1.269)	9.631 (1.013)	1.160	*P* = 0.912
Mood									
Alert	61.994 (2.601)	63.799 (2.152)	1.805	63.547 (3.135)	61.821 (2.116)	- 1.726	64.022 (2.861)	65.816 (2.212)	1.794	*P* = 0.392
Calm	65.671 (2.723)	67.069 (2.804)	- 1.788	65.569 (2.28)	68.213 (2.775)	- 2.443	66.986 (2.454)	63.985 (2.857)	- 6.781	*P* = 0.294
Content	68.857 (2.899)	69.128 (2.466)	0.271	70.656 (2.681)	70.297 (2.453)	- 0.359	70.766 (2.754)	71.918 (2.491)	4.932	*P* = 0.278
Fatigue									
VAS - Not at all <> Extremely	51.114 (3.251)	51.430 (3.613)	0.316	53.907 (3.369)	49.437 (3.597)	- 4.470	49.886 (3.553)	48.073 (3.629)	- 1.813	*P* = 0.271, Visit*

△ Changed scores compared to the baseline: (post-intervention - pre-intervention)

[1]Values are baseline-adjusted means ± SEs unless otherwise indicated; [2] Only P values for treatment effect was reported; adjusted for pairwise comparison: least significant difference (LSD) P ≤ 0.0167 for all pairwise comparison significance and effects observed. RT, reaction time; VAS: Visual Analogue Scale.

5.3.4 Working memory

No effect of treatments was shown for the serial 3 subtraction and the serial 7 subtraction tasks. There was a significant visit effect on the correct number of serial 3 subtraction (F (2, 224.248) = 7.527, *P* = 0.001). There was significantly higher correct number at the 2nd post-intervention visit compared to the 1st post-intervention visit (95% CI of 2.427 [0.845, 4.009], *P* = 0.003) and a significantly higher correct number at the 3rd post-intervention visit compared to the 1st post-intervention visit (95% CI of 3.154 [1.485, 4.823], *P* < 0.001). For serial 3 and serial 7 subtraction tasks, participants tried for 3 repetitions for each task during the assessment of working memory. There was also a significant treatment*visit*repetition interaction effect for the total number of serial 7 subtraction (F (20, 256.024) = 1.614, *P* = 0.049). On the 1st post-intervention visit and the 3rd repetition, total number of serial 7's task was significantly higher after receiving the placebo intervention compared to the blueberry intervention (mean difference 8.343, 95% CI [4.050, 12.635], *P* < 0.001) and the blueberry powder intervention (mean difference 8.290, 95% CI [4.269, 12.310], *P* < 0.001); for the 3rd repetition in the placebo intervention, the total number of serial 7 subtraction task was significantly higher on the 1st post-intervention visit compared to the 2nd post-intervention visit (mean difference 8.329, 95% CI [4.034, 12.635], *P* < 0.001) and the 3rd post-intervention visit (mean difference 5.812, 95% CI [1.721, 9.903], *P* = 0.006); for the placebo intervention on the 1st post-intervention visit, the total number of serial 7 subtraction was significantly higher on the 3rd repetition compared to the 1st repetition (mean difference 6.831, 95% CI [2.425, 11.236], *P* = 0.003). **Figure. 5.4** displays the effect of interventions on selected domains including serial 7 subtraction scores which assessed working memory.

5.3.5 Episodic memory

No effect of treatment, visiting day or the interaction effects of treatment*visit were shown for immediate, delayed word recall and word recognition after adjusting for baseline.

5.3.6 Mood and mental fatigue

Calm, alert and content were not affected by treatments, visits and the treatment*visit interactions with baseline covariance adjustment. Mental fatigue was not affected by treatments or the treatment*visit*repetition interactions. There was a visit day effect ($F(2, 239.553) = 4.564$, $P = 0.011$). There was a significantly lowered fatigued score on the 3rd post-intervention visit compared to the 2nd post-intervention visit (95% CI of -5.589 [-9.509, -1.669], $P = 0.005$). **Figure. 5.4** also displays the effect of interventions on fatigue score.

5.4 Post hoc power calculation

The standard deviations and mean values of the primary endpoints as identified in section 4.7 were obtained from **Table. 5.4** and **Table. 5.5** to perform retrospective post hoc power calculations [230]. The observed power for all primary endpoints are summarised in **Table. 5.6**. The hypothesis as described in section 1.5 included detecting significant treatment difference compared to the control for at least one primary endpoint. As shown in **Table. 5.6**, the observed powers ranged between 61 % - 100 %. The assessments for SBP, Correct Serial 3s and Correct Serial 7s were underpowered according to the initial sample size calculation with 80 % power as described in section 4.3.

Table. 5.6 Retrospective post hoc power calculation

Primary endpoints	Observed Power[a]	
	Blueberry intervention	Blueberry powder intervention
PWV	1.000	1.000
SBP	0.681	0.976
DBP	0.971	0.833
Correct Serial 3s	0.762	0.705
Correct Serial 7s	0.610	0.656
Delayed word recall - Correct	1.000	0.998
Immediate word recall - Correct	0.991	0.848
Digit vigilance - % Correct	0.934	0.804
Digit vigilance - Correct RT	0.935	0.684

[a]: Computed using $\alpha = 0.05$

5.5 Effects of the blueberry interventions on urinary metabolite profiles

5.5.1 PCA and PLS-DA analysis pre- and post-interventions

Following LC-MS analysis and peak picking, 5,523 mass spectral features were successfully detected. The resulting peak table was sequentially filtered to include only stable and reproducible peaks that resulted in a final 3327 features in positive ionisation mode (negative ionisation mode not performed due to COVID-19 restrictions). Multivariate analysis was applied to this peak table. In order to assess whether there were metabolomic differences at baseline prior to receiving interventions, pre-intervention data and post-interventions data were both analysed. QC samples were checked that clustered together and provided in **Appendix. 4.1.** The PCA was performed first to screen out any outliers and 3 samples were removed prior to PLS-DA. The PLS-DA scores plot showed no obvious clustering among blueberry treatment samples (N = 38), blueberry powder treatment samples (N = 34) and placebo treatment samples (N = 36) pre-intervention **(Figure. 5.5a).** PLS-DA also validated the significance of variable class discrimination in the dataset by a permutation test (*P* = 0.69) and demonstrated no significant difference among the interventions at baseline (R^2 = 0.02, Q^2 = -0.04, accuracy 22 %).

There were 6 outliers removed by PCA for post-interventions data. The PLS-DA scores plot displayed noticeable overlap in the clustering among post-intervention groups across Component 1 and 2, where Component 2 only accounted for 6.9 % of variation and was increased only by 4.4 % compared to the baseline data **(Figure. 5.5b)**, suggesting only moderate changes to the metabolome post-interventions compared to the baseline. The discrimination between variables was assessed by a permutation test (*P* = 1). Although there was some clustering, no significant difference was reported by the PLS-DA model (R^2 = 0.049, Q^2 = -0.07, accuracy 30 %).

Figure. 5.5 PLS-DA differentiation between intervention groups (a) pre- and (b) post-interventions

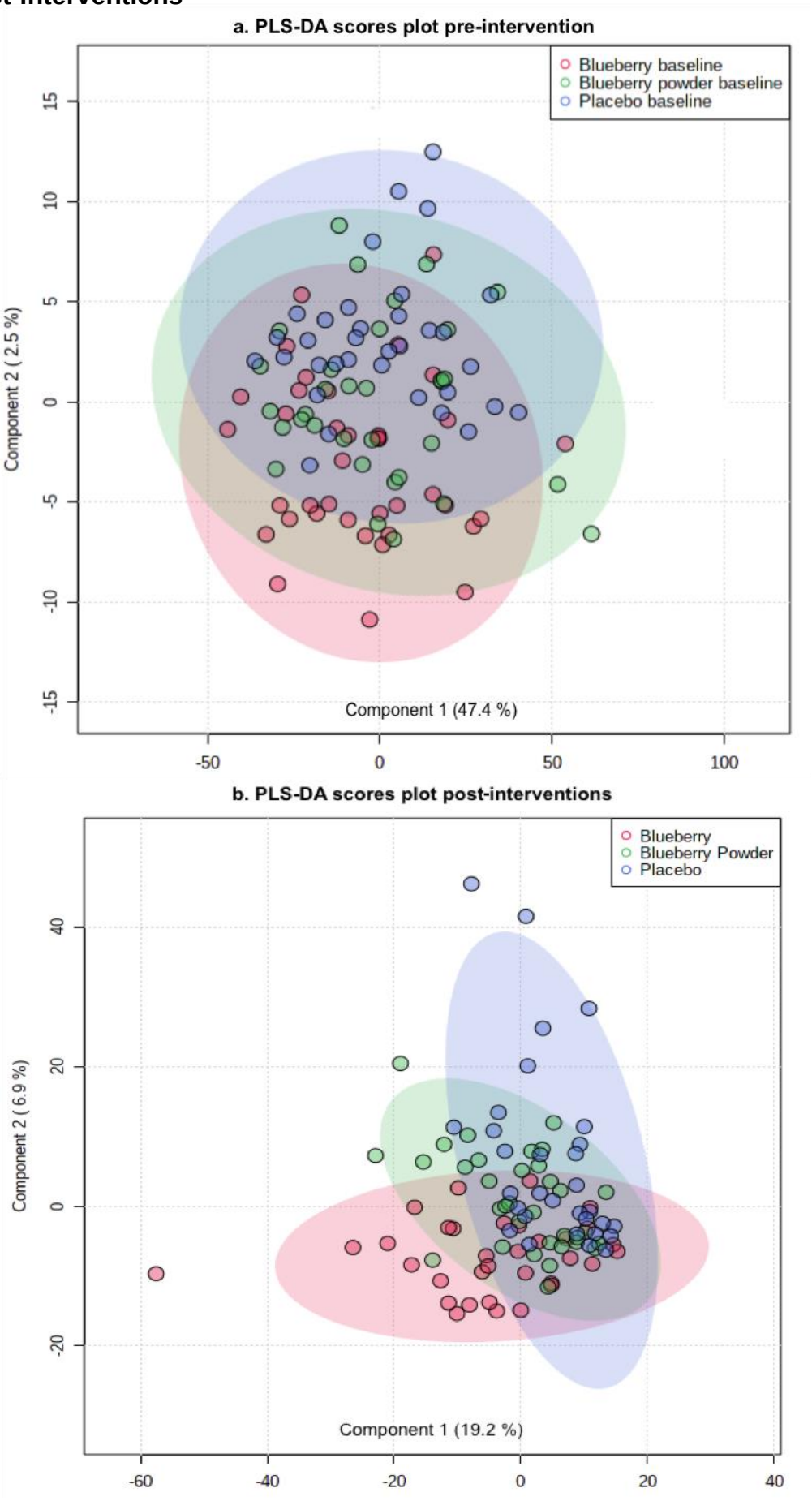
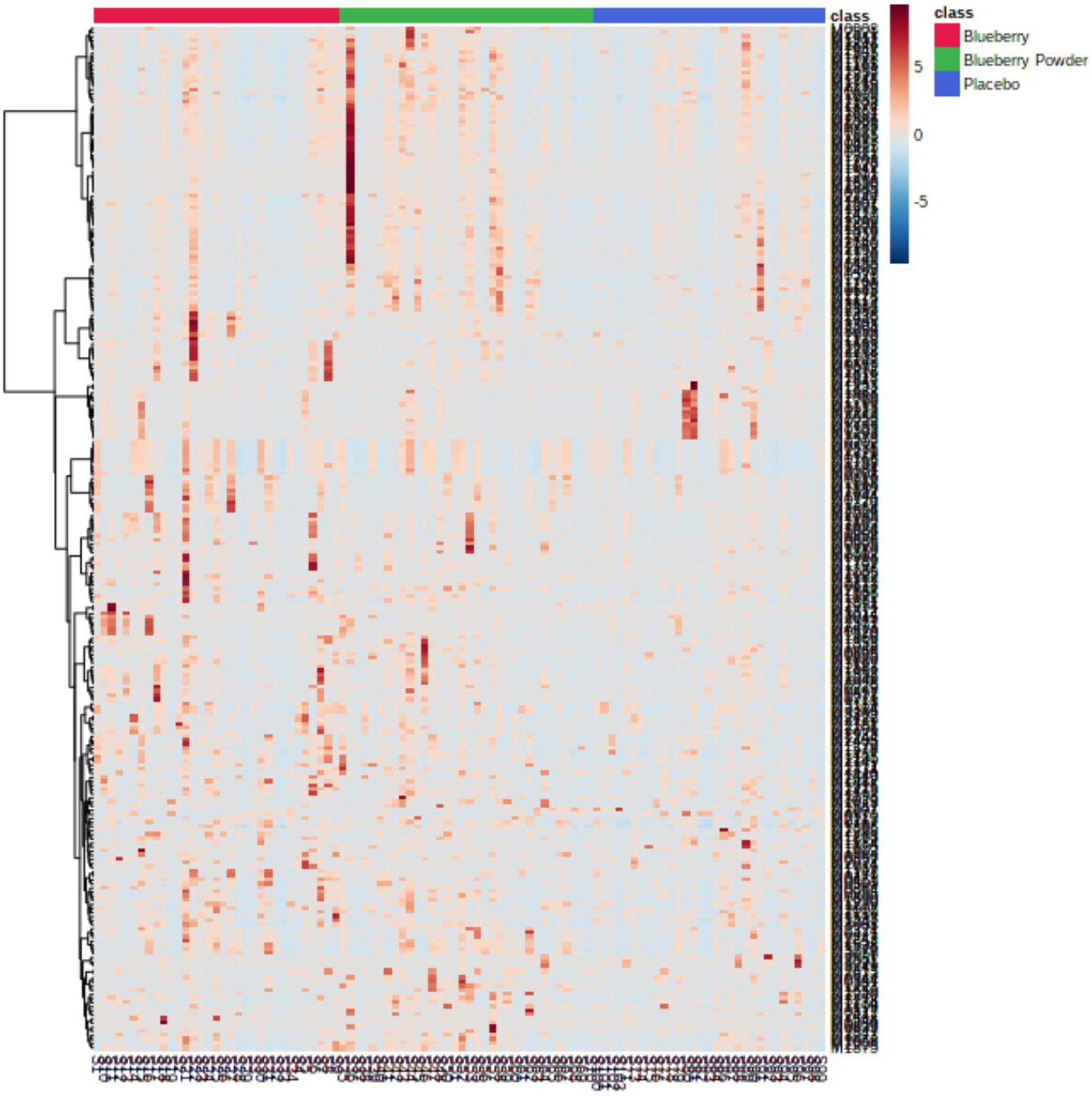


Figure. 5.6 presents a heatmap of ion feature intensities for each post-intervention group.

Figure. 5.6 Hierarchical clustering heatmap for the post-intervention groups



*The colour corresponds to an intensity level. Individual samples (horizontal axis) and features (vertical axis) are separated using hierarchical clustering (Ward's algorithm), with the dendrogram being scaled to represent the distance between each branch (distance measure: Pearson's correlation). The clusters corresponding to post-blueberry, post-blueberry powder and post-placebo interventions are highlighted in red, green and blue, respectively.

5.5.2 Data mining of important discriminating features

Random forest (RF) was used as a metric for determining discrimination among the interventions. RF shows the most important features ranked by their contributions to the classification accuracy shown as Mean Decrease Accuracy (MDA) for post-intervention groups. Features showing 2-fold increases or decreases in intensities after the interventions were further ranked by MDA. Ion features shown as discriminatory in the placebo intervention were excluded for whole blueberry and blueberry powder interventions. **Table. 5.7** and **5.8** display the top discriminating features selected by RF and MDA after whole blueberry intervention and blueberry powder intervention respectively and P values of comparing feature intensities among post-intervention groups (between group analysis). **Table. 5.7** and **5.8** also present univariate analysis for feature intensity changes with P values of each intervention comparing with baseline (within group analysis). The PCA and PLS-DA sores plots for within group analysis were provided in **Appendices. 4.2 - 4.4.**

There were approximately 57 % of ion features showing higher intensity after receiving whole blueberry intervention compared to blueberry powder (85 %) intervention. As shown in **Table. 5.7** and **5.8**, only features M2075, M2974, M1526, M2961, M2353 and M1448 showed increases in intensity following both blueberry and blueberry powder interventions, feature M1738 showed decreases in intensity following both interventions. But these findings were not significant. Feature M1223 increased intensity significantly following blueberry powder but not whole blueberry intervention, the significant difference was also detected among post-intervention groups ($P < 0.0001$).

Table 5.7 Discriminating features ranked by random forest following whole blueberry intervention

Feature ID	m/z	Between-group analysis		Within-group analysis	
		MDA[a]	*P* value[b]	Log2(FC)[c]	*P* value[c]
M1223*	233.163	1.788×10^{-3}	**1.267×10^{-6}**	-0.400	0.987
M1472	257.106	5.775×10^{-4}	0.723	0.689	0.256
M3014	152.051	1.476×10^{-4}	0.040	1.377	0.761
M3058	185.062	1.160×10^{-4}	0.324	-1.203	0.466
M1819	298.151	1.151×10^{-4}	0.398	-1.197	0.247
M3056	358.117	1.100×10^{-4}	0.028	0.664	0.076
M1448	163.100	-1.026×10^{-4}	0.821	1.270	0.475
M1738	844.535	-1.043×10^{-4}	0.552	-1.407	0.723
M1839	182.058	-1.427×10^{-4}	0.183	0.745	0.016
M3279	179.058	-2.935×10^{-4}	0.044	0.516	0.077
M2961	578.305	-4.954×10^{-5}	0.747	3.448	0.630
M1290	320.124	-5.405×10^{-5}	0.149	-1.048	0.282
M3123	129.079	-6.061×10^{-5}	0.307	-1.227	0.319
M1950	161.094	-7.245×10^{-5}	0.603	-1.16	0.663
M3290	159.090	-1.587×10^{-6}	0.572	-1.054	0.848
M2974	289.153	-5.357×10^{-6}	0.774	3.377	0.580
M1526	569.303	-5.623×10^{-6}	0.980	1.359	0.833

[a] : Mean decrease accuracy scores for all post-intervention groups

[b] : For univariate analysis of post-intervention groups comparing among groups; The false discovery rate(FDR) is 1.000 for all the features except for M1223 (FDR = 2.527×10^{-5})

[c] : For univariate analysis of post-intervention groups compared to baseline only; Log_2(FC): log transformed fold changes; FC: fold changes were the ratio of ion intensity post-intervention compared to baseline; the FDR is 1.000 for all the features

*: Significant for univariate analysis of post-intervention groups comparing between groups

Table. 5.8 Discriminating features ranked by random forest following blueberry powder intervention

Feature ID	m/z	Between-group analysis		Within-group analysis	
		MDA[a]	*P* value[b]	Log2(FC)[c]	*P* value[c]
M1223*	233.163	1.788×10^{-3}	**1.267×10^{-6}**	2.453	**1.060×10^{-5}**
M0645	250.132	8.898×10^{-4}	0.179	0.635	0.045
M0320	114.032	4.519×10^{-4}	0.727	1.029	0.425
M2358	276.169	3.476×10^{-4}	0.446	1.808	0.044
M2075	170.069	3.273×10^{-4}	0.245	1.404	0.086
M2021	253.132	2.867×10^{-4}	0.782	4.05	0.396
M2242	182.092	1.871×10^{-4}	0.016	1.577	0.001
M0968	456.270	6.452×10^{-5}	0.457	1.123	0.031
M2388	340.162	-1.055×10^{-4}	0.635	2.442	0.028
M3244	286.190	-1.187×10^{-4}	0.887	1.823	0.291
M1839	182.058	-1.427×10^{-4}	0.183	0.313	0.121
M3279	179.058	-2.935×10^{-4}	0.044	0.762	0.008
M1135	295.063	-4.860×10^{-5}	0.009	1.093	0.003
M2353	231.057	-5.714×10^{-5}	0.740	2.799	0.192
M3123	129.079	-6.061×10^{-5}	0.307	1.477	0.086
M3290	159.090	-1.587×10^{-6}	0.572	1.252	0.094
M2974	289.153	-5.357×10^{-6}	0.774	1.441	0.137

[a] : Mean decrease accuracy scores for all post-intervention groups

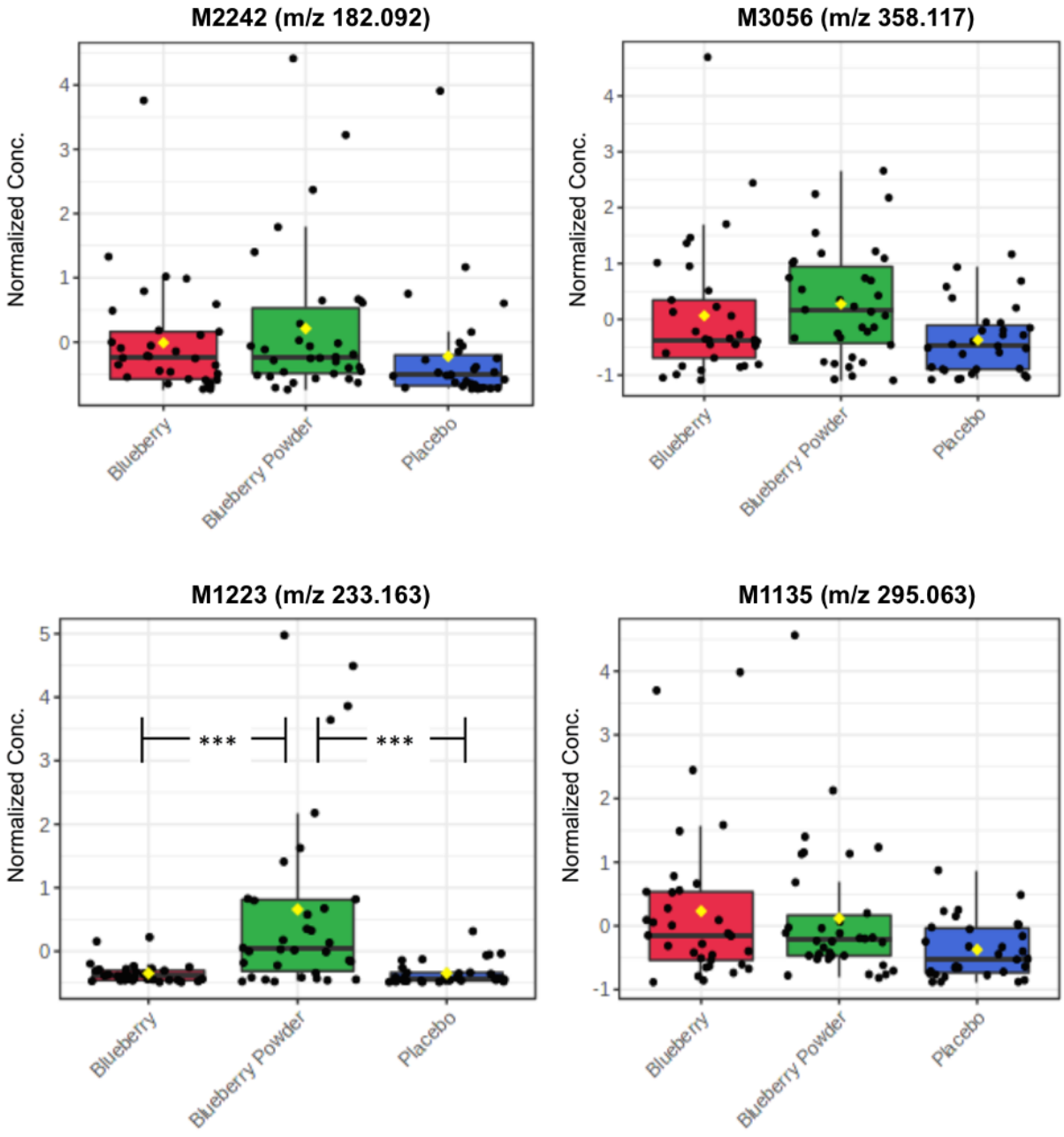
[b] : For univariate analysis of post-intervention groups comparing among groups; The false discovery rate(FDR) is 1.000 for all the features except fort M1223 (FDR = 2.527×10^{-5})

[c] : For univariate analysis of post-intervention groups compared to baseline only; Log_2(FC): log transformed fold changes; FC: fold changes were the ratio of ion intensity post-intervention compared to baseline; the FDR is 1.000 for all the features except for M1223 (FDR = 0.021)

*: Significant for univariate analysis of post-intervention groups comparing between groups

Figure. 5.7 displays ion intensities comparing post-intervention groups for selected features.

Figure. 5.7 Selected ion features with different intensity profiles for each intervention group



***: Significant for univariate analysis comparing normalised intensity between post-intervention groups

5.5.3 Putative annotations of important discriminating features

The discriminatory features ranked by MDA and shown in both blueberry and blueberry powder interventions were annotated where available basing on respective accurate mass using Thermo Fischer Compound Discovery Software. KEGG database was incorporated for annotations where available as summarised in **Table. 5.9.** It should be noted that all the annotations made here are putative and thus need further confirmation. Unfortunately, due to COVID-19 restrictions further fragmentation work was not possible.

Five putative annotations were made including adducts of 3-Dehydrocarnitine (M3290), O-Adipoyl carnitine (M2974), D-Pipecolic acid (M3123), (R)-3-(4-Hydroxyphenyl)lactate (M1839) and Hippuric acid (M3279). Univariate analysis of one-way ANOVA has shown one significant feature M1223 between post-intervention groups but has no closest match in the database.

Table. 5.9 Annotation of putative metabolites that discriminate blueberry interventions

Features ID	m/z	MDA[a]	Mass error (ppm)	Formula	Putative annotation	KEGG ID and pathway
M1223	233.163	1.788×10^{-3}	-5.250	C12 H19 N5	N/A	N/A
M1839	182.058	-1.427×10^{-4}	1.980	C9 H10 O4	(R)-3-(4-Hydroxyphenyl)lactate	Phenylpropanoic acids; C03964, map00130 Ubiquinone and other terpenoid-quinone; map00350 Tyrosine metabolism; map01100 Metabolic pathways; map01110 Biosynthesis of secondary metabolites
M3279	179.058	-2.935×10^{-4}	1.080	C9 H9 N O3	Hippuric Acid	Benzoic acid; C01586, map00360 Phenylalanine metabolism; map01100 Metabolic pathways
M3123	129.079	-6.061×10^{-5}	1.090	C6 H11 N O2	D-Pipecolicacid	D-alpha-amino acids; C00408, map00310 Lysine degradation; map00960 Tropane, piperidine and pyridine alkaloid biosynthesis; map01060 Biosynthesis of plant secondary metabolites; map01064 Biosynthesis of alkaloids derived from ornithine, lysine and nicotinic acid; map01100 Metabolic pathways; map01110 Biosynthesis of secondary metabolites
M3290	159.090	-1.587×10^{-6}	1.190	C7 H13 N O3	3-Dehydrocarnitine	Short-chain keto acids and derivatives; C02636
M2974	289.153	-5.357×10^{-6}	1.620	C13 H23 N O6	O-Adipoyl carnitine	Acyl carnitines

*: Mean decrease accuracy for variable importance ranked in random forest

5.6 Discussion

5.6.1 Principal findings of the effect of blueberry interventions

To our best knowledge, this is the first cross-over RCT investigating the efficacy of improving endothelial function, blood pressure, blood lipids and inflammatory biomarkers of vascular function, and cognition after whole blueberry (160 g) or freeze-dried blueberry powder (20 g) in a 7-day chronic intervention. We found that increasing daily blueberry consumption to 160 g/d or equivalent blueberry powder in healthy adults for 1 week did not influence blood pressure, endothelial function, plasma lipids, glucose, vascular inflammatory biomarkers and cognition. Plasma nitrite levels were improved 68.66 % and 4.34 % separately following whole blueberry and blueberry powder supplementations compared to the baseline whereas the placebo supplementation reported a decrease (- 9.10 %), although not statistically significant. There were significant effects of visit order due to randomisation of treatment sequence and interaction effects between treatments and visits were also observed in the study. For cognition, an interaction effect with task repetition and treatment was also observed for some cognitive tasks. The participants displayed significantly slowed reaction time in the assessment of attention after 3 repetitions of the assessment, suggesting that the interventions did not alleviate fatigue over time during the cognitive assessment.

5.6.2 Comparison between whole blueberry and blueberry powder

As discussed in the above section, there was no major effect of the interventions supplementing whole blueberry compared to blueberry powder in both vascular and/or cognitive function. There is a lack of studies comparing metabolomic differences between whole blueberry and blueberry powder consumptions. In the current study, there are some similarities in the metabolite profiles between blueberry interventions **(Fig. 5.5)** but differences were also observed. Feature M1223 (m/z 233.163) was increased in intensity solely after blueberry powder intervention, and because of that it is logical to speculate that it may originate from the freeze-drying processing of blueberry to powder, even though freeze-dried blueberry powder should arise from 100 % whole blueberry.

Even though limited metabolomic differences were found between whole blueberry and freeze-dried blueberry powder in the current study, there could be differences in bio-accessibility between freeze-dried powder and whole blueberry in the study that were likely induced from freeze-dried processing for powders. In terms of the nutritional value, the blueberry powder supplemented in the current study had lower sugar content (22 % less) compared to the whole blueberry and glucose load in the fruit matrix has been shown to delay anthocyanins absorption, which could result from the competitive action of glucose and anthocyanin on the sodium-dependent glucose cotransporter SGLT-1 [80]. The blueberry skin is rich in anthocyanins and usually kept during the freeze-drying processing of the blueberry to powder [81]. It has also been shown that fibre content is contained during the processing from blueberry to powder [82]. The technological processing for blueberry powder in relation to the whole blueberry could also cause increased bio-accessibility and absorption of bioactives [78].

Indeed, there were more ion features with increased intensity following blueberry powder (85 %) intake compared to whole blueberry (57 %), but the differences were not statistically significant. In another study comparing between blueberry and blueberry juice interventions (2-h post consumptions only) approximately 15 % of metabolites were higher in intensity after whole blueberry compared with juiced (approximately 3 %) blueberry [55]. In the current study, there could be differences in bioactives and nutrient values between freeze-dried blueberry powder and whole blueberry, but the metabolomic differences were not significant and neither was any treatment effect on improving endpoints, so it has not been possible to demonstrate if whole or powdered blueberry is the most effective at health promotion.

5.6.3 Urinary metabolite profiling

Metabolomics was used here to add mechanistic evidence for the role of blueberry. Metabolomics is of interest in nutritional studies [105], for instance, in the exploration of dietary patterns [231], biomarkers for specific fruit [232] or carbohydrate [233]. Here, only very modest changes to the urinary metabolome following blueberry interventions were found compared to placebo, the variation described was only 7 % and PLS-DA models showed poor discriminating ability (**Fig. 5.5)**. Nevertheless, the use of both

univariate and multivariate methods are encouraged to complement the findings in biomarker studies [234]. The current study was able to show discriminatory features that were increased in intensity following both blueberry interventions relative to control. Blueberry metabolites that increased in intensity following the interventions could be also used as markers of compliance.

Discriminatory ion features shown in the placebo intervention were excluded from the analysis so that the remaining discriminating features reported were likely induced by the blueberry interventions. Most features with putative annotations were shown to generate from metabolism (endogenous). 3-Dehydrocarnitine (M3290) and O-Adipoyl carnitine (M2974) are involved in the metabolism of fatty acids in the mitochondria for the generation of metabolic energy [235]. D-Pipecolic acid (M3123) is an endogenous metabolite of amino acids and originates from the metabolism of intestinal bacteria but could also originate from dietary sources [236]. A number of fatty acyl derivatives following acute blueberry consumption have being found previously, confirming a possible consequence of endogenous metabolism of acyl derivatives 3-Dehydrocarnitine and O-Adipoyl carnitine found in the current study [55].

Here, putative metabolites of phenolic acids (R)-3-(4-Hydroxyphenyl)lactic acid (M1839) and hippuric acid (M3279) were increased in intensity after both blueberry and blueberry powder interventions and are likely to be of exogenous origin and indicators of blueberry intake. (R)-3-(4-Hydroxyphenyl)lactate, or 3-(4-Hydroxyphenyl)lacetic acid a phenolic acid that is likely derived from phenolic or polyphenolic compounds in the diet [237]. Hippuric acid levels can rise with the consumption of phenolic compounds including berry polyphenols; the phenols are first converted to benzoic acid, and then to hippuric acid and excreted in urine [238]. There has been other research that has investigated the metabolomic differences induced by dietary blueberry or blueberry polyphenol interventions [73, 239]. Urinary hippuric acid has been found following 22 g blueberry powder (200 g blueberry equivalent) consumption in an acute 2-h post dose study [73, 239]. Hippuric acid, including its derivatives, were responsible for the major urinary and plasma metabolites changes after 1 month following 22 g blueberry powder daily consumptions [239]. Urinary 3-(4-Hydroxyphenyl)lacetic acid has been found increased in intensity following 150 g blueberry supplementation after 2 - 4 h and the metabolites have shown to originate

from procyanidins (flavonoids contained by blueberry) in culture models [73, 240, 241]. Phenylacetic acid derivatives have also been detected following 22 g (200 g blueberry equivalent) blueberry powder consumption metabolites [239]. For chronic consumptions, hippuric acid has previously been found in both serum and urinary metabolites following 150 g blueberry consumption for 6 months and also found in plasma metabolites following strawberry drink intake after 45 and 90 days [239, 242]. Compared to hippuric acid, hydroxyphenylacetic acid was also found following chronic consumption (45, 90 days) but was not increased in intensity compared to the baseline [242]. The current study was not acute and only observed the presence of putative annotations of hippuric acid and (R)-3-(4-Hydroxyphenyl)lactic acid after chronic consumption of both whole and powdered blueberry (1 week), which does suggest better retention of these compounds among a wide array of blueberry metabolites after sustained anthocyanin metabolism compared to other blueberry phenolic acids. In addition to phenolic acids, glucuronide, sulphate and glycoside derivates of anthocyanins, a range of major metabolites following phase II metabolism, have been reported previously following blueberry intake in both positive and negative ion mode but the current study did not find increased intensity of these compounds solely in positive mode [51, 55].

The bioavailability of dietary polyphenols could also interact with several factors including the metabolic process mediated by the liver (phase I and II metabolism) [243]. Variations could be caused by phase II metabolism for blueberry anthocyanins and the involved enzymes all could play a role at microbial, age, smoking, sex, and genetic levels to induce variability between individuals affecting anthocyanin metabolism [53, 54]. These metabolic differences between individuals thus may lead to differences in the biological effects of the dietary polyphenols on the health risk factors [52]. Therefore, the determination of the inter-individual variations in response to dietary intervention is important to explore towards a more personalised dietary recommendation. The inter-individual variation in response to the current interventions is further explored in Chapter 6.

Metabolomics was included in this study to provide mechanistic evidence. However, as no overall effect of the treatments were found and limited effects on metabolite profiles were observed, it is not possible to go further into detail. However, it should

be noted that due to COVID-19 disruptions, the metabolomic profiling in this study was not complemented with negative ionisation mode nor further fragmentation analysis to confirm metabolite identifications, which is unfortunate as negative ion mode is more favourable for excreted metabolites [233] and especially polyphenol metabolites [55]. Furthermore, the multivariate analysis model for differences between interventions was not robust (R^2 = 0.049, Q^2 = - 0.07, accuracy 30 %). The lack of significant metabolomic changes during the interventions are consistent with the lack of interventional effects shown in the trial.

5.6.4 Study limitations and implications

The lack of major effects of the interventions may also explain the limited changes to the urinary metabolome and also without confirmatory analysis then mechanisms of action could not be reported. The metabolomic analysis was also limited to LC-MS positive mode to provide more features for identification and for checking compliance of participants following the blueberry interventions. The total polyphenol content, but not anthocyanins, in blueberry and freeze-dried blueberry powder was measured and reported in this study. Nevertheless, the excreted anthocyanin levels usually are very low and metabolised to low molecular weight phenolic acids and several factors affecting response to the interventions are discussed in Chapter 6. Potential covariances of sex, age, BMI, ethnicity. socioeconomic status, and physical activity were not included in the analyses, but there were mostly young participants (25.86 ± 6.81 years old), white ethnicity (N = 31) and all healthy with non-smoking status during the trial. There were significant effects of visit order observed due to randomisation of treatment sequence and interaction effects between treatments and visits were also observed in the study. A cross-over design includes repeated visits and has the limitation of carryover effect of different treatments, which may explain the influence of visit order on treatment effects in the study [244]. There was low retrospective power (< 80 %) for some of the endpoints, therefore certain endpoints were underpowered which could impact on the ability to detect significant treatment effects for some of the primary endpoints in the current intervention.

It should be noted that harvest condition and post-harvest processing could lead to differences in the phytochemical profile and nutrition value between the whole

blueberry and freeze-dried blueberry powder [245]. There were also batch to batch variations in the phytochemical profile of supplied whole blueberry throughout the study timeline and the variations were not assessed in the study [246]. The nutritional difference and anthocyanins concentrations for both the two interventions were not analysed in the study. The total polyphenol content for the two interventions were analysed using Folin-Ciocalteu colorimetric assay, but many chemical compounds may act as interfering agents such as ascorbic acid in this method, producing inaccurate estimations of the real phenolic compounds concentrations in the matrix [247, 248]. The dietary energy intake of participants were analysed pre- and post - interventions but no significant difference was found for the dietary intake between and within the whole blueberry and blueberry powder intervention arms.

Prevention of cognitive impairment and vascular diseases, including CVD, are key priorities for both NHS England and the Government [249, 250]. A recent science-based report of the U.S. Dietary Guidelines Advisory Committee, also recommended dietary intervention for the preventing chronic (non-communicable) diseases including vascular diseases and dementia [60, 251]. However, the study here was performed on healthy people rather than participants with developed disease risks. Previous RCTs also implemented a longer duration of dietary intervention, whereas a more realistic intervention duration should be taken into account to assess their impact in a faster period and also assure good compliance from participants [252]. Here, all assessed endpoints for study participants were in normal range, which could account for the absence of the improvement on assessed vascular function and cognitive function. The presence of blueberry metabolites (hippuric acid) post-interventions may be used to confirm the compliance but the metabolite intensity was not significantly higher compared to the baseline and limited to only positive mode from LC-MS analysis. A validation of bioavailability may be necessary.

The length of each treatment arm was relatively short with 1 week compared to an average of 8 weeks with long-term interventions of blueberry assessing vascular function and 13 weeks assessing cognition and mood, as reviewed in Chapters 2 & 3. The current study was informed from the reviews. A moderate dosage from the range of whole blueberry dosages was applied and it was more applicable for participants in real life consumption. Therefore, the dosage of blueberry was equivalent to doubled

daily adult portion size as recommended by NHS [229], which was expected to augment efficacy within a shorter term. In addition to this, the logistics including the delivery and storage of blueberry during a long-term intervention were also considered.

It also should be noted that berry polyphenols and phytonutrients, not isolated anthocyanins, appear to be either more or less effective depending on the synergistic effect within the food matrix on improving blood pressures, plasma nitrite, lipid, and glucose controls [253]. This may be possible depending on the type of berry or the food matrix consumed and both the aforementioned interventions and the current study predominantly used fruit supplementations containing rich phenolic compounds instead of isolated anthocyanins. Furthermore, these blueberries could be ingested either before, after or with meals during the interventions. Therefore, the variations of findings between studies may be partially explained by the different food matrix and bio-accessibility of ingested blueberries.

Chapter 6. Inter-individual variation and metabotypes of response

6.1 Introduction

Understanding inter-individual variation in response to dietary interventions is important for understanding the efficacy for improving health at the individual level. Multi-dimensional data including molecular signatures of genes, transcripts, proteins, metabolites, and microbes among individuals have been applied in the areas of disease detection, prognosis and treatment [254]. To this end, the integration of these multi-dimensional data also allows transforming -omic approaches towards nutritional science by characterising the phenotypes or the susceptibility to the disease risk under controlled dietary conditions [255]. However, challenges lie with the complexities of individual responses to diet and their phenotypic variations. However, in order to achieve the ultimate goal of integrating an accurate and predictive approach for a sustained and effective health maintenance, these challenges need to be conquered.

A high inter-individual variability was observed in the present and most dietary interventions [256-258]. However, there is a lack of evidence to identify if individual's response is consistent across biological phenotypes or if the response is inconsistent; for example if a certain participant responds well to an intervention in terms of blood pressure, do they also respond similarly to other endpoints? A consistent response will help to identify a sustainable approach to improve health and prevent disease. Therefore, the current study aimed to characterise and identify consistent responder (RS) and non-responder (NRS) groups following the blueberry interventions in the assessment of vascular function and cognition; two important clinical endpoints for health maximisation. We have also attempted to identify putative metabolites at baseline that could act as predictors of response to the intervention. A quartile division was used as approach for RS and NRS characterisation. In order to demonstrate the approach of characterising RS and NRS, the identification of features predicting RS and NRS groups in response to the blueberry powder intervention is also shown in this chapter.

6.2 Results

6.2.1 Inter-individual variations in the assessment of endpoints following the interventions

A high range of variation was found for both vascular and cognitive endpoints. **Table. 6.1** displays the proportion of participants that showed either improved, worsened, or no changes in scores compared to the baseline in the assessed endpoints following the blueberry interventions.

Table. 6.1 Proportion of participants showing improved, worsened scores or no changes respectively in the endpoints after the blueberry interventions

Outcome	Blueberry intervention			Blueberry powder intervention		
	↑	–	↓	↑	–	↓
PWV	49 %	6 %	46 %	53 %	0 %	47 %
SBP	52 %	9 %	39 %	43 %	14 %	40 %
DBP	56 %	6 %	38 %	49 %	14 %	37 %
Nitrite (NO_2^-)	71 %	0 %	29 %	37 %	0 %	63 %
Glucose	45 %	0 %	55 %	50 %	3 %	47 %
TAG	34 %	0 %	66 %	59 %	0 %	41 %
Total cholesterol	36 %	0 %	64 %	42 %	0 %	58 %
HDL-C	55 %	0 %	45 %	55 %	0 %	45 %
LDL-C	31 %	3 %	66 %	42 %	0 %	58 %
Working memory*	57 %	12 %	31 %	48 %	14 %	36 %
Episodic memory*	48 %	14 %	37 %	45 %	16 %	38 %
Attention*	47 %	11 %	42 %	50 %	8 %	42 %
Alert	71 %	0 %	29 %	47 %	0 %	53 %
Content	54 %	11 %	35 %	33 %	6 %	61 %
Calm	62 %	3 %	35 %	58 %	3 %	39 %
Mental fatigue	45 %	3 %	52 %	55 %	4 %	41 %

*: ↑ Improved score after the intervention; – No changes after the intervention; ↓ Worsened score after the intervention; TAG: triglycerides; HDL-C: High density lipoprotein cholesterol; LDL-C: Low density lipoprotein cholesterol

*: Multiple tasks were used assess working memory, episodic memory and attention, so combined mean value of the proportions of participants in responding to those multiple tasks under each assessed cognitive domain were calculated

For the assessment of vascular function, all participants showed changes in plasma nitrite and lipids levels (triglycerides, total and HDL - C) whereas approximately 3 % - 14 % of participants did not show changes in the other endpoints. For cognitive function, 3 % - 16 % of participants have shown no changes in the majority cognitive endpoints except the alert assessment. The response level was further calculated and presented with percentage as described in section 4.9.1. **Table. 6.2** summarises the range of the response level (%) of participants in the assessment of vascular and cognitive endpoints following blueberry and blueberry powder interventions.

Table. 6.2 Range of responses (%) in the endpoints after the blueberry interventions

Outcomes	Response level (%)	
	Blueberry intervention	**Blueberry powder intervention**
*PWV	- 48 % - + 27 %	- 51 % - + 31 %
SBP	- 16 % - + 17 %	- 20 % - + 11 %
DBP	- 34 % - + 16 %	- 28 % - + 12 %
Nitrite (NO_2^-)	- 141 % - + 525 %	- 111 % - + 215 %
Glucose	- 33 % - + 66 %	- 32 % - + 25 %
TAG	- 105 % - + 95 %	- 94 % - + 132 %
Total cholesterol	- 30 % - + 62 %	- 68 % - + 43 %
HDL-C	- 51 % - + 85 %	- 90 % - + 52 %
LDL-C	- 34 % - + 79 %	- 58 % - + 64 %
Working memory	- 39 % - + 61 %	- 21 % - + 51 %
Episodic memory	- 30 % - + 30 %	- 12 % - + 21 %
Attention	- 33 % - + 18 %	- 34 % - + 14 %
Alert	- 57 % - + 48 %	- 70 % - + 59 %
Content	- 41 % - + 42 %	- 33 % - + 28 %
Calm	- 39 % - + 60 %	- 29 % - + 40 %
Mental fatigue	- 65 % - + 96 %	- 114 % - + 80 %

*PWV, pulse wave velocity; BP, blood pressure; TAG: triglycerides; HDL - C: high density lipoprotein cholesterol; LDL - C: low density lipoprotein cholesterol

As shown in **Table. 6.2**, following the dietary blueberry interventions, participants presented dynamic variations for plasma vascular biomarker levels including nitrite, glucose and lipids with responses ranging from - 141 % to + 525 %. The non-invasive assessment for vascular function including PWV and blood pressures presented relatively lower variations with response level ranging from - 51 % - + 31 %. A range of moderate to high inter-individual variations were also shown for cognition and self-rated mental fatigue presented the most dynamic changes relative to other cognitive endpoints varying from - 114 % + 96 %. To better demonstrate the variations, **Figures. 6.1 - 6.8** depict the inter-individual variations in response to a range of clinical endpoints assessing vascular function and cognition.

It can be noticed that most participants showed random responses across vascular and cognitive endpoints following the interventions. For example for PWV, participant S3 responded the highest following the whole blueberry intervention but was the lowest responder for the blueberry powder intervention. For mental fatigue, participant S31 responded high following whole blueberry intervention but responded the lowest for blueberry powder. However, participant S2 improved responses to most vascular and cognitive endpoints, except to plasma nitrite level after both two interventions. Participant S2 also showed a high consistency of improved responses to cognitive assessments after the blueberry powder intervention, but not to the vascular function. Participant S19 and S25 showed a high consistency of improved responses to vascular function following both blueberry and blueberry powder interventions, but their responses to cognitive function appeared random.

Figure. 6.1 Inter-individual variation in pulse wave velocity (PWV) following the interventions

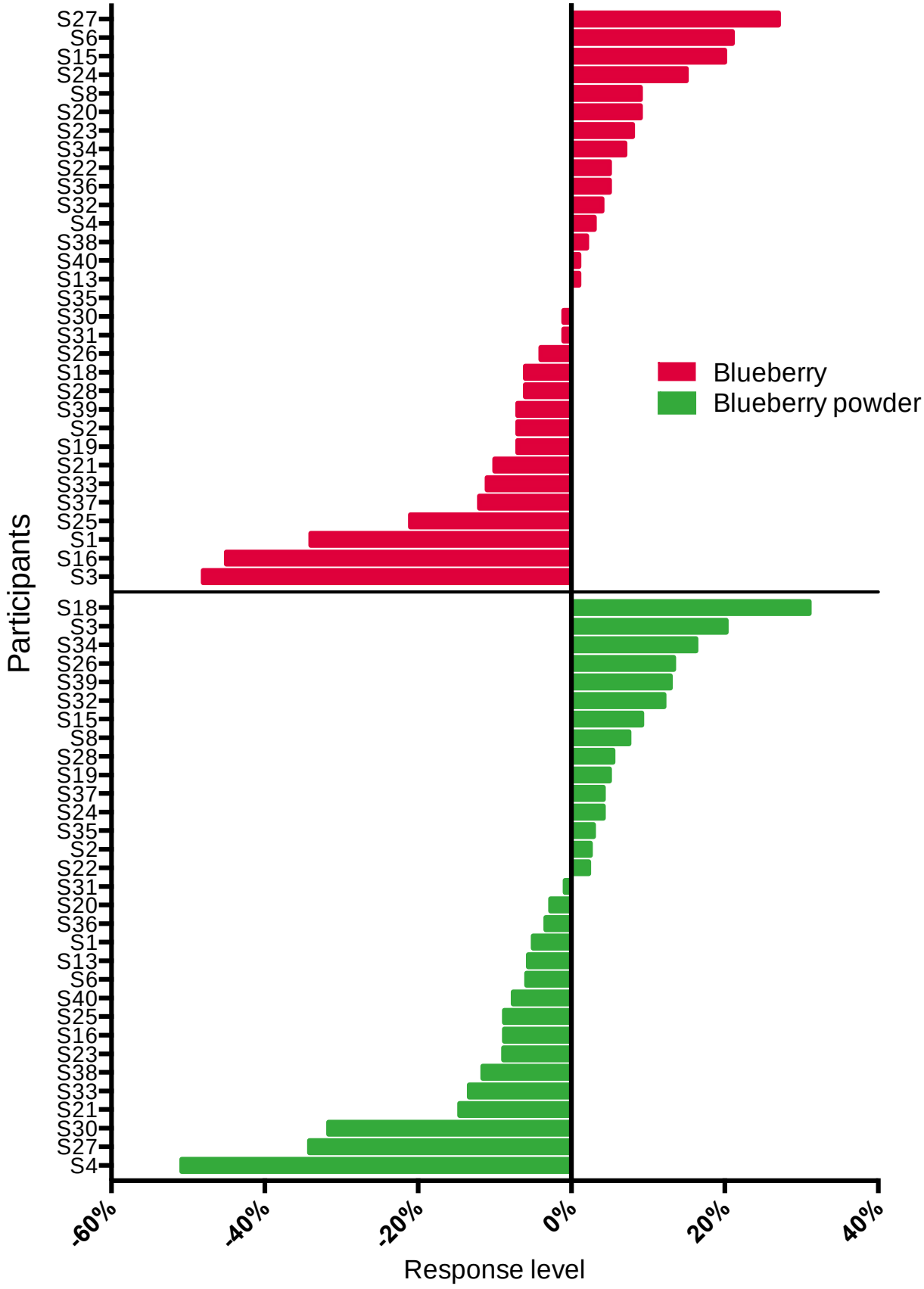


Figure. 6.2 Inter-individual variation in systolic blood pressure (SBP) following the interventions

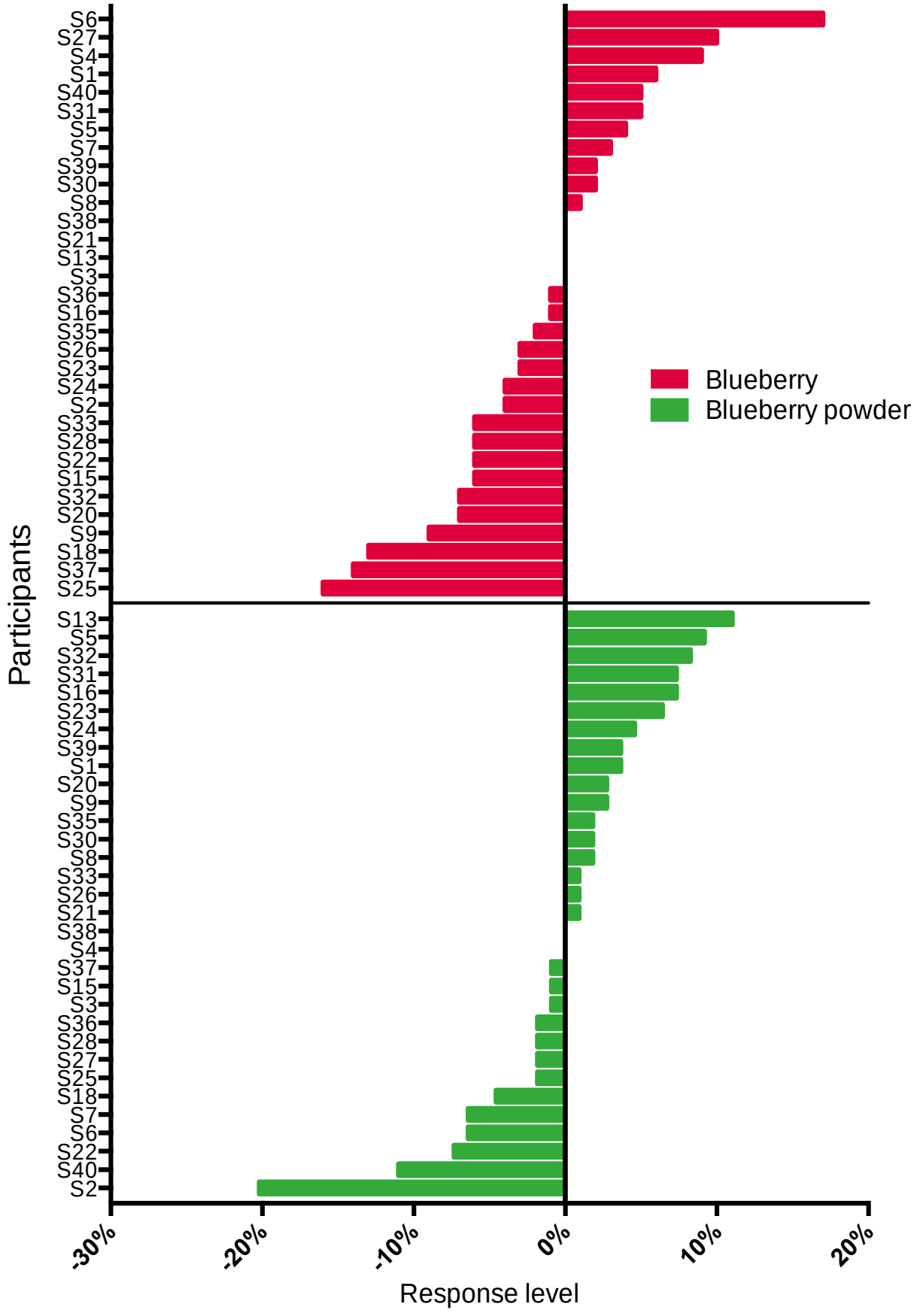


Figure. 6.3 Inter-individual variation in plasma nitrite (NO_2^-) following the interventions

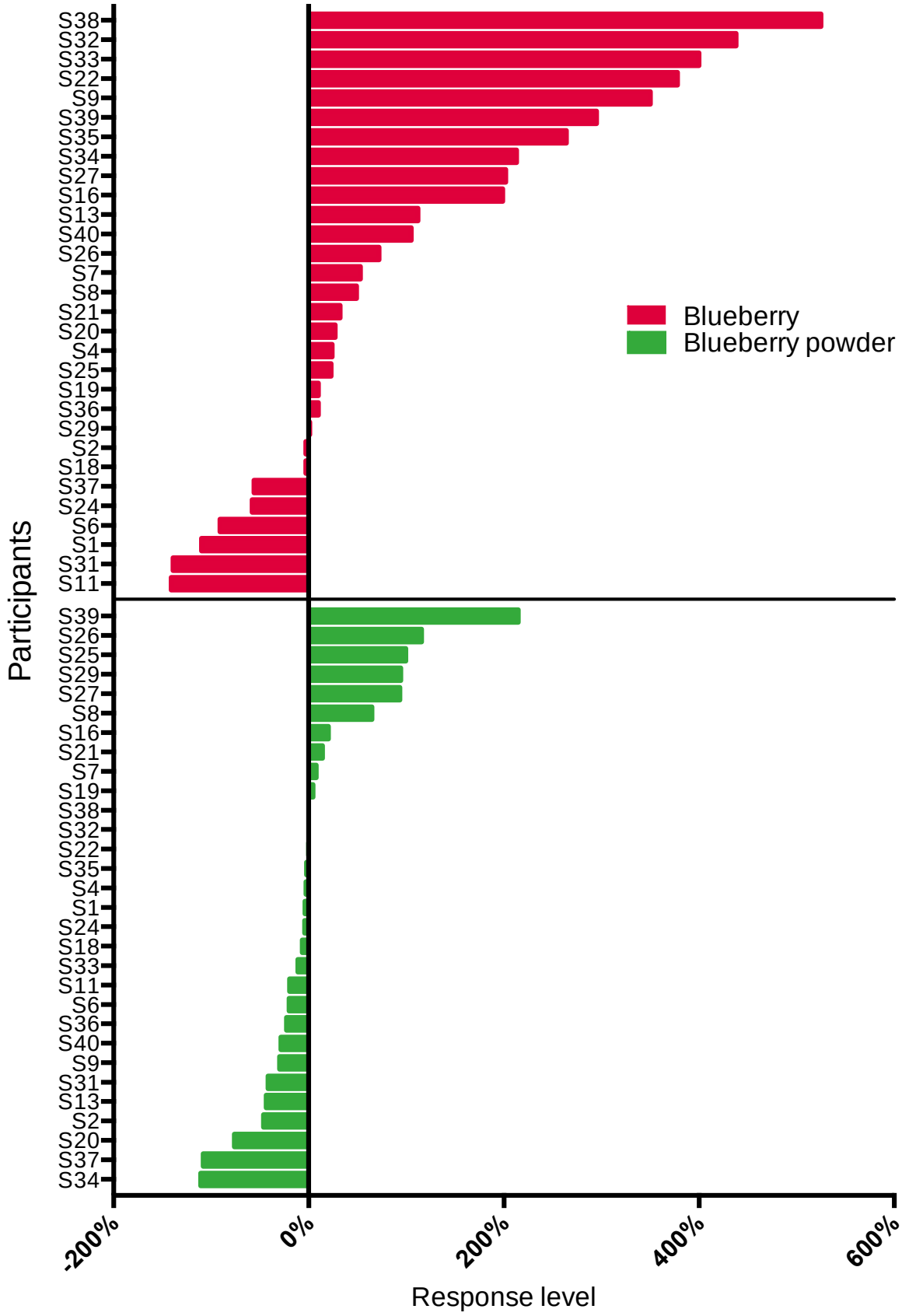


Figure. 6.4 Inter-individual variation in plasma low-density lipoprotein cholesterol (LDL - C) following the interventions

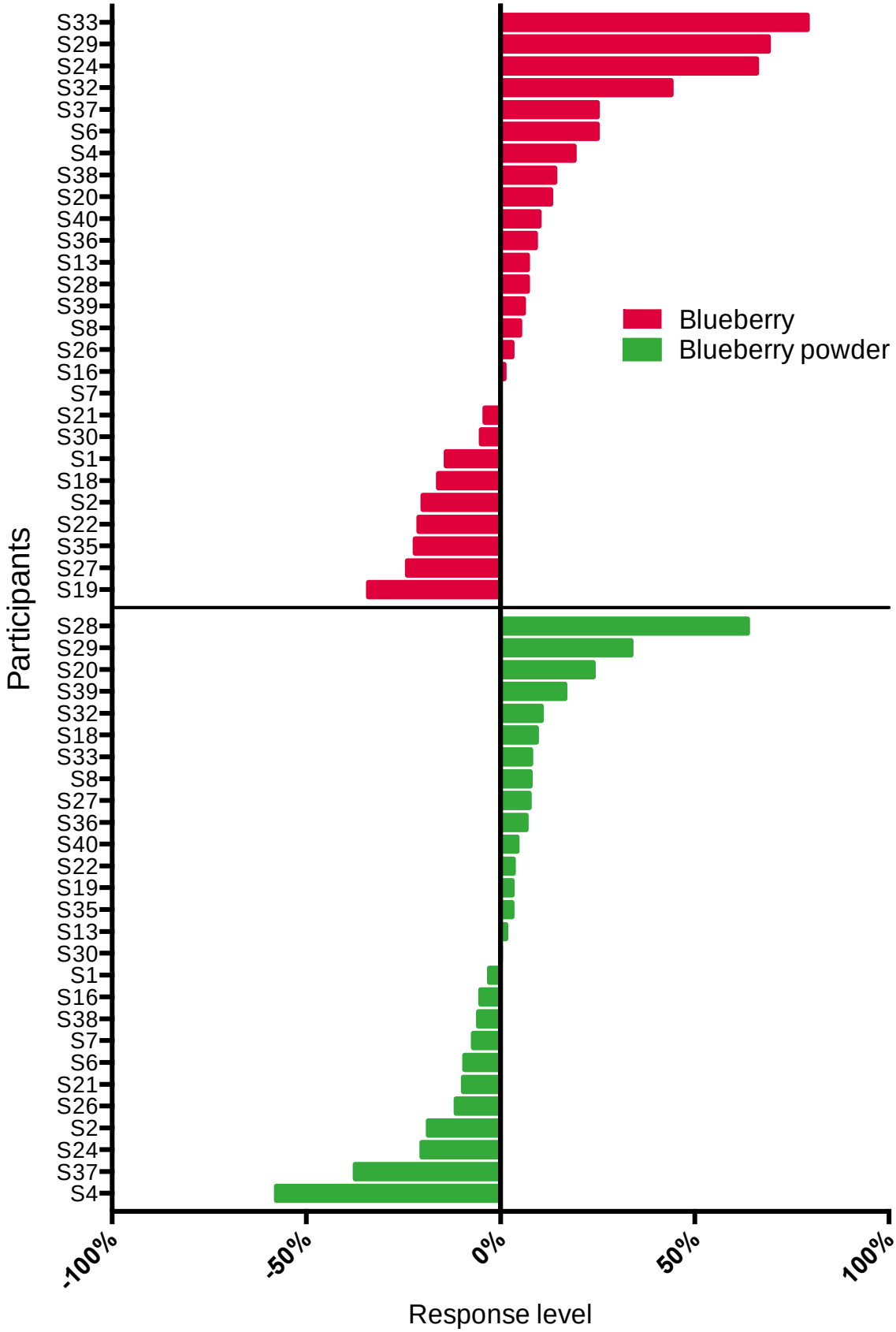
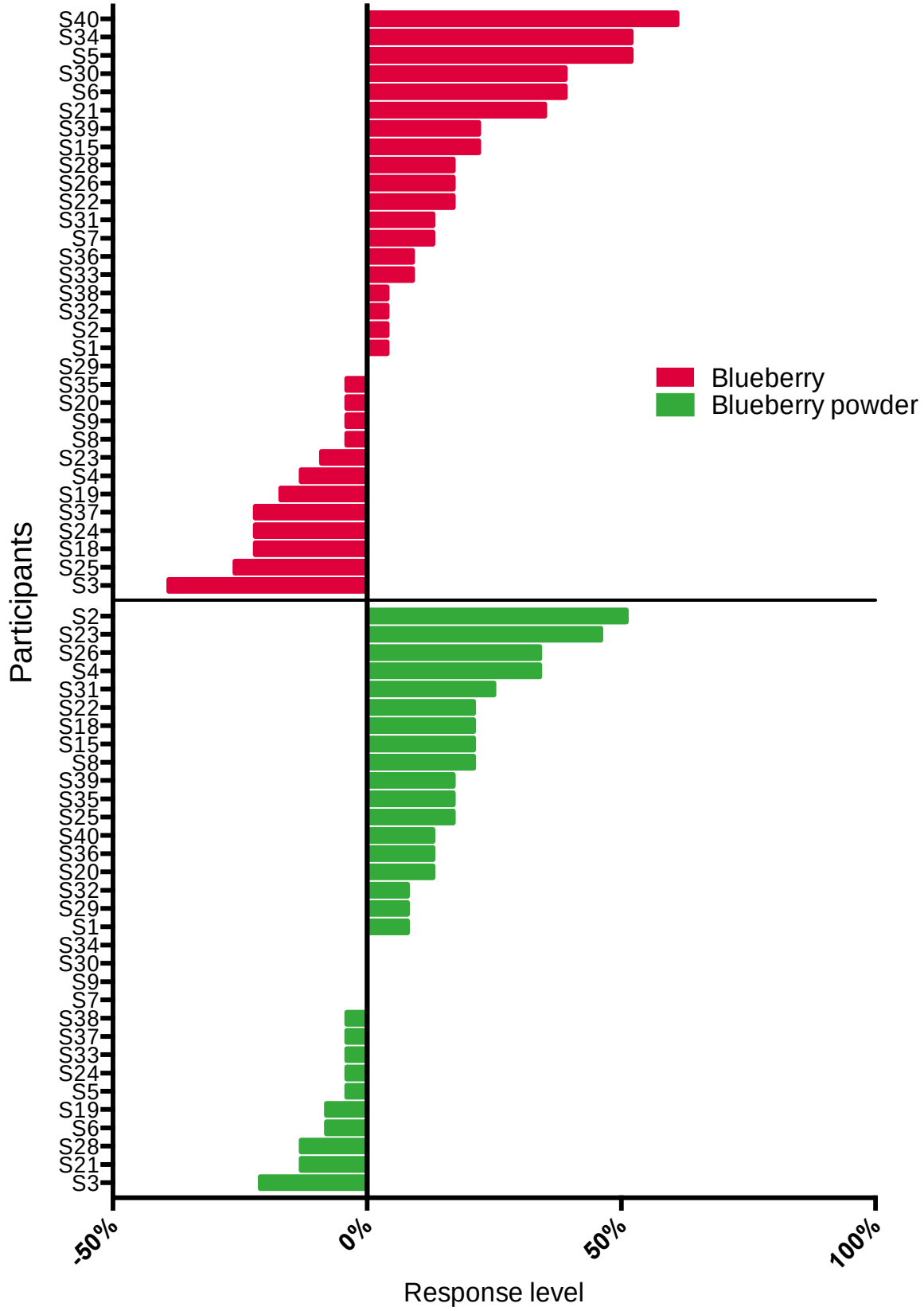
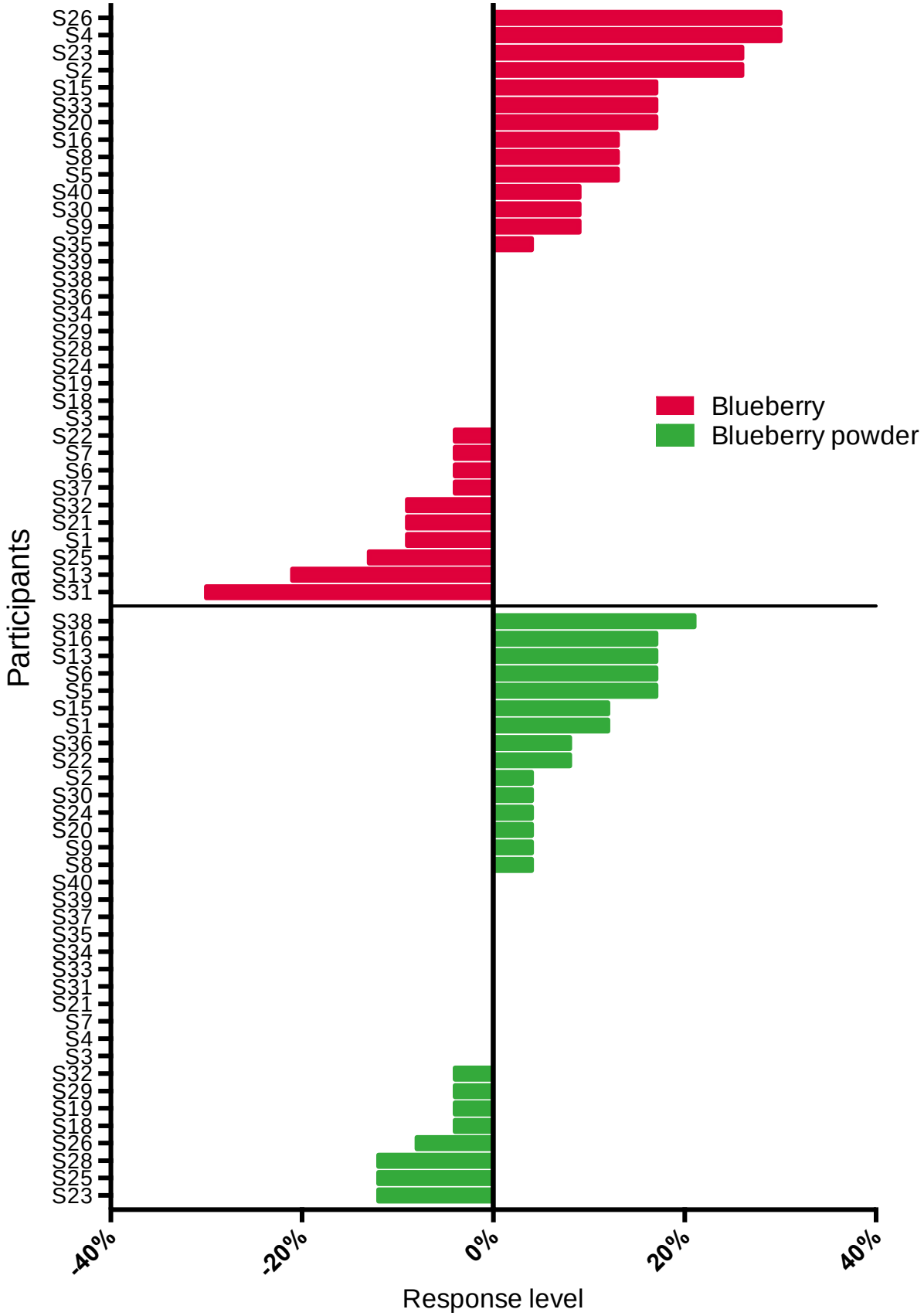


Figure. 6.5 Inter-individual variation in working memory following the interventions



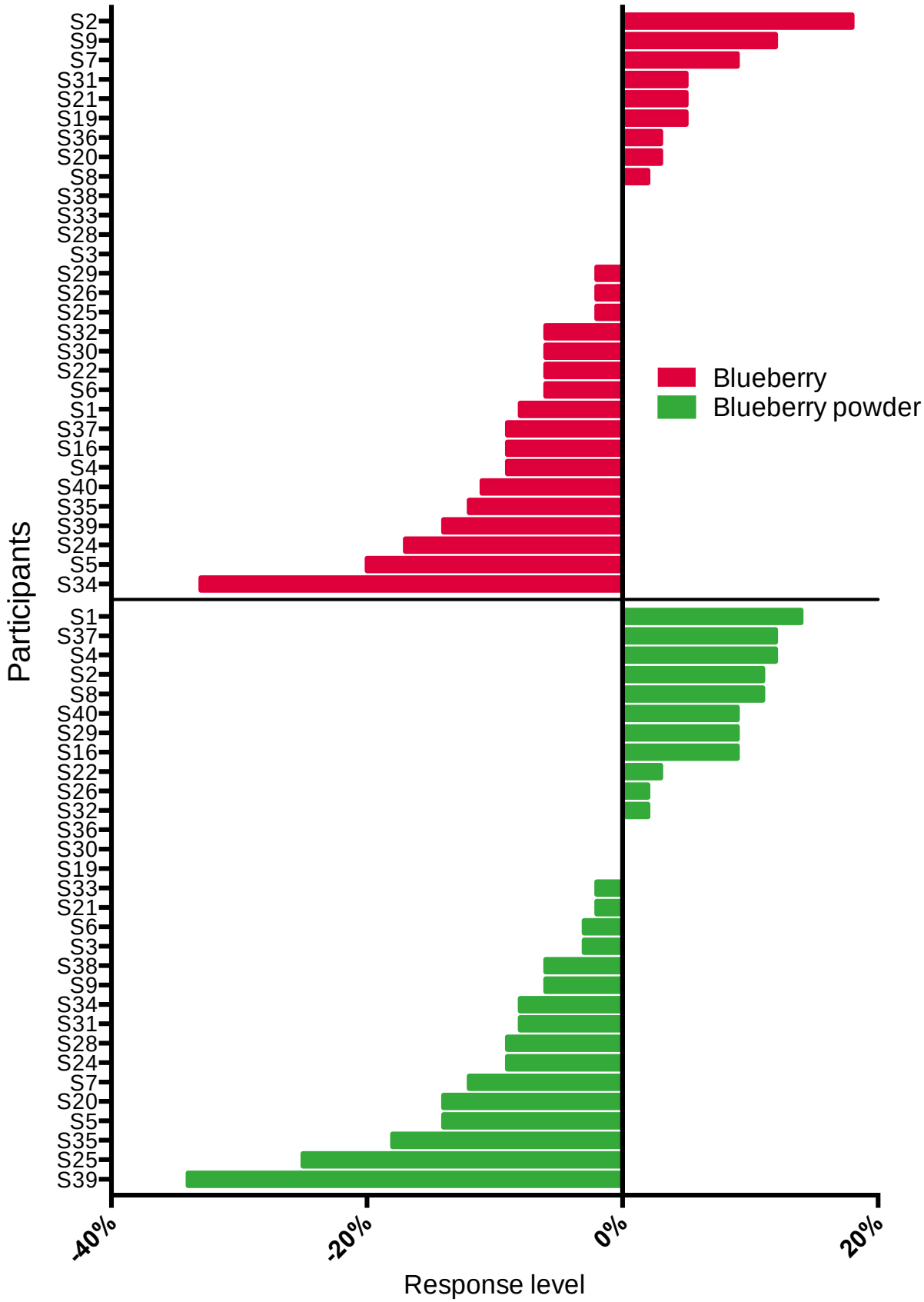
*Working memory assessed by the correct number of seral 7 subtraction task

Figure. 6.6 Inter-individual variation in episodic memory following the interventions



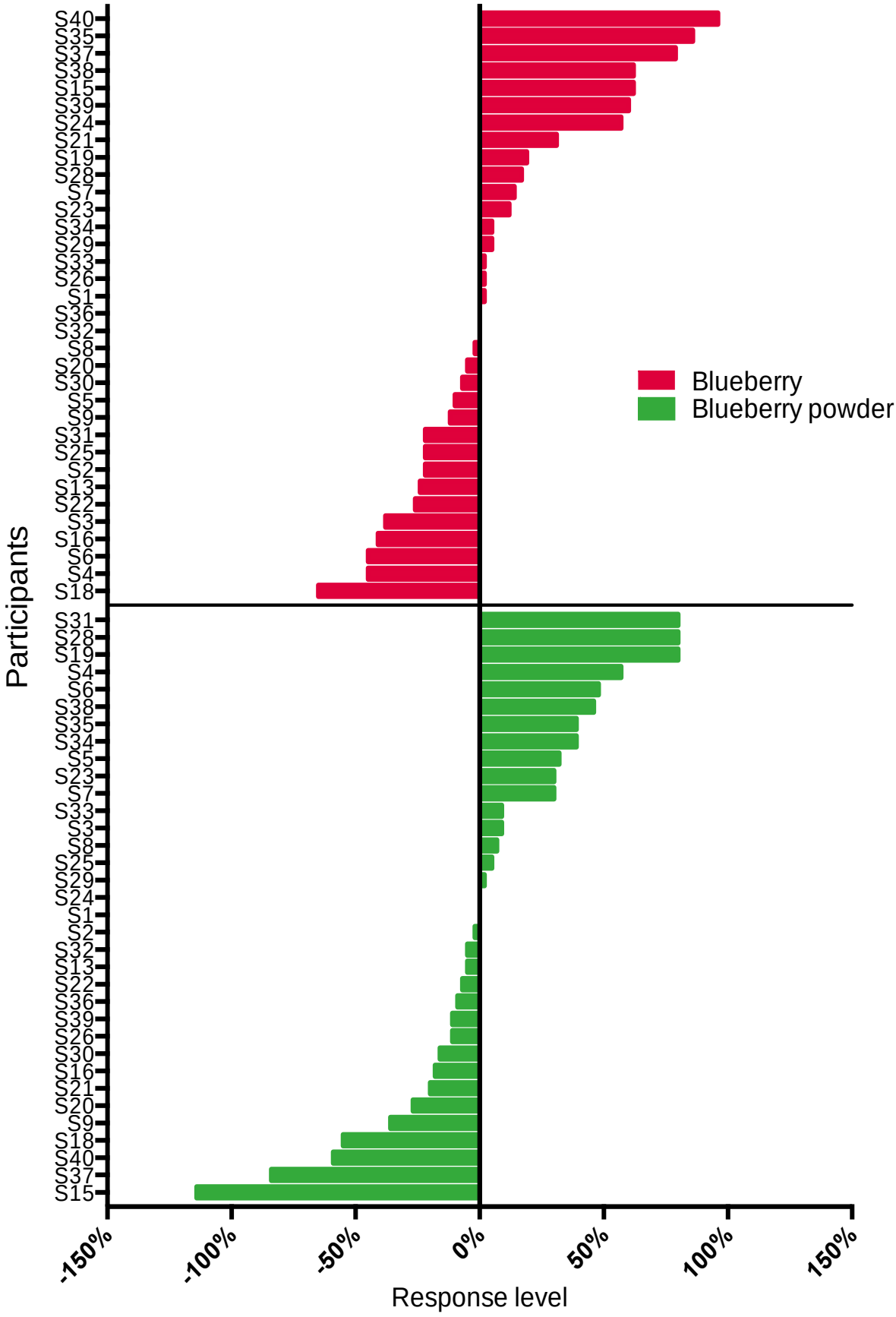
*Episodic memory assessed by the correct number of word recognition task

Figure. 6.7 Inter-individual variation in attention following the interventions



*Attention assessed by the correct number of digit vigilance task

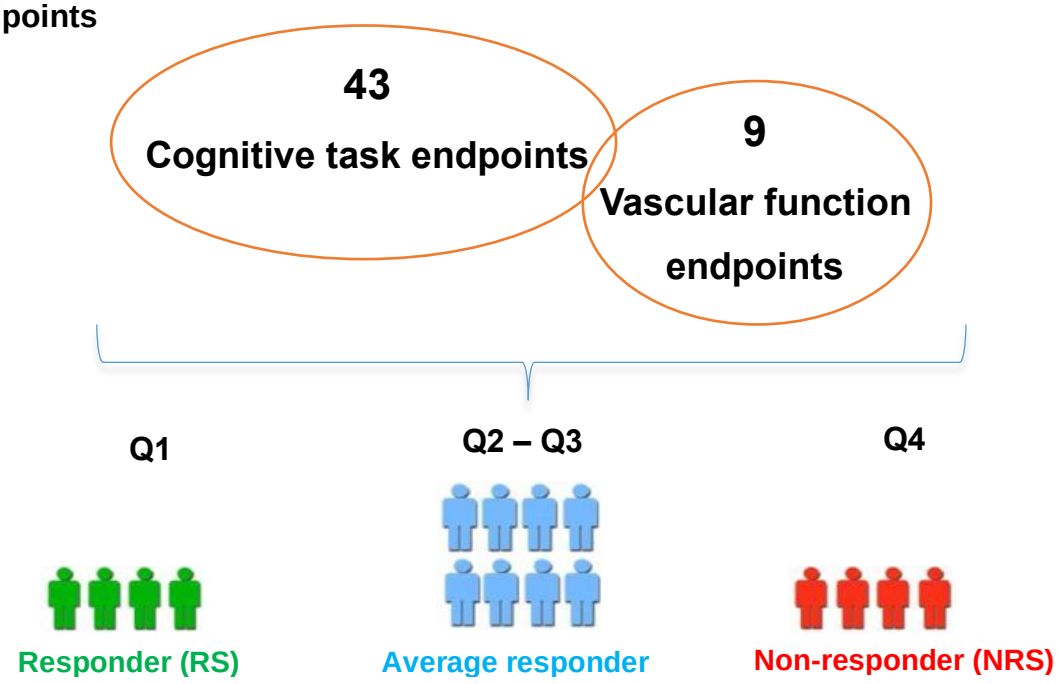
Figure. 6.8 Inter-individual variation in mental fatigue following the interventions



6.2.2 Characterisation of responder (RS) and non-responder (NRS) groups

The response level for each assessed endpoint was calculated from post - interventions compared to baseline and presented as a percentage (%). For each endpoint, particpants were ranked basing on their response level from highest to lowest. Then, participants that ranked at the upper quartile (Q1) or the lower quartile (Q4) were identified, considering the effect direction of each assessed endpoint. There were 43 endpoints assessing cognition, 9 endpoints assessing vascular function and 52 endpoints in total. Finally, based on the rank of the number of the endpoints that participants responded to from the largest to the smallest, the Q1 of participants were identified as the RS group. Respectively, based on the rank of the number of endpoints that participants non-responded to from the largest to the smallest, the Q1 of participants were identified as NRS group. **Figure. 6.9** shows the number of endpoints for identifying RS and NRS.

Figure. 6.9 RS and NRS identification based on the number of assessed endpoints



*Q1: the upper quartile basing on the rank of response level: Q2 - Q3: the second to third quartiles basing on the rank of response level; Q4: the lower quartile basing on the rank of response level

Table. 6.3 Characterisation of responder (RS) and non-responder (NRS) groups in response to the blueberry interventions

Participant ID	Blueberry intervention						Blueberry powder intervention					
	RS			NRS			RS			NRS		
	All endpoints*	Vascular function	Cognition	All endpoints	Vascular function	Cognition	All endpoints	Vascular function	Cognition	All endpoints	Vascular function	Cognition
S1	16	3	13									
S2	16	4	12								2	12
S3				18		18					3	
S4	15		15		3		16	4	12			
S5	14		14							19		18
S6			11	14	6			4				
S7	16		13							16		16
S8												
S9												
S11								3				
S13								3				
S14							23		20			
S15							23		21			
S16		3										
S18		4		15		13	16		14			
S19					3							
S20		3								17	5	12
S21								3				
S22	16	5										
S23	13		12							15		13
S24				18	6					25	3	22
S25						14		4		16		15

Table. 6.3 (Continued)

S26					3		16		14			
S27								4				
S28					3					24	4	20
S29					4			4				
S30								3		17		15
S31	15		14							15	4	
S32		3									5	
S33												
S34		4				13					4	
S35		3		15		14						
S36				17		14	15		14			
S37				19	4	15	23		21			
S38	15		14				16		15			
S39				17		15					5	
S40				21	4	17	22		20			

*Total number of endpoints: 52; number of vascular function endpoints: 9; number of cognitive endpoints: 43
RS in the total endpoints after whole blueberry intervention: S1, S2, S4, S5,S7, S22, S23, S31, S38
NRS in the total endpoints after whole blueberry intervention: S3, S6, S18, S24, S35, S36, S37, S39, S40
RS in the vascular endpoints after whole blueberry intervention: S1, S2, S16, S18, S20, S22, S32, S34, S35
NRS in the vascular endpoints after whole blueberry intervention: S4, S6, S19, S24, S26, S28, S29, S37, S40
RS in the cognitive endpoints after whole blueberry intervention: S1, S2, S4, S5, S6, S7, S23, S31, S38
NRS in the cognitive endpoints after whole blueberry intervention: S3, S18, S25, S34, S35, S36, S37, S39, S40
RS in the total endpoints after blueberry powder intervention: S4, S14, S15, S18, S26, S36, S37, S38, S40
NRS in the total endpoints after blueberry powder intervention: S5, S7, S20, S23, S24, S25, S28, S30, S31
RS in the vascular endpoints after blueberry powder intervention: S4, S6, S11, S13, S21, S25, S27, S29, S30
NRS in the vascular endpoints after blueberry powder intervention: S2, S3, S20, S24, S28, S31, S32, S34, S39
RS in the cognitive endpoints after blueberry powder intervention: S4, S14, S15, S18, S26, S36, S37, S38, S40
NRS in the cognitive endpoints after blueberry powder intervention: S2, S5, S7, S20, S23, S24, S25, S8, S30

Table. 6.3 presents the participants that were characterised into either RS or NRS groups for the separate vascular or cognitive endpoints following the interventions based on the number of endpoints they responded or non-responded to. Following whole blueberry intervention, 2 participants (S1, S2) were identified as RS and another 2 participants (S37, S40) were identified as NRS across both vascular and cognitive endpoints. Following blueberry powder intervention, 1 participant (S4) was identified as RS and 4 participants (S2, S20, S24, S28) were identified as NRS across both vascular and cognitive endpoints. For the assessment of total 52 endpoints, there were 2 out of 9 participants (S4, S38) identified as RS following both whole blueberry and blueberry powder interventions. The remaining identified RS and NRS demonstrated random responses to either the total endpoints, or the separate vascular and cognitive endpoints.

6.2.3 Effects of gender, BMI, and study visit order on response

There was RS/NRS with different gender and with either normal (18.5 - 24.9 kg/m^2) or overweight (25.0 - 29.9 kg/m^2) BMI. Study visit effect (treatment sequence) was shown in intervention effect for some of the endpoints. Therefore, to assess the effect of gender, BMI and visit order on the response group, chi-square test was applied. The age group recruited in the current study is mostly young (97 % of pariticipants being less than 42 years old) so the association of age and response to clinical endpoints was not assessed. No relation between gender and response, BMI and response, or visit order and response was shown **(Table. 6.4).**

Table. 6.4 The asscoaition of gender, BMI, and study visit order with response

		Chi square test statistics*			
	Factor	Response following blueberry intervention		Response following blueberry powder intervention	
All endpoints	Gender	X^2 =1.000	*P* = 0.620	X^2 =1.286	*P* =0.576
	BMI	X^2 =0.000	*P* =1.000	X^2 =0.000	*P* =1.000
	Visit	X^2 =0.000	*P* =1.000	X^2 =1.067	*P* =0.587
Vascular Function	Gender	X^2 =0.234	*P* = 1.000	X^2 =1.000	*P* =0.620
	BMI	X^2 =0.234	*P* = 1.000	X^2 =0.000	*P* =1.000
	Visit	X^2 =0.533	*P* =0.766	X^2 =0.900	*P* =0.638

Table 6.4 (continued)

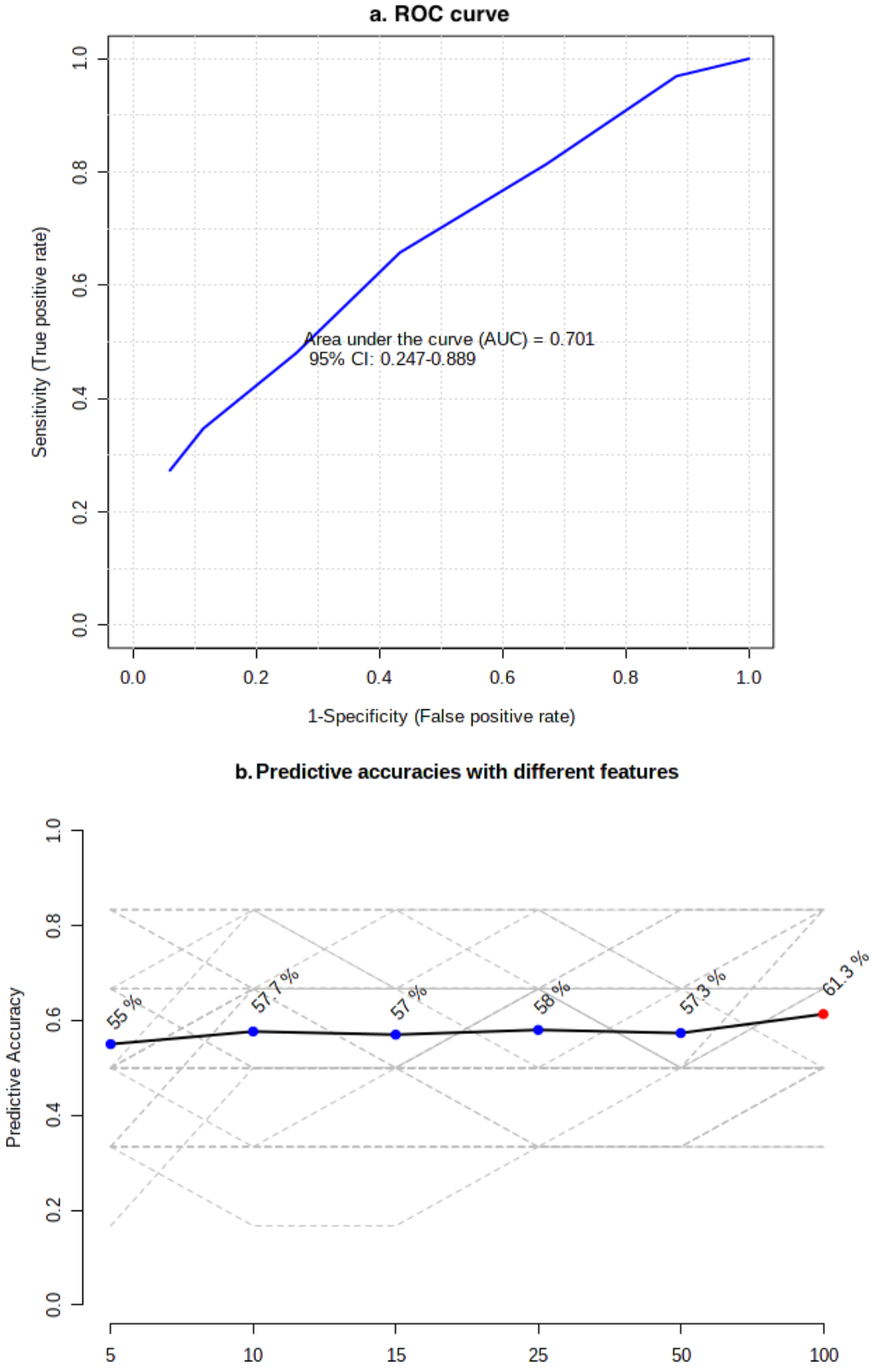
Cognition	Gender	X^2 =1.000	*P* =0.620	X^2 =3.600	*P* =0.206
	BMI	X^2 =0.234	*P* =1.000	X^2 =0.000	*P* =1.000
	Visit	X^2 =1.111	*P* =0.574	X^2 =1.067	*P* =0.587

*: Degree of freedom =1; sample size (N) =18; 2-sided alpha level of 0.05 significance

6.2.4 Receiver operating characteristic (ROC) curve analysis for RS and NRS groups to blueberry powder

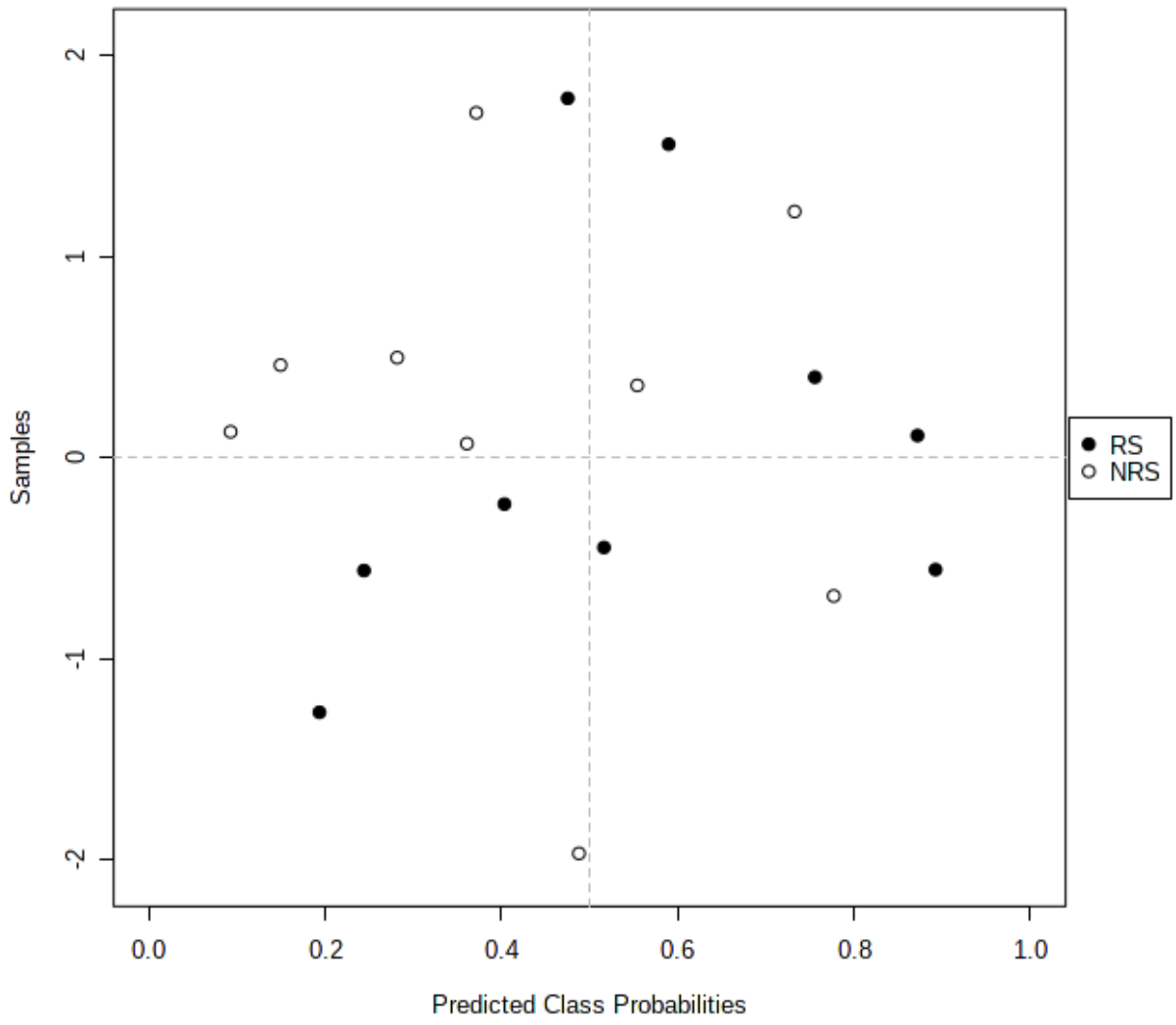
There were more ion features increased in intensity following blueberry powder intervention (85 %) compared to whole blueberry intervention (57 %) as shown in Chapter 5. Therefore, only the blueberry powder group was chosen for the ROC analysis in order to identify predictive biomarkers for response. Responder (RS) and non-responder (NRS) in response to blueberry powder intervention underwent biomarker analysis in MetaboAnalyst 5.0. Baseline data only was used to identify predictive features as any predictor of response should be found pre-intervention. **Figure. 6.10a** shows the ROC curve for a random forest classification model created using a subset of 100 features selected by the random forest ranking. The area under the curve (AUC) value was 0.701 and 95 % confidence interval (CI) was 0.247 - 0.889. Meanwhile, the predictive accuracy for the variables was 61.3 % **(Figure. 6.10b).** The predicted class probability for each RS and NRS sample based on AUC is shown in **Figure. 6.11.** The predication results demonstrated that in the 9 RS samples, 5 were predicted correctly, and in the 9 NRS samples, 6 were predicted correctly. The features were further ranked by their contribution to discriminate between RS and NRS as shown in **Figure. 6.11.** There were 79 % of total features higher in intensity for NRS relative to RS. Feature intensity in RS group was also indicated whether was higher or lower relative to NRS group in **Figure. 6.12.**

Figure. 6.10 The ROC curve (a) and the predictive accuracy (b) discriminating between RS and NRS



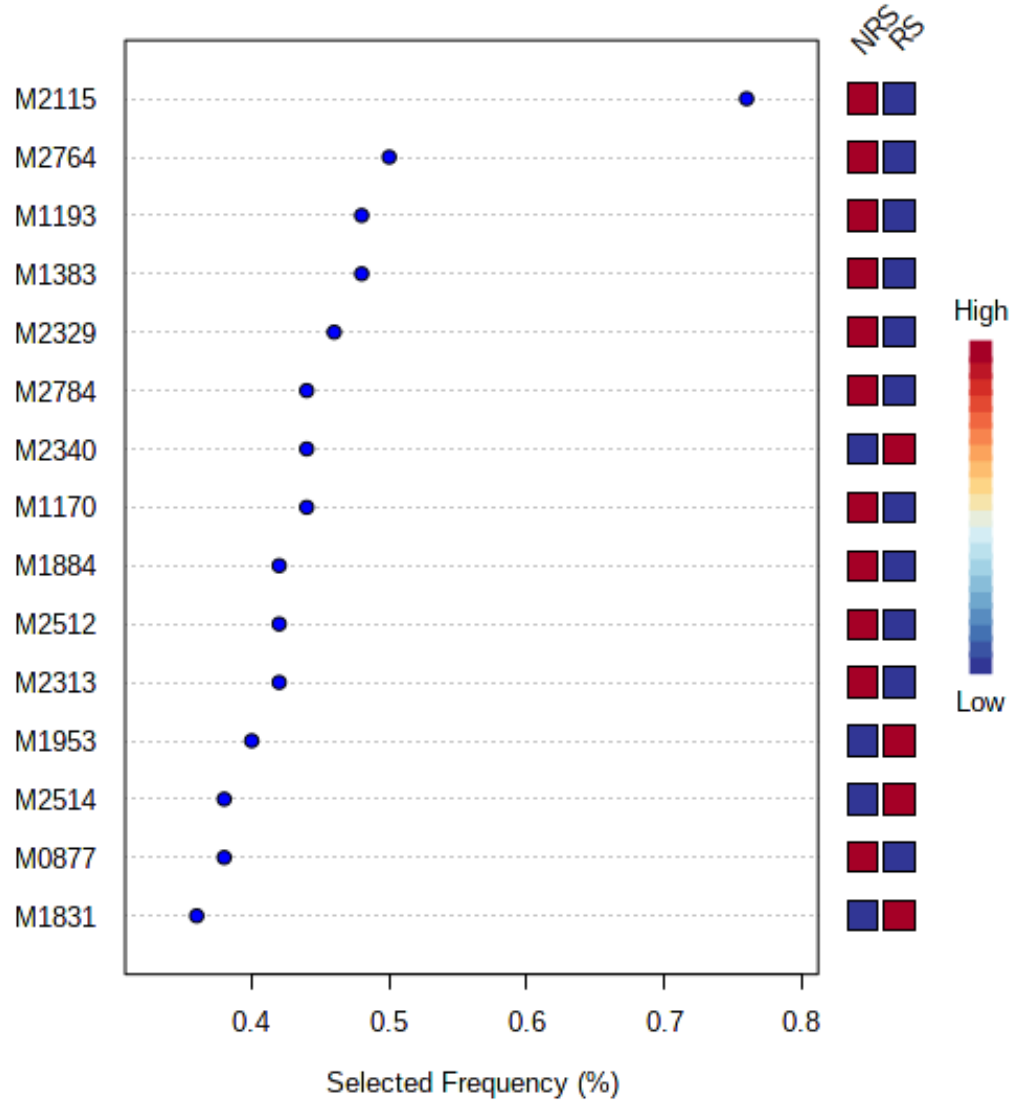
*a. the corresponding sensitivity and specificity indicate the true positive rate and 1-false positive rate respectively for the prediction and AUC represents the degree of discriminability of the RS and NRS; b: the predictive accuracy changes with the number of features for permutation in the RF model

Figure. 6.11 Predicted class probabilities for RS and NRS



*The image shows the prediction probabilities of RS and NRS classes using Monte-Carlo cross validation (MCCV) analysis for each sample using the best classifier (based on AUC); 4 RS samples and 3 NRS samples were classified to the wrong groups

Figure. 6.12 Important features ranked by random forest classification



*The selected frequency of the top features was based on random forest (RF) built-in method; importance of features were presented with selected frequency (%) in the predictive model from 0.3 to 0.8 with the right axis indicating the relative concentrations of the corresponding metabolite in each group.

As shown in **Figure. 6.12**, features were ranked based on a RF model in descending order of importance. However, actual intensities for those ranked features were higher in NRS group and thus cannot be used to predict response. There are some features with higher intensity in RS but all ranked low in the RF model and had low AUC values. Therefore, **Table. 6.5** summarises the important features identified from the ranking and also a number of features that showed higher intensity in RS relative to NRS. The performance details of ROC curve analysis are also summarised for the selected features including area under individual ROC curve, sensitivity and specificity, T-statistics and Log2 fold change (FC).

Table. 6.5 ROC analysis details for individual features

Feature ID	m/z	AUC[a]	Cut-off value[b]	Specificity (%)	Sensitivity (%)	*P* value	Log2(FC)[c]
M2115	340.059	0.951, 95 % CI (0.815 -1)	- 0.248	100 %	90 %	0.002	5.307
M1193	184.085	0.926, 95 % CI (0.741 -1)	- 0.116	90 %	90 %	0.002	2.454
M1383	435.247	0.926, 95 % CI (0.753 - 1)	0.130	90 %	90 %	0.001	2.228
M2340	152.059	0.926, 95 % CI (0.728 - 1)	- 0.145	100 %	80 %	0.004	1.703
M2514	296.077	0.914, 95 % CI (0.722 -1)	0.289	90 %	90 %	0.002	3.675
M1170	144.042	0.914, 95 % CI (0.747 -1)	0.205	90 %	90 %	0.002	1.552
M2313	320.075	0.901, 95 % CI (0.679 - 1)	- 0.409	100 %	80 %	0.005	1.811
M1953	511.278	0.901, 95 % CI (0.716 - 1)	- 0.523	90 %	80 %	0.001	4.150
M2512	302.124	0.889, 95 % CI (0.697 - 1)	0.241	90 %	90 %	0.014	1.684
M0877	317.148	0.889, 95 % CI (0.667 - 1)	- 0.185	100 %	80 %	0.002	1.499
M2764	280.143	0.889, 95 % CI (0.704 - 1)	0.178	90 %	90 %	0.009	1.709
M2784	151.029	0.889, 95 % CI (0.670 - 1)	0.080	100 %	80 %	0.018	1.271
M1884	405.200	0.840, 95 % CI (0.598 - 1)	0.280	90 %	90 %	0.037	1.097
M2075	170.069	0.728, 95 % CI (0.457 - 0.914)	- 0.291	70 %	80 %	0.113	-0.919
M0323	244.997	0.605, 95 % CI (0.304 - 0.864)	- 0.376	80 %	60 %	0.650	-0.952
M1950	161.094	0.556, 95 % CI (0.222 - 0.815)	- 0.496	80 %	60 %	0.889	-1.009
M2579	1310.866	0.543, 95 %CI (0.247 - 0.827)	- 0.420	90 %	40 %	0.367	-2.261

[a] : AUC: area under the curve with 95 % of confidence interval;
[b]: Cut-off value indicates the criterion value of feature intensity that predicts RS;
[c]: Log2(FC): log transformed fold change in intensity; FC: fold changes were the ratio of ion intensity in NRS to RS

The cut off value here refers to a threshold for individuals being identified as either NRS or RS when the intensity level is lower than the cut of value. The corresponding sensitivity and specificity indicate the true positive rate and 1-false positive rate respectively for the prediction and AUC represents the degree of discriminability of the RS and NRS [259]. The higher these values are the better the prediction is. As shown in **Table. 6.5,** on the basis of cut-off values ranging from - 0.523 to 0.289 for predicting RS versus NRS using important features (AUC > 0.800) based on the RF method and the model exhibited sensitivities ranging from 90 % to 100 % and specificities ranging from 80 % to 90 %. However, the actual intensities for all these features were higher in NRS in relative to RS (Log2 fold changes 1.097 - 5.307), therefore these highest ranked features by RF model could not be used to predict RS.

For individual features that presented higher intensity in RS compare to NRS, there were smaller AUCs ranging from 0.543 to 0.728, and moderate sensitivities ranging from 70 % to 90 % and smaller specificities ranging from 40 % to 80 %. M2075 and M3203 presented slightly higher AUCs but the intensity differences between RS and NRS were not significant. Therefore these features did not present the ability to predict RS. **Figure. 6.13 and 6.14** illustrate the ROC curves and intensity bar graphs for selected features.

Figure. 6.13 ROC curve details for selected features with higher intensity in RS

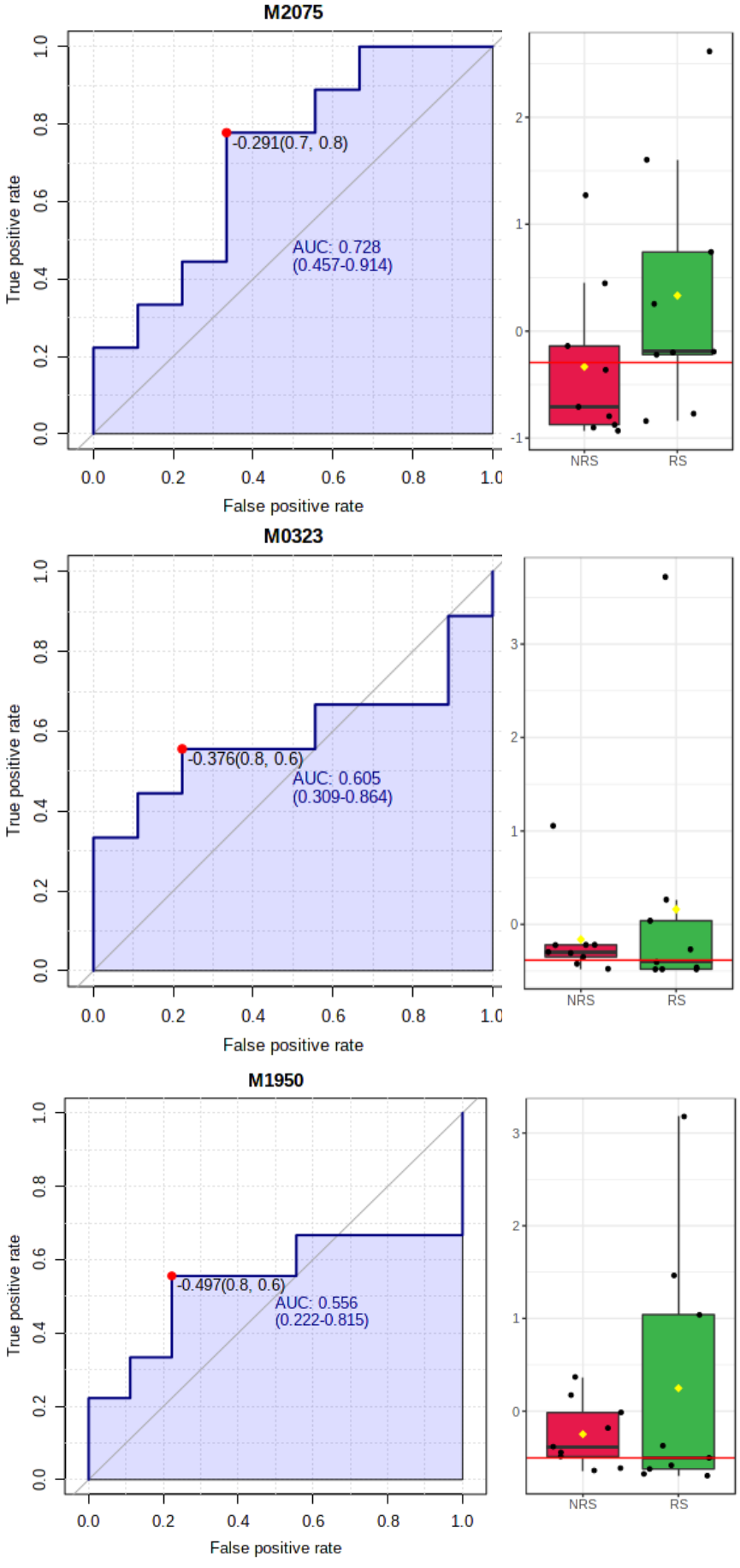
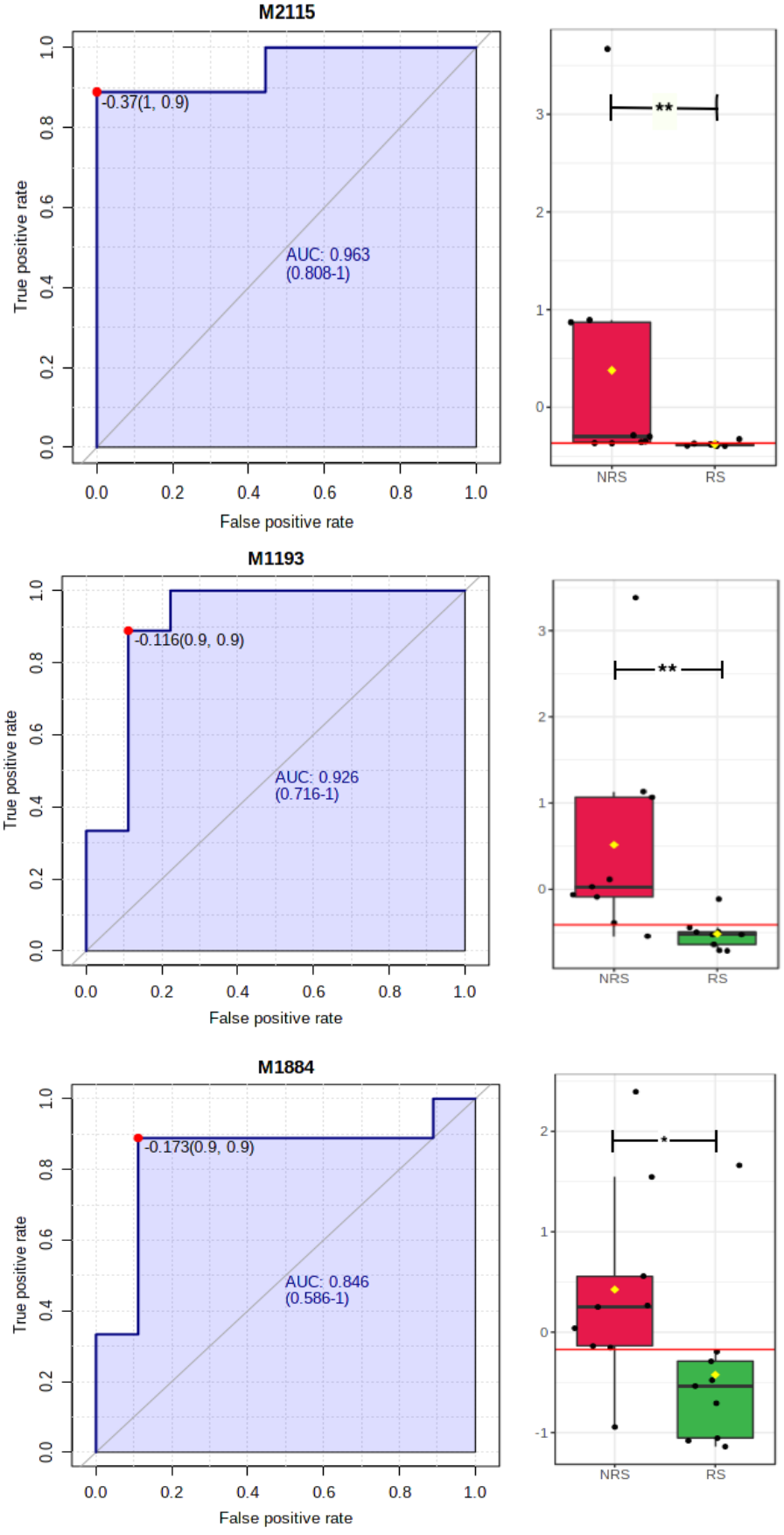


Figure. 6.14 ROC curve details and intensity bar graphs for RF highest ranked features



*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$

6.2.5 Putative annotations of important features predicting RS and NRS

Putative annotations for above features with KEGG compound identifiers (with C letter prefix) and putative KEGG biological pathways where available **(Table. 6.6).** Four putative annotations were made for features that had relatively higher intensity in RS including N-Carbamoylputrescine (M2075), 3,5-dimethoxy-2-methylphenol (M0323) Dihydrothymine (M1950), and Ganglioside GA1 (M2579). However, the features could not be used to predict RS because of low ROC analysis scores.

Table. 6.6 Annotated features from ROC analyses

Features ID	m/z	AUC, 95 % CI*	Mass error (ppm)	Formula	Putative annotation	KEGG ID and pathway
M2115	340.059	0.951, 95 % CI (0.815 -1)	- 3.120	C12 H16 N6 S3	N/A	N/A
M1193	184.085	0.926, 95 % CI (0.741 -1)	1.080	C8 H12 N2 O3	N/A	N/A
M1383	435.247	0.926, 95 % CI (0.753 - 1)	- 2.110	C21 H33 N5 O5	N/A	N/A
M2340	152.059	0.926, 95 % CI (0.728 - 1)	0.320	C7 H8 N2 O2	N/A	N/A
M2514	296.077	0.914, 95 % CI (0.722 -1)	- 0.100	C9 H17 N2 O7 P	N/A	N/A
M1170	144.042	0.914, 95 % CI (0.747 -1)	1.490	C6 H8 O4	2,3-Dimethylmaleate	C00922, map00760 Nicotinate and nicotinamide metabolism; map01120 Microbial metabolism in diverse environments
M2313	320.075	0.901, 95 % CI (0.679 - 1)	1.680	C12 H16 O10	N/A	N/A
M1953	511.278	0.901, 95 % CI (0.716 - 1)	- 2.170	C27 H37 N5 O5	N/A	N/A
M2512	302.124	0.889, 95 % CI (0.697 - 1)	4.900	C13 H25 N2 P3	N/A	N/A
M0877	317.148	0.889, 95 % CI (0.667 - 1)	0.750	C14 H23 N O7	N/A	N/A
M2764	280.143	0.889, 95 % CI (0.704 - 1)	1.030	C14 H20 N2 O4	N/A	N/A
M2784	151.029	0.889, 95 % CI (0.670 - 1)	N/A	N/A	N/A	N/A
M1884	405.200	0.840, 95 % CI (0.598 - 1)	3.540	C22 H33 N O2 P2	N/A	N/A
M2075	170.069	0.728, 95 % CI (0.457 - 0.914)	0.790	C7 H10 N2 O3	N-Carbamoylputrescine	C00436, map00330, Arginine and proline metabolism; map01100, Metabolic pathways
M0323	244.997	0.605, 95 % CI (0.304 - 0.864)	1.540	C4 H3 N7 O4 S	3,5-dimethoxy-2-methylphenol	N/A
M1950	161.094	0.556, 95 % CI (0.222 - 0.815)	N/A	N/A	Dihydrothymine	C00906
M2579	1310.866	0.543, 95 % CI (0.247 - 0.827)	- 0.550	C69 H120 N10 O10 P2	Ganglioside GA1	C06136

*: AUC: area under the curve, 95 % confidence interval

6.3 Discussion

6.3.1 Principal findings

To our best knowledge, this is the first known study characterising inter-individual variations based on clinical endpoints assessing both vascular and cognitive health comparing the supplementation of dietary blueberry and blueberry powder. In the current study, 31 % - 71 % of participants presented improved responses and 29 % - 66 % of participants presented worsened responses for the vascular and cognitive endpoints. A higher proportion (71 %) of participants presented improved responses to plasma nitrite concentration and self-rated alertness and these high proportions were only observed following the whole blueberry intervention. There were also more participants (3 % - 16 %) showing no changes in cognitive endpoints that assessed aspects of working memory, episodic memory, attention, and mood compared to vascular endpoints. In regards to the inter-individual differences in response levels, plasma nitrite and triglyceride concentrations and mental fatigue were observed to have the most dynamic changes. For example, plasma nitrite showed responses ranging from 525 % increase to 141 % decrease following the blueberry interventions. A dynamic response level from - 114 % to + 96 % for mental fatigue was observed compared to other cognitive endpoints following the interventions. There was no association found between participants characteristics (gender and BMI) or the study visit and the response.

No consistent responders (RS) across multiple endpoints were identified in the current study. When characterising the inter-individual differences shown in the assessed endpoints, only a small number of RS (2 out of 9) demonstrated consistent responses to the assessment of the total number of endpoints following both of the blueberry interventions, but the response was not shown across both vascular and cognitive functions. This finding could be expected as the assessed endpoints are in different biological systems [260]. Among 52 assessed endpoints, the consistency of improved responses was not high for most of the identified RS and no consistent responses were shown for vascular and cognitive endpoints. Only 3 participants (S14, S15 and S37) showed the most consistent response as they all were identified as RS in the assessment of 44 % of endpoints. However, their responses to cognitive and vascular

functions were not consistent. The comparison of response across more similar or closely associated endpoints may be necessary to observe a consistent response for individuals. Furthermore, most of the ion features presented higher intensity in NRS (79 %) relative to RS and there was no metabolic predictor for RS identified in the study. Therefore, the identified responses across clinical endpoints in the study were random.

6.3.2 Inter-individual variations in the response of clinical endpoints

For the endpoints assessing vascular function, non-invasive assessment of vascular function including PWV, BP and lipid profiles have been reported previously with SD ranging from 1.2 - 7.7 but reached up to 23.6 for plasma nitrite concentrates following either blueberry powder or juice interventions [16]. Similar to these findings, in the current study, the plasma nitrite response demonstrated the highest inter-individual variation (- 141 % - + 525 %) following blueberry interventions compared to other vascular assessments. The highest trend of intervention effect shown on nitrite level may also explain this observation. Plasma nitrite (NO_2^-) is converted from nitrate (NO_3^-) and body nitrate (NO_3^-) stores can be boosted via diet (e.g. from leafy vegetables and beetroot) quickly and approximately 25 % of NO_3^- can be absorbed and concentrated in the saliva to be converted to NO_2^- [261]. Therefore, apart from the potential effects following blueberry interventions, the dynamic conversions for nitrite in the circulating plasma can account for the fluctuations shown for the nitrite response levels. Pulse wave velocity (PWV) and blood pressure reductions have been shown to associate with NO_3^- - NO_2^- - NO pathway, and because of that it is logical to speculate similar response variations in PWV, SBP, and DBP [262]. However, the current study observed relatively moderate response variations for PWV (- 51 % to + 31 %) and small variations for SBP (- 20 % - + 17 %) and DBP (- 34 % - + 16 %) compared to nitrite. In order to further explore the consistency of response between participants for PWV, BP and plasma nitrite level, the current study compared participants for their response but no consistent response was identified. For instance, following the whole blueberry intervention, participant S25 showed improved response across PWV, SBP, and plasma nitrite endpoints but not DBP and participant S2 showed improved response across PWV, SBP, DBP but not plasma nitrite. No other participants were identified with a consistent response across vascular endpoints including PWV, SBP,

DBP, plasma nitrite, glucose and lipids. Therefore, no consistency was observed for the response across the vascular endpoints.

For the endpoints assessing cognition and mood, there were also larger proportions of participants presenting with no changes in the cognitive endpoints relative to other endpoints. The susceptibility of participants in response to different assessments may lead to different extent of response but it's worth noting that the cognitive tasks for the current study were selected on the evidence of previous sensitivity to nutritional interventions [204, 263]. The complex regulatory mechanisms of cerebral blood flow and cognition may explain the lack of intra-individual changes on cognitive performances following the interventions [156]. It's interesting to notice that following the Mediterranean diet, the inter-individual difference in response to alert score was also higher (+ 6.93 of response change) compared to content (+ 5.35 of response change) and calm (- 0.28 of response change) based on the visualised rating scale [95]. The current study observed a similar trend for alertness (- 70 % - + 59 %) compared to content (- 41% - + 41%) and calm (- 39 % - + 60 %). The inter-individual variations were not limited to mood assessment though. The inter-individual differences in cerebral blood flow dynamics, neural correlates, heritability, physical, social environment, and personality all constitute complex associations between individual response and specific cognitive domain phenotype. The review in Chapter 2 demonstrated a larger variation across studies assessing memory (SD of 2.2) relative to executive function (SD of 0.08 - 0.16) following either blueberry powder or blueberry concentrate intervention [77, 93, 94]. However, in the current study, working memory, episodic memory and attention all demonstrated moderate to high variations (- 39 % - + 61 %) and only participant S36 showed improved response across working memory, episodic memory and attention but not mood assessments after the blueberry powder intervention. No other participants had a consistent response across cognitive endpoints so that the response in cognitive abilities also seemed random across domains.

Unfortunately, there are limited studies characterising response in vascular and cognitive endpoints at the individual level. Most studies reported average response for group effects only and the variation for response were reported utilising mostly SD and coefficient of variation [88, 89]. No other study was found that has compared the

individual response across clinical endpoints. The current study assessed response variations at the individual level for both the vascular and cognitive endpoints following the blueberry interventions. A high range of inter-individual variations was observed but no consistent response of individuals was identified across the assessed endpoints. It is worth noting that a trend of improved plasma NO_2^- level was observed following the blueberry intervention (Chapter 5) and more people responded with high levels in the endpoint of NO_2^-. Therefore, if significant treatment effects were observed with longer duration and/or larger dosage, the responses might show more consistency across endpoints. It is also logical to presume that the response is random if no consistency was found even a significant treatment effect was shown following the same type of intervention. Therefore, the inter-individual variation may be due to the fact of either the absence of treatment effect or the random effect and similar studies to the current one may be necessary for further exploration.

6.3.3 Predictors of response

The inter-individual variations in response to diet have been widely studied complementing the investigation of metabolomic differences induced by nutritional interventions. The first step in characterising the response is important. Characterisation of individuals that could benefit the most from a dietary intervention will help to target the phenotype in favour of the intervention. Currently, most studies characterised response (RS/NRS) including the development of a model or score based on the health outcomes assessment or the combination of metabolomics, genomics, gut metagenomics, body composition, and/or glycaemic responses for predicting response variations [89, 264-266]. Due to the development of '-omic' technologies, most dietary interventions have been phenotyping participants for their genotypes, plasma, urine metabotypes, and gut microbiome or at least one of these factors [89, 264, 265]. A number of interventions also utilised quartiles or tertiles to characterise high or low response (RS/NRS) [267-269] or used a threshold number of improved level (e.g. ≥ 5 % compared to baseline) to report high response in the assessment of clinical endpoints [270].

The INRA POSITIVe COST action has addressed inter-individual variations in bioavailability and physiological responses to consumption of plant food bioactives in

relation to cardiometabolic endpoints across Europe [101]. One randomised controlled trial investigated the metabotype in order to explain the inter-individual variability in the assessment of cardiovascular biomarkers following pomegranate polyphenol extracts [257, 271]. The microbial-derived urolithin metabotype UM-B was shown to solely associate with individual improvement in the response of blood lipid profile including total cholesterol (- 15.5 ± 3.7 %) and LDL - cholesterol (- 14.9 ± 2.1 %) [271]. Although the individual response was not described, the metabotype could be used to predict RS that could benefit from pomegranate supplementation.

Another study investigated the lipid metabolome in response to dietary interventions enriched in unsaturated fatty acids and a metabotype consisting of triacylglycerol, phosphatidylcholine lipid and apolipoprotein B fractions was identified to discriminate among the 3 participants [272]. Their study utilised a targeted investigation to identify lipid metabolic phenotype in an acute postprandial manner but with a very small sample size (n = 3), nevertheless if a large number of participants were included then the measurement of metabotype could be reproducible.

In a similar approach, Garcia-Perez *et al.* developed a prospective dietary metabotype score (DMS) to directly map individual urinary metabolites to differentiate metabotypes [273]. A ranking was used with DMS to further classify individual responses into either a healthy or unhealthy diet based on WHO guidelines [273]. Their DMS score consisted of nutrient responsive metabolites including hippurate, 3-methylhistidine, carnitine that derived from intake of specific foods. When the metabotypes following the highly controlled healthy or unhealthy diet were profiled, other individual's metabotypes in the DMS score could be used to predict the diet they were adhering to. A significant association between urinary metabotypes and glycaemic and lipid profile was shown but the variation was not quantified in their study. Compared to their work, a DMS score could not be applied in the current study as only one unknown feature was found that significantly increased in intensity (m/z 233.163) following blueberry interventions and no metabotype predicting RS was found that would enable the development of a similar ranking scheme. Both their study and the current characterised urinary metabolites in order to identify RS that could present different metabolomic profiles but the current study could not identify neither one or a group of predictive metabolites that could distinguish different responses at the clinical

endpoints. The current study did not measure blueberry polyphenol metabolites and this may have been beneficial to improve metabotyping of response as demonstrated by Rodriguez-Mateos *et al*. that 21 plasma metabolites of blueberry anthocyanins were observed to correlate with improved endothelial function assessed by flow-mediated dilation (FMD) chronically [189].

Nevertheless, currently the in-depth characterisation of inter-individual variation in response to vascular and/or cognitive endpoint following blueberry-based interventions is still scarce. Rodriguez-Mateos *et al*. combined different human trials and animal models to provide evidence that blueberry anthocyanins metabolites in 10 healthy humans (e.g. gallic acids) were linked to cellular gene expression variation (FOXO3 towards an cardiovascular protective profile) [189]. The investigators found 74 % - 99 % of the variability in gene expression changes were explained by blueberry (poly)phenol metabolites including 3-O-ß-D-glucuronide and homovanillic acid using multivariate linear regression. The current study used a different approach of untargeted profiling and ROC analysis was utilised to explore the accountability of urinary untargeted metabolites for the inter-individual variability in response to vascular and cognitive endpoints. However, 79 % of features showed increased intensities in non-responder (NRS) group and thus cannot be used to predict response. Several features (i.e. m/z 170.069, 244.997, 161.094) demonstrated higher intensities in RS relative to NRS but the differences in the intensities were not significant and the area under the curves (0.56 - 0.72) and specificities (40 % - 60 %) did not achieve a high enough level to make them predictive for RS.

6.3.4 Potential factors influencing inter-individual variation

The influence of visit order (treatment sequence) on individual response was explored and no association was found. The inter-individual variation in the baseline level has been considered as fixed factor and covariate in the analysis of intervention effects on assessed endpoints and the baseline levels of each endpoint could also influence the individual response following the intervention. Even though the response level calculated in this chapter was presented as percentage change from baseline, participants with relatively higher cognitive level at the baseline were unlikely to achieve higher cognitive response further following a dietary intervention due to

‘ceiling effects’ [274]. Similarly, participants with relatively lower levels of vascular dysfunction risks, such as a low BP, maybe unlikely to achieve a reduction further [275]. Therefore, a larger sample group covering participants with both low and high health risks at the baseline may be necessary to investigate the association between baseline health risks with the response of vascular and cognitive endpoints.

A large range of factors including SNPs, bioavailability and gut microbiota are known to influence the biological response [101]. Inter-individual variations induced by genetic mutations including ABCG5, NPC1L1, ABCA1, APOE that modulate response to a range of diet and food bioactive (i.e. flavonoids, plant-sterols, and Mediterranean diet) have been shown [257, 258, 276]. For instance, after ingestion in the gastrointestinal tract, enzyme catechol-O-methyltransferases (COMT, AA or GG genotype) at the genetic level is involved in anthocyanins metabolism and could play a role to induce variability in response to the intervention [277]. A range of polymorphism variations have been shown to associate with clinical phenotypes directly. For example, the COMT genetic variation (rs4646312) was shown to involve in the biological pathway of presynaptic depolarization and calcium channel opening across neuronal synapsis [278]. The SNP was reported with increased risks developing schizophrenia [279]. Gene ITPR1 (rs6798160) was shown to be associated with reduced levels of blood pressure via the NO pathway and maintenance of platelet homeostasis [280]. The genetic polymorphism variations such as GRIA1 (rs12153160), FADS1 (rs174546), APOE (rs429358), PTPN11 (rs11066301, rs11066320) that could be used to partially explain for the variations affecting responses to dietary intervention and physiological homoeostasis via other biological pathways of i.e. synapses protein-protein interactions, lipid metabolism regulations, molecule signal transductions, and vascular cell surface interactions [281].

The variation in the bioavailability of blueberry polyphenols between individuals can lead to inter-individual variations in response to vascular function and cognition [190, 282]. Generally, urinary and plasma anthocyanins metabolite levels are found low and the variation in urinary levels was between 0.009 ± 0.002 and 0.79 ± 0.90 % of the dose consumed, as measured in previous studies [45, 283]. However, some associations were found for polyphenol bioavailability with the clinical endpoints. For vascular function, Jin *et al.* have shown no improvement in vascular reactivity targeting

healthy participants supplementing with anthocyanin-rich blackcurrant juice and hypothesized that was due to the low concentrations (20 %) in juice and thus low bioavailability [284]. Rodriguez-Mateos *et al.* observed an improvement in endothelial function following acute blueberry juice intake related to the peak abundance of plasma metabolites vanillic acid, benzoic acid, hippuric acid, hydroxyhippuric acid and homovanillic acid, that may partially predict the improvement in FMD [285]. Compared to their work, no improvement in the endpoints was shown in the current study, but metabolomic profiling exhibited 85 % and 57 % of features with increased intensities following whole blueberry and blueberry powder interventions separately but only 21 % features increased intensity in RS. The inter-individual variations in response to the interventions could not be explained by excreted hippuric acid and (R)-3-(4-Hydroxyphenyl)lactate identified from the current interventions as these were not validated for distinguishing between RS and NRS in the current study.

For the association between polyphenol bioavailability and cognitive function, Ammar *et al*. recently reviewed acute and chronic effects of polyphenol-rich supplementations on cognitive function and brain health in young and middle-aged adults [286]. An acute and/or chronic intervention including a range of 70 - 747 mg polyphenols with bioavailability rates of ≥ 30 % may be necessary to improve brain and cognitive function assessing working memory and mental fatigue [286]. Compared to their findings, the current study supplemented 220 - 288 mg blueberry polyphenols but no improvement was shown for working memory and mental fatigue with response level ranging from - 58 % - + 74 % and - 50 % - + 74 % respectively, which may suggest less than 30 % of average bioavailability rate among participants in the current study. Singh *et al.* also suggested the difference between polyphenols or their metabolites that can cross blood-brain barrier (BBB) toward neuroprotective action [287]. Specifically, lipophilic and methylated flavonoids (e.g. naringenin and hesperetin) can readily cross the BBB compared to their phenolic counterparts [287]. Here, the putative annotation of metabolites discriminating between RS and NRS did not observe blueberry polyphenols and/or their derivatives and there may also be a low bioavailability of phenolic compounds crossing BBB in the peripheral blood circulation.

Gut microbial metabolites could explain inter-individual variation in the response to dietary intervention also [257]. Manach *et al*. have summarised potential factors

including gut microbiota that are responsible for inter-individual variability in the bioavailability and the physiobiological responsiveness to the consumption of plant food bioactive enriching in polyphenols, carotenoids, and glucosinolates in relation to cardiometabolic health [257]. After ingestion, enzymes including β-glucosidases at the small intestine or the gut microbial level could induce variability between individuals affecting anthocyanin metabolisms [53, 54]. For direct association of gut microbial products with clinical endpoints, one dietary intervention found that the gut microbial metabolite 3-(4-hydroxyphenyl)propionic acid explained 8 % of the reduction in fasting plasma total cholesterol levels among healthy adults following an intervention with Aronia berry extract for 12 weeks [288]. Another study found lower levels of gut microbial metabolites short chain fatty acids (SCFAs) and butyrate in Parkinson disease (PD) to be significantly associated with poorer cognition and low BMI via energy homeostasis modulations or gut-brain pathways involving immune, endocrine, neural and humoral routes [289, 290].

Apart from above factors, age, BMI, sex, physical activity, smoking, *Helicobacter pylori* infection, blood lipids, medication intake have also been suggested to be responsible for inter-individual differences in the response to dietary intervention [101]. The current study did not find associations of sex, BMI with response, still, the relative importance for each factor to overall inter-individual differences is unknown. Those factors adding to the diversity and activity of gut microbiota and the metabolising phase I, II enzymes along with genetic variations (i.e. SNPs) all could affect the bioavailability in dietary polyphenols and thus play important roles in individual response of clinical phenotypes [257].

6.3.5 Limitations and implications

The mapping for RS predictive features was built using urinary metabolites alone. Nevertheless, metabolites have been circulating in plasma prior to being excreted in the urine and those metabolites could represent the homoeostatic signatures of metabolism [273]. Only positive ion mode was analysed using LC - MS and the platform has certain limitations too that it has high sensitivity but requires metabolites to be efficiently ionized to be detected and tends to be more susceptible to variability [291]. In the current study, quartile division basing on the response level calculated at

the endpoints was utilised to quantify the number of responder (RS) and non-responder (NRS) following the dietary blueberry interventions. Unfortunately, there was no consistent response identified, thus the characterisation of response was not differentiated at the metabolomic level between RS and NRS. This could be partially explained by a relatively small sample size for RS and NRS groups (9 each) and the inter-individual variation at the baseline. Therefore, the predictive features should be explored and validated in a larger population so that a larger number of response and a wider range of individuals can be classified as RS and NRS. However, it is worth noting that previous therapeutic studies have applied a similar quartiles calculation to characterise different responses for non-diabetic essential hypertensives following short-term thiazide treatment [217], or for physical fitness following training exercise [218] or for coronary syndrome patients following an antiplatelet therapy [219]. Furthermore, a genetic mapping for nutrient and physiological responsive variants may help to characterise RS and NRS in an intervention based on the genotypic feedback. A recent trial (Food4Me) has screened nutrient-responsive genes for participants to characterise participants for personalised dietary plans [292].

The study here did not assess other factors, e.g. ethnicity, that may induce variations in the bioavailability of (poly)phenol metabolites from dietary interventions [293]. The ethnicity data was collected but there was not enough non-white participants to allow an assessment of its effects on the inter-individual variation. The study consisted of young adults only (97 % of participants being less than 42 years old) and the association of BMI, gender or the study visit order with response was not significant. The compliance of participants was assessed using a 1-day food diary that confirmed 92 % of compliance, the presence of blueberry metabolites (e.g. hippuric acid) could have been used to confirm the compliance but the metabolite intensity was not significantly higher compared to the baseline. Gut microbial metabolites of blueberry (poly)phenols could induce variations in response following the dietary interventions [257]. Human gut microbes have also been explored that was combined with plasma and urinary metabolome in the studies of inter-individual variations in response to dietary polyphenols [293-295]. The non-invasive method of collecting faecal specimens for gut microbial analysis have been widely used [273]. To complement the investigation in the future, gut microbial metabolites could be used for studying inter-individual differences in response to absorption, distribution, metabolism and excretion

(ADME) of blueberry or blueberry polyphenol interventions in combination with other '-omics' methods.

In conclusion, the novelty of the current study is the assessment of the consistency of individual response across endpoints and demonstrated that no consistency in response of vascular and cognitive endpoints was found. To our best knowledge, this is the first known study showing inconsistency of individual response across and within vascular and cognitive endpoints. The current study could not identify urinary metabolites for predicting RS in the understanding of the inter-individual variations manifested through vascular and cognitive endpoints. However, there remains many gaps in the knowledge. More approaches characterising responses in human interventions studies, data coupling with genotypic and lifestyle behaviour feedback will be needed to unravel how they contribute to inter-individual variation in the physiological responses.

Chapter 7. General discussion

7.1 Introduction

The aim of the thesis was to characterise inter-individual variations in response to vascular and cognitive endpoints following the consumption of blueberries. Within each chapter, a range of relevant issues have been discussed, therefore, the following sections mainly focuses on the principal findings from the current thesis in the context of existing studies, limitations and potential implications for future research work.

7.2 Principal findings

7.2.1 Effect of blueberry interventions on vascular function

The findings on vascular function are in disagreement with most non-acute blueberry interventions investigating outcomes including blood pressure (BP) and lipids, arterial stiffness and nitric oxide (NO) [69, 70, 72, 73]. Even though BP is commonly evaluated as an indicator of vascular function, studies exploring the ingestion of berries or their polyphenol bioactive compounds are not unequivocal on BP [16]. There have been interventions supplementing freeze-dried blueberry powder (22 - 45 g, 4 - 8 weeks), blueberry juice (480 ml, 8 weeks) that have reported either no effects [71-73] or improved effects on SBP, DBP [69, 70, 74]

A trend for increased NO_2^- levels was observed following the blueberry and blueberry powder interventions in the current study. The NO synthesis system has been shown to benefit both peripheral and cerebral peripheral blood flow (CBF) [191]. Anthocyanins, a rich polyphenol compound found in blueberry has been associated with blood pressure modulation by 3 predominant mechanisms including the regulation of endothelial nitric oxide NO synthases (eNOS) expression and activity to increase endothelial derived NO (a vasodilator for improving BP), the prevention of NO oxidative damage and radical-induced NO conversion such as the reaction caused by NADPH oxidative, or the inhibition of the angiotensin-converting enzyme (ACE) activity, endothelin-1, and thromboxane via inhibition of the cyclooxygenase (COX) pathway to eliminate synthesis of vasoconstricting molecules [296, 297]. Clinical trials with dietary polyphenols have also shown benefits on vascular function, with one of the proposed mechanisms via the NO_3^- - NO_2^- - NO pathway [298]. Despite these, the

current study found no significant effect of the interventions on SBP, DBP, and plasma nitrite (NO_2^-). During NO synthesis, the production of NO by iNOS (an NOS isoform) has been shown to be maintained for a longer duration and found in much higher concentrations in the cell than from the other isoforms of NOS [299]. So any regulatory effects on NO systems due to blueberry flavonoids could be beneficial over a longer term supplementation in addition to the acute (1 - 6 h) postprandial mechanisms [299]. Therefore, a longer intervention duration may be needed for a beneficial observation on nitrite levels and manifestation to blood pressure.

However, one chronic blueberry intervention did not show an improvement in endothelial function and NO levels after receiving a blueberry drink (25 g freeze-dried powder) for 6 weeks [71]. Similar to the current study, blood samples were taken after fasting (approximately 12 h) following the blueberry ingestion, when Anthocyanins were possibly cleared from the circulation. Currently there is limited data of acute freeze-dried blueberry powder interventions investigating vascular function, the blueberry powder interventions reviewed in Chapter 2 were all longer-term interventions (22 - 50 g, 6 - 8 weeks) and have shown inconclusive results including the effect on NO levels [69-73]. Only one 6 h study supplied blueberry powder (equivalent to 240 - 560 g blueberry) and demonstrated an improvement in endothelial function in healthy humans within 1 - 6 hours after consumption [300]. Compared to blueberry powder interventions, there are several acute whole blueberry interventions (300 g, 24 h) that also showed positive results on improving peripheral vascular function but were from the same author [17, 18, 137]. Nevertheless, the vascular protective effects of whole fresh blueberry and blueberry powder supplementations have not been compared in a study before, despite the study duration.

The health status of participants could also impact the study findings. Previous blueberry interventions (22 - 45 g blueberry powder, 150 - 300 g whole blueberry equivalent) assessing adults with high vascular (dys)function risks such as MetS and hypertension ranging from 6 weeks - 6 months have reported an improvement in at least one of the outcomes (i.e. PWV, BP, lipids, NO levels) compared to either the control or the baseline [16]. On the contrary, blueberry interventions targeting generally healthy people have reported limited changes to outcomes. An exercise intervention combined with blueberry treatment (150 g) among healthy people for 4

weeks, demonstrated inconclusive evidence regarding the effect of blueberry treatment on vascular (dys)function risk factors, where the HDL-cholesterol level was increased in the blueberry group but the TAG level was reduced in the control group compared to the baseline [301]. It should also be noted that McAnulty *et al.* assessed a healthy group and observed improved arterial function assessed by augmentation index (Aix), although no improvement was shown for PWV [70]. There was also significant reductions to DBP following the blueberry supplementation compared to baseline, however an improvement was exhibited in a subset of hypertensive participants (N = 9), the blood pressure of overall cohort was not affected [70]. However, there was one study reporting positive findings on vascular health in healthy adults following blueberry consumption. The intervention supplementing 22 g of blueberry powder daily that contained 300 mg of Anthocyanins for 1 month to low-risk healthy adults demonstrated significantly improved both FMD and 24-h SBP, the benefits to the vascular system were shown in healthy adults following both acute and chronic blueberry consumptions [189]. However, this was also the first study known to show both improved SBP and endothelial function in young healthy adults after chronic blueberry interventions (1 month). Thus, in humans, populations with elevated baseline inflammatory, hypertensive, hyperlipidaemia, and endothelial (dys)function risk factors may be necessary to observe a risk-reduction of blueberry consumption.

7.2.2 Effect of blueberry interventions on cognition

For cognition and mood assessment, long term interventions (100 mg extract powder - 200 g whole blueberry equivalent, 6 - 24 weeks) have shown inconclusive results on cognitive performance as reviewed in Chapter 3 [29, 68, 77]. The potential effect of polyphenols and their impact on cognitive performances via alleviation of neuroinflammation, oxidative stress, neuroplasticity in animal models of neurodegeneration and aging have also been discussed in other systematic literature reviews [36, 299]. Despite this, the results on cognitive changes in current study are in disagreement with most acute studies assessing cognition and/or mood that mainly included elderly participants.

In addition to vascular effects, the timings of cognitive effects observed also appear related closely to the absorption and metabolism rates of the supplemented fruit and

its phenolic compounds [299, 302]. This may also explain why more acute studies following blueberry supplementations reported positive influence on cognition and mood compared to long-term studies. The timings of plasma polyphenol metabolites reaching peak intensities at 1 - 2 h and 6 h following blueberry consumption have been observed to correspond with 2 peak timings of cognitive effects [300]. This corresponding association between peak timings (1- 6 h) of other fruit flavonoids including quercetin, myricetin, and kaempferol and their metabolites have also been observed for the positive cognitive findings on memory, executive function and mood [299].

There were more acute blueberry interventions assessing cognition and/or mood. Currently there is correlation suggested between working memory and executive function but no correlations of the improvements for cognitive domains following fruit interventions were found [152]. There were more acute (25 - 30 g freeze-dried powder, 2 - 8 h) interventions reported positive cognitive findings on at least one of the aspects of episodic memory, executive function and mood compared to the chronic interventions. There have been no acute studies supplementing whole blueberry though. Blueberry flavonoids have shown acute vasodilatory effects by increasing both peripheral and cerebral blood flow (CBF) postprandial [191]. Greater CBF velocity usually corresponds with a lower rate of cognitive decline and lower risk of dementia in healthy ageing [299]. Glucose control, neuronal enhancement by inhibiting monoamine oxidase (MAO) for monoaminergic neurotransmission during cognitive performances and improved visual function have also been shown to associate extensively with blueberry or flavonoids supplementations acutely [299]. In the current study, all drawn blood samples and cognitive assessments were completed following overnight fasting (> 12 h), it's possible that the circulating metabolites, including polyphenol metabolites, remained in the CBF circulation and exerted any effect acutely and were eliminated from the body on the testing days. However, this was not confirmed as bioavailability was not measured in the current study.

It is also worth noting that most blueberry interventions have targeted older participants and reported positive findings on cognition or mood in participants with cognitive impairment or older participants with age-related cognitive decline. Improved executive function, working memory, functional mobility and episodic memory have

been reported in older participants (60 - 92 years) following either 100 mg extract - 24 g blueberry powder, 30 ml blueberry concentrate or 200 g frozen blueberry for daily consumptions during 6 weeks to 24 weeks [29, 68, 77, 93]. Only one study supplementing 25 g freeze-dried blueberry powder (equivalent to 148 g whole blueberry) daily to older people (68 - 92 years) with mild cognitive impairment has found no improvement in working memory and mood [94]. For healthy participants, cognitive improvements have been observed in an acute study (2 - 8 h) in younger adults (< 60 years) following blueberry interventions (25 - 30 g freeze-dried powder, 2 - 8 h) [303, 304]. The acute postprandial effect of blueberry may contribute to the difference as discussed in the previous section.

From the above, interventions targeting high-risk populations consistently demonstrated positive findings on the improvement to vascular health and/or cognitive health. The current study intended to include older individuals but only younger populations were recruited. Results from the current study are consistent with many blueberry interventions targeting low-risk populations. In conclusion, low-risk populations (< 60 years old and healthy) receiving either blueberry powder or whole blueberry (100 mg extract - 560 g whole blueberry equivalent) or concentrate (30 ml) supplementations both acutely (1 - 24 h) and chronically (6 - 24 weeks) have shown less effects compared to high-risk populations towards vascular- and cognitive-protective benefit following blueberry consumptions independent of the various dosage and duration implemented.

7.2.3 Difference in metabolite profile between whole blueberry and blueberry powder interventions

The metabolomic difference and inconsistency of response between whole blueberry (57 % of features increased intensity) and blueberry powder (85 % of features increased intensity) was also found here despite there being no interventional effects on clinical endpoints from both of the interventions. Although freeze-dried blueberry powder usually arises from 100 % whole blueberry, the technological processing of blueberry powder from the whole blueberry could also change the bio-accessibility and absorption of bioactives [78]. However, as both the metabolomic differences were not significant and either was any improvement in clinical outcomes, the current thesis

could not conclude if whole fresh or powdered blueberry is the most effective at improving vascular and/or cognitive health.

The present study observed individual differences in response to the whole blueberry and blueberry powder interventions. The high inter-individual variation may account for the non-significant changes in the results, despite the potential benefit of dietary blueberries for improving vascular and cognitive functions. In this study, the clinical endpoint of participants at baseline were in the normal, healthy physiologic range, therefore, the effects of whole blueberry or blueberry equivalent powder should be evaluated further on subjects with high health-risk levels.

The inter-individual variation following the blueberry interventions was further explored by untargeted metabolomics. Putative metabolites of phenolic acids were only detected post- blueberry consumption and blueberry powder consumption, which helped differentiate the untargeted metabolite profiles post- placebo consumption. Furthermore, putative metabolites hippuric acid and hydroxyphenolacetic acid, as found in our study showed differences in intensity between the two blueberry interventions, suggesting possible biokinetic differences post- whole blueberry and blueberry powder intakes. One acute dose-response study examined whether the bioavailability of freeze-dried blueberry powder (poly)phenols is intake - dependant in 9 healthy volunteers, where plasma hippuric acid was among the most abundant metabolites in different dosages consumed, followed by metabolites including 3- and 4- hydroxyphenylacetic acids [41]. In a blackcurrant juice post- dose study, the hydroxyphenylacetic acid derivatives were detected in a minor group of participants, whereas hippuric acid derivatives were detected in all participants, suggesting metabolically inter-individual differences in the circulating levels of polyphenol metabolites [305]. Among hippuric acid derivatives, hippuric and 4-hydroxyhippuric acid reached max amount post-intake at 2 hours, where 3-hydroxyhippuric acid reached max amount post intake during 8 - 16 hours, suggesting different metabolic pathways for different berry polyphenols [305].

Biokinetic differences are usually assessed after a single dose and were not measured in our study, no studies comparing biokinetics of metabolites between whole blueberry and freeze-dried blueberry powder have been compared, as far as we know. It is

possible that there could be differences between whole blueberry or freeze-dried blueberry powder polyphenols biokinetics in terms of Cmax in the current study. Individuals could metabolically respond differently with different phenolic metabolite pathways due to complex kinetic mechanisms. Therefore, inter-individual differences in metabolomic pathways could account in part for the inconsistent responses found across the endpoints following the blueberry interventions.

7.2.4 Consistency and prediction of response across endpoints

Dietary interventions have been shown to reduce cardiovascular disease (CVD) risks by 60 % [306] and reduce Alzheimer's disease (AD) risks by 35 % - 54 % [307]. However, individuals usually do not benefit from the full potential of the protective effects of a healthy diet because the phenomenon of inter-individual variability in the response [101]. It has also been calculated previously that approximately 40 % of participants could benefit from an intervention [308]. The current thesis observed up to 71 % of participants benefiting in their vascular function (+ 105 % - + 525 % of improved response level) and up to 71 % of participants benefiting their cognition (+ 61 % - + 114 % of improved response level) following the consumption of whole blueberry (Chapter 6).

Apart from the common benefit from polyphenol-rich fruit interventions on vascular and cognitive health, the two endpoints may not be directly linked but some associations were highlighted. For example, age-related impairment in cardiovascular function may disrupt neuronal micro-environmental homeostasis and lead to impaired downregulation of cerebral blood flow (CBF) [309]. Vascular dysfunction is also an important contributor to Alzheimer's disease (AD) and treatments targeting vascular health may contribute to preserving cognitive function [310]. Therefore, the consistency of high response to both endpoints were of interest.

Despite the moderate to dynamic inter-individual variations observed, several participants, for example participants S22 showed high responses to vascular function following whole blueberry intervention, S38 showed high response to cognition following both whole blueberry and blueberry powder interventions but the responses were not consistent across endpoints. No consistent response across endpoints

following both two interventions was identified. The assessment of response across endpoints is important because knowing if one individual will continue responding at high levels to another endpoint can help to maximise individual health benefits and give personalised nutrition guidelines.

The current thesis also could not identify a predictive biomarker predicting RS group following blueberry interventions. Studies predicting individual response in the clinical endpoints following blueberry interventions are still scarce but the response variations have been explored previously in therapeutic trials that have undertaken quartile division to characterise high and low responses [217-219], similar to the present study. Currently, interventional studies that predicted responses to a range of clinical endpoints can be divided into either a prospective [89, 264, 265, 272, 273] or retrospective [267, 271, 311] design. For the first type of studies, the responder/non-responders (RS/NRS) are usually identified with known conditions and a range of biomarkers and response was further characterised post-intervention. These types of studies are usually able to report strong predictors that are associated with RS either downstream or upstream in a biological pathway such as the investigation of predictive biomarkers for CVD [312] and type 2 diabetes [313].

The current study falls under the latter category that required the characterisation of RS/NRS prior to applying biomarker analysis to search for predictors for response. The characterisation of RS and NRS has previously included development of a model or score based on the health outcomes assessment or the combination of metabolomics, genomics, gut metagenomics, body composition, and/or glycaemic responses for predicting response variations [89, 264-266]. To identify candidate prognostic metabolites, various statistical approaches have been used. Receiver operating characteristic (ROC) curve analysis is usually used to identify signature biomarkers [265, 314], linear mixed model and logistic regression models have also been applied to obtain associations between the response and the predictive biomarkers [265, 273].

However, to our knowledge, there have been no previous studies investigating the use of predictive markers for screening potential responders to maximize the benefits of

blueberry interventions improving vascular and cognitive function. In addition, we tested whether there are predictive metabolite(s) validated by ROC analysis. However, the ROC curve for the top 100 metabolites basing on random forest model did not confirm any single feature to be predictive for RS, highlighting that more work including larger sample size and further fragmentation identification need to be done to identify predictive metabolites. The association between the response and the biomarkers could not be explored further due to the lack of predictive biomarkers.

7.3 Limitations and implications for future work

To our best knowledge, this is the first study that has compared effects on vascular and cognitive health coupled with untargeted metabolite profiling between whole blueberry and freeze-dried blueberry powder interventions in the same study. A number of other covariates should be considered apart from what were discussed in Chapters 5 & 6 that the use of a standardised blueberry interventions, populations and standardised control formulations should be considered when comparing with other similar studies. Those covariates include prescribed medications [315], participants' sleep and exercise patterns [316, 317] apart from the variations in the anthocyanin content of whole blueberries as these may affect cognitive performances and vascular function. Those covariates should be better controlled in an RCT. The sample size calculation also should be carried out for each primary endpoint to decrease the risk of insufficient statistical power to detect a significant treatment effect [230]. However, the efficacy of improving vascular and cognitive health cannot be adequately established on the basis of a single endpoint, the number of endpoints analysed in the current thesis increased (n = 52). Therefore, the type II error rate of falsely concluding the intervention is ineffective is also increased [318].

Attention should be drawn to the fact that the intake of dietary blueberry supplementation could induce glucose loads and synergise with the postprandial effect of blueberry polyphenols. Dietary polyphenols, and vitamins act on cognition via pathways such as intracellular neuronal and glial signalling, energy metabolism, regulation of cerebral blood flow, and protection against neurotoxins and neuroinflammation [153-155]. Glucose loads could induce cognitive changes via sympathetic activation, glucocorticoid secretion, and pancreatic β-cell function, rather than simply boosting neuronal activity [319]. Nevertheless, the carbohydrate intake

was analysed and no significant changes were found between and within the intervention groups.

There are other limitations in our study. Plasma and urinary metabolites of flavonoids and phenolic acids from blueberries can be found in *vitro* and in *vivo* [56]. However, blueberry polyphenols, and especially anthocyanins, are well-known for their low bioavailability in human plasma following ingestion [320]. The levels of polyphenol metabolites may also saturate after absorption and distribution as time increases [321]. Furthermore, circulating plasma polyphenol concentrations are much lower than urinary concentrations, whereas the plasma is essential to deliver the polyphenols and their metabolites to the targets exerting their physiological effect [320]. Although the biokinetics post-interventions in the current study are unknown, the dosage the subjects consumed in our study may not be able to maintain polyphenols levels within the body after absorption and overnight fasting (> 12 h) prior to the study visit. It also should be noted that the batch to batch variations in the nutrition value and polyphenol content, especially anthocyanins for whole blueberry, were not assessed in the current study [246]. There were also differences in the nutritional value and polyphenol content between freeze-dried blueberry powder and whole blueberry, arising from different cultivars, harvest and post-harvest processing [245]. Even though the total polyphenol content were assessed for the whole blueberry and blueberry powder, the Folin-Ciocalteu method has limitations that may lead to inaccurate estimation of the phenolic compounds in the fruit [247, 248].

The LCMS profiling in negative ion mode and further fragmentation work were also absent in the current work, which could help to obtain a wider range of urinary metabolites. Furthermore, currently very few studies have combined genomics, epigenetics metabolomics, and gut microbiome and anthropometric characteristics together to assess individual response in the assessment of clinical endpoints following dietary interventions. The current study recruited mainly females (71 %) and there was no association suggested between gender and response. The above factors all could influence the absorption, distribution, metabolism and excretion (ADME) processes known to influence metabolic phenotypes [101]. However, a range of studies under the scheme of COST Action POSITIVe revealed a lack of knowledge about the carriers, enzymes, isoforms, and gut bacteria involved in ADME, making it

difficult to identify a key biomarker of ADME variability [101]. In addition, the genetic variations are not independent of the gut microbiome, although there is study indicating that the contribution of genetics to the microbiome is small (1.9 - 8.1 %) [322], this interaction in the influence on inter-individual variation in response must also be considered [266]. It is important to investigate how much each factor contribute to the inter-individual variations in response (i.e. via linear mixed method or multivariate analysis) following a dietary intervention in future work.

It is also important to take social, cultural and personal dietary habit into account when tailoring dietary advice to the individual. Although the most challenging part for precision nutrition is to characterise individual response to diet from a population, the adherence from individuals to follow the dietary intervention is important. Therefore, factors including religion, ethnicity, socioeconomic status and lifestyle should be considered when designing interventions assessing individual responses that individuals can follow through [266]. The field of precision nutrition is still in its infancy, the current study did not identify a consistent response and predictive biomarker for response but demonstrated one approach for charactering individual response to blueberry consumptions in the assessment of vascular and cognitive health, the two important health endpoints. The challenges in the field still need to be conquered but the knowledge arising from studies similar to this could contribute to individuals and public health collaboratively.

7.4 Conclusion

To conclude, the systematic reviews and meta-analyses of RCTs spanning at least 1 week for the assessment of berry, citrus and cherry dietary interventions for improving vascular and cognitive health have suggested the potential for berry fruits overall to significantly improve SBP and DBP, and thus to benefit vascular function. However, the current study supplementing either 160 g whole fresh or 20 g freeze-dried powdered blueberry daily to healthy adults for 1 week found no significant effect in improving vascular and/or cognitive health. No significant difference of the efficacy of improving vascular and cognitive health was found neither between the whole blueberry and blueberry powder interventions. Moderate to high inter-individual variation for each endpoint was found and the response across endpoints following

the blueberry interventions was inconsistent between and within participants. No predictive biomarker for discriminating responders to the endpoints following the blueberry interventions was identified. Nevertheless, the findings from the current thesis suggests that a novel approach characterising response across endpoints following a dietary intervention beyond the ‘one size fits all’ dietary strategy is necessary prior to defining a beneficial food or food groups.

Appendices

Appendix. 1.1 Jadad scores of interventions assessing vascular function

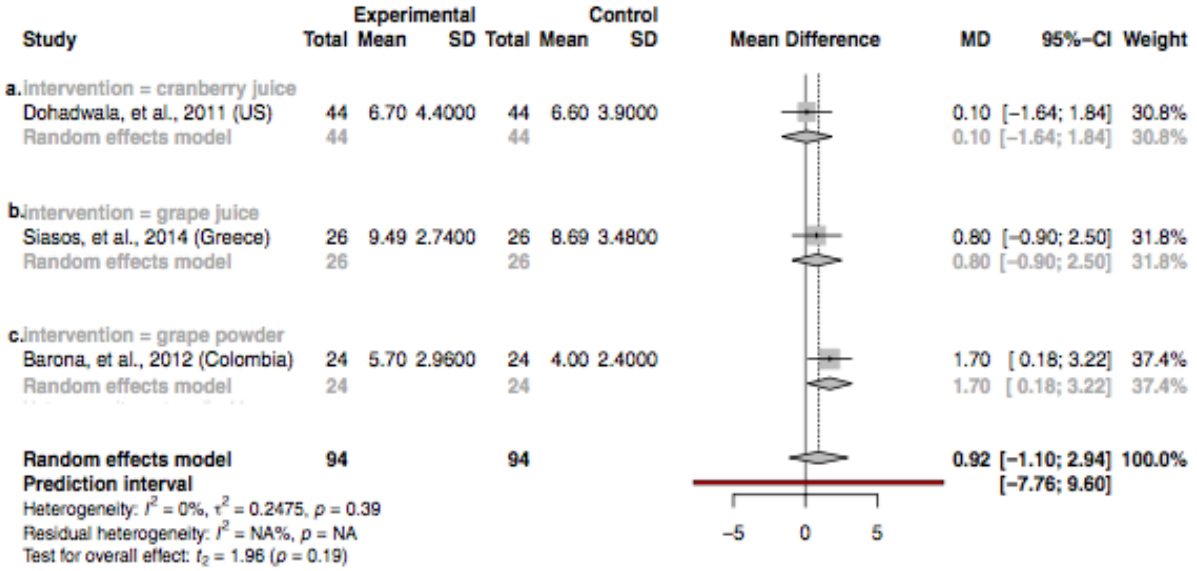
(Author/ year) country of origin	Jadad Score - Randomisation (1 or 0)	Jadad Score - blinding (1 or 0)	Jada score - withdrawals (1 or 0)	Jadad score extra point (1 or 0)	Jadad score extra point (1 or 0)	Total Jadad score (0 to 5)
Alquarashi, R. et al. 2016 (UK)	1	1	0	1	1	4
Barona, et al. 2012 (Colombia)	1	1	0	0	0	2
Buscemi, et al. 2012 (Italy)	1	1	0	0	0	2
Constans, et al. 2015 (France)	1	1	0	1	0	3
Del Bo, et al. 2013 (Italy)	1	0	1	0	0	2
Dohadwala, et al. 2011 (US)	1	1	0	1	1	4
Draijer, et al. 2015 (UK)	1	1	1	0	1	4
Habauzit, et al. 2015 (France)	1	1	0	1	1	4
Hampton, et al. 2010 (UK)	1	1	1	0	1	4
Jin, et al. 2011 (UK)	1	1	1	0	0	3
Keane, 2016 (UK)	1	1	1	0	0	3
Kean et al. 2016 (UK) (2)	1	1	0	0	0	2

Lamport, et al. 2016 (UK)	1	1	0	1	1	4
Lamport et al. 2016 (2) (UK)	1	1	0	1	0	3
Morand, et al. 2011 (French)	1	1	1	1	1	5
Rendeiro, et al. 2017 (UK)	1	1	1	1	1	5
Riso et al. 2013 (Italy)	1	0	0	1	0	2
Rodriguez. Et al. 2013(UK)	1	1	0	1	1	4
Ruel, et al. 2013 (Canada)	1	1	1	0	0	3
Schaer et al. 2015 (US)	1	1	0	1	1	4
Siasos, et al. 2014 (Greece)	1	1	1	0	1	4
Willems, et al. 2015 (UK)	1	1	0	0	0	2
Basu, A. et al. 2014 (UK)	1	0	1	0	0	2
Basu, et al. 2010 (US)	1	1	0	0	0	2
Basu, et al. 2010 (US)	1	1	0	0	1	3
Basu, et al. 2011 (US)	1	1	0	0	1	3

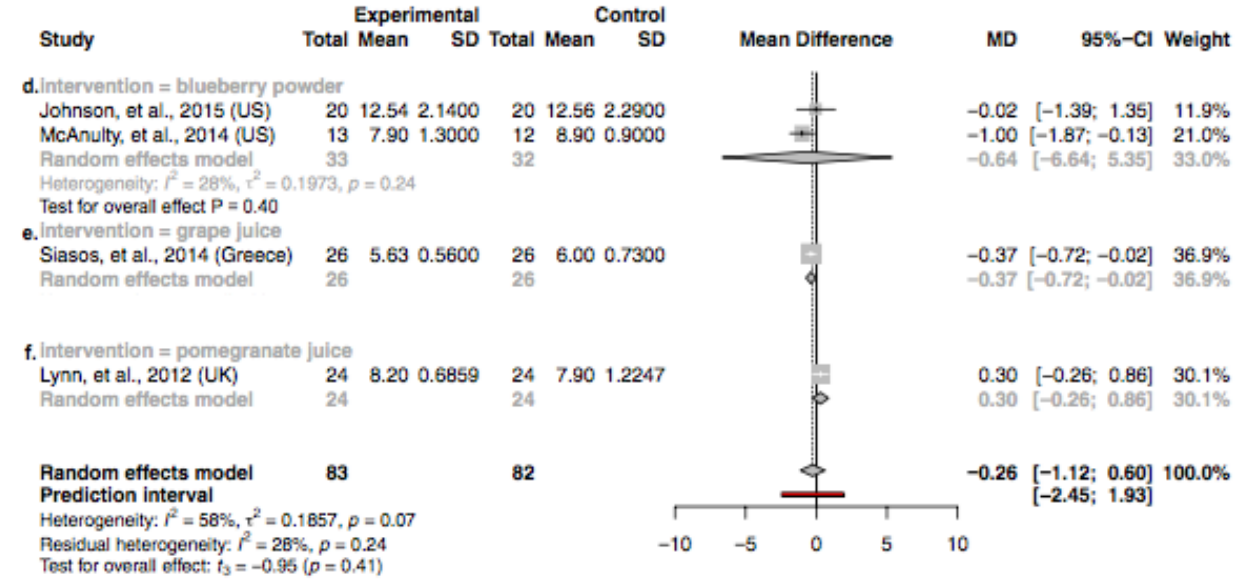
Cerda 2006a (Spain)	1	1	1	0	0	3
Duthie 2006b (Scotland)	1	0	1	0	0	2
Del Bo, et al. 2014 (Italy)	1	0	1	1	0	3
Dow, et al. 2013 (US)	1	0	0	0	0	1
Flammer, et al. 2013 (US)	1	1	0	0	1	3
Gonzalez-Ortiz 2011b (US)	1	1	1	0	0	3
Hollis 2010a (US)	1	1	1	0	0	3
Jeong, et al. 2016 (Korea)	1	1	0	1	0	3
Jeong, et al. 2016 (Korea) (2)	1	1	0	1	0	3
Jeong, et al. 2014(Korea)	1	1	0	0	1	3
Johnson, et al. 2015 (US)	1	1	0	1	0	3
Kent, et al. 2017 (Australia)	1	1	0	1	1	4
Khan, et al. 2014 (UK)	1	1	0	1	0	3
Lekakis, et al. 2005 (Greece)	1	1	1	0	1	4

Lynn, et al. 2012 (UK)	1	0	1	1	0	3
Lynn, et al. 2014 (UK)	1	0	0	1	0	2
McAnulty, et al. 2014 (US)	1	0	1	0	0	2
Murkovic, et al. 2004b Australia	1	1	1	0	0	3
Novotny et al. 2015 (US)	1	1	0	1	1	4
Park, et al. 2009 (Japan)	1	1	1	0	0	3
Rodriguez. et al. 2016 (UK)	1	1	0	1	1	4
Sumner, et al. 2005 (US)	1	1	0	1	1	4
Stull, et al. 2015 (US)	1	1	0	1	1	4

Appendix. 1.2 Example forest plot of berry interventions including (a) cranberry juice, (b) grape juice and (c) grape powder assessing FMD and (d) blueberry powder, (e) grape juice and (f) pomegranate juice assessing PWV



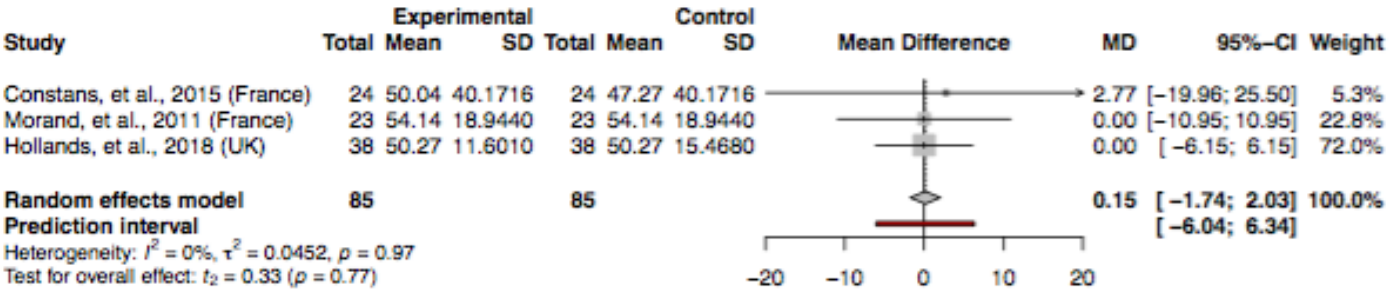
A. Forest plot of the effect of fruit interventions on FMD



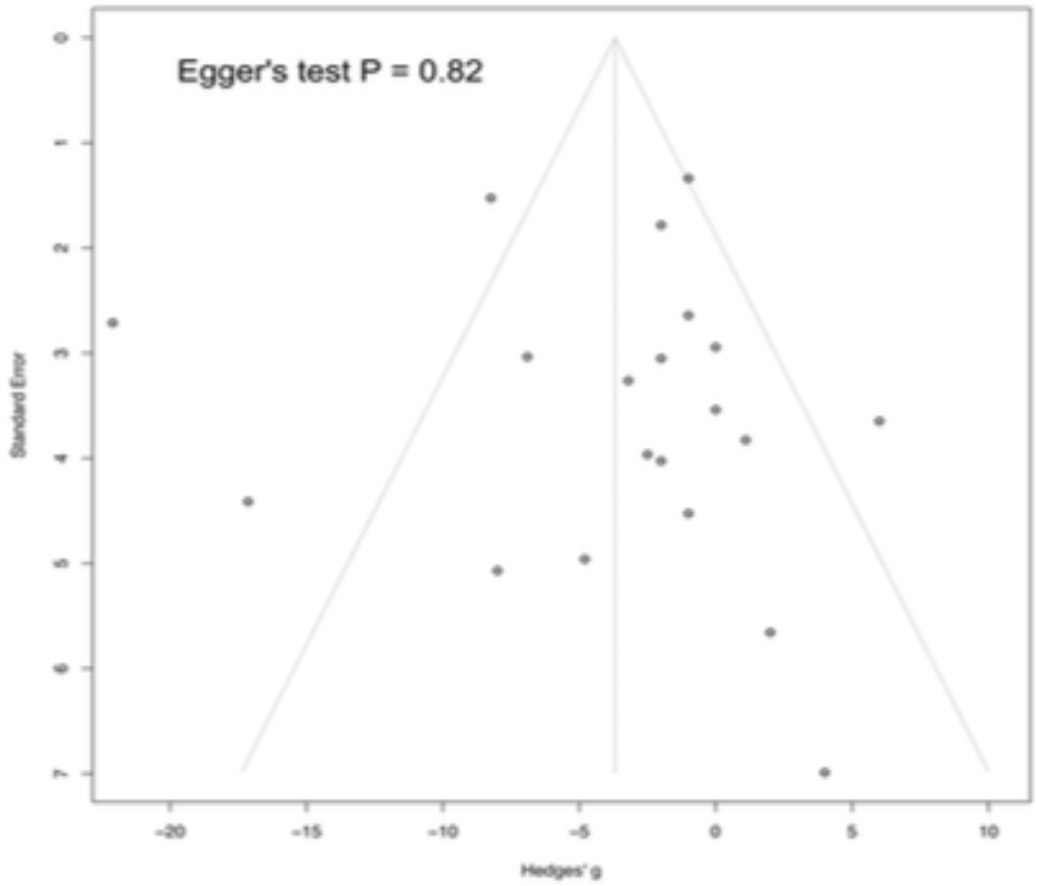
B. Forest plot of the effect of fruit interventions on PWV

***Notes**
a. 480ml, 28 days
b. 240ml, 14 days
c. 46g, equivalent to 2 cups of grape, 30 days
d. 22g or 38g, equivalent to 1or 1.5 cups of blueberry, 56 days or 42 days
e. 240ml, 14 days
f. 330ml, 28 days

Appendix. 1.3 Example forest plot of orange juice interventions assessing HDL -C



Appendix. 1.4 Example funnel plot of berry studies assessing SBP

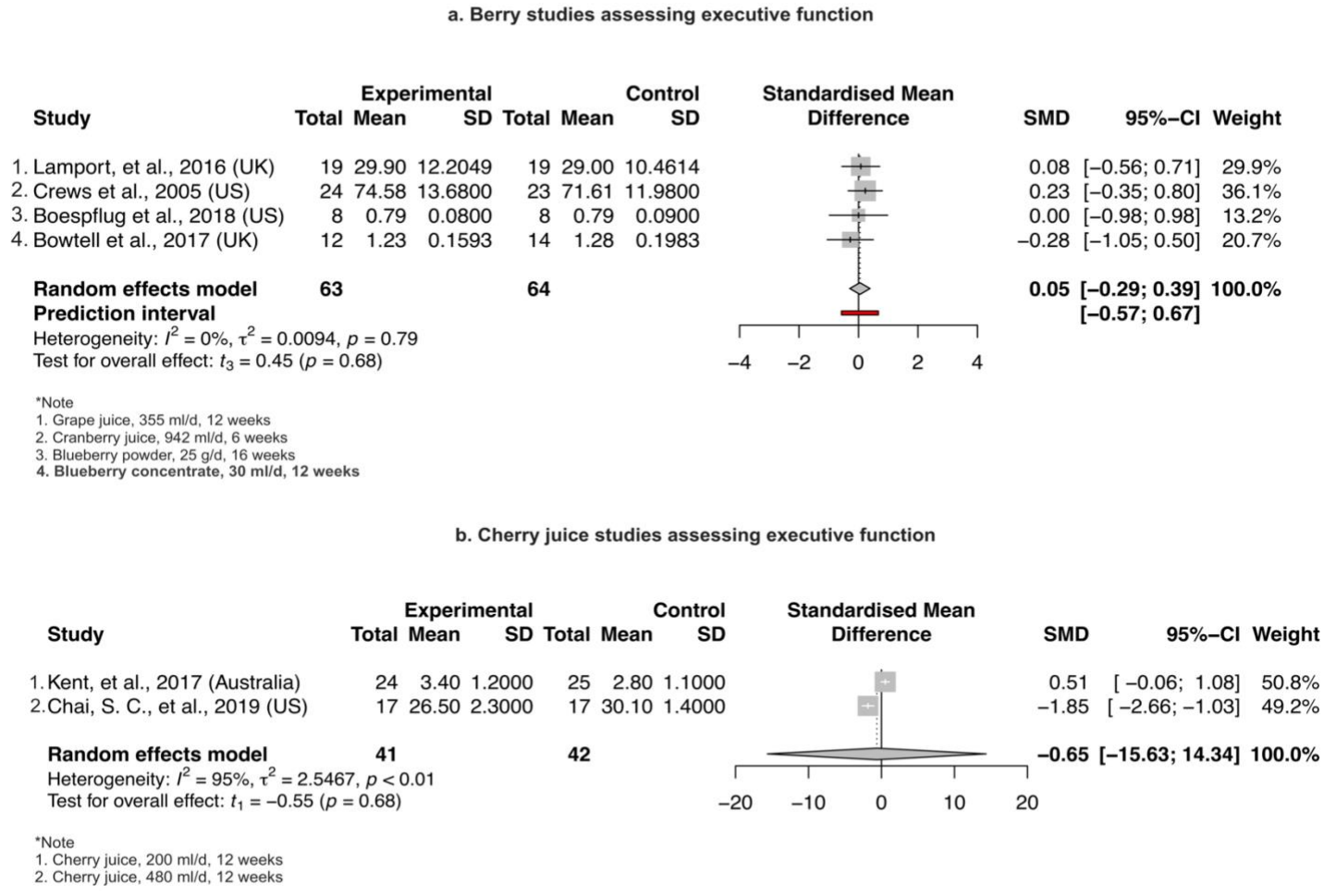


Appendix. 2.1 Jadad scores of interventions assessing cognition

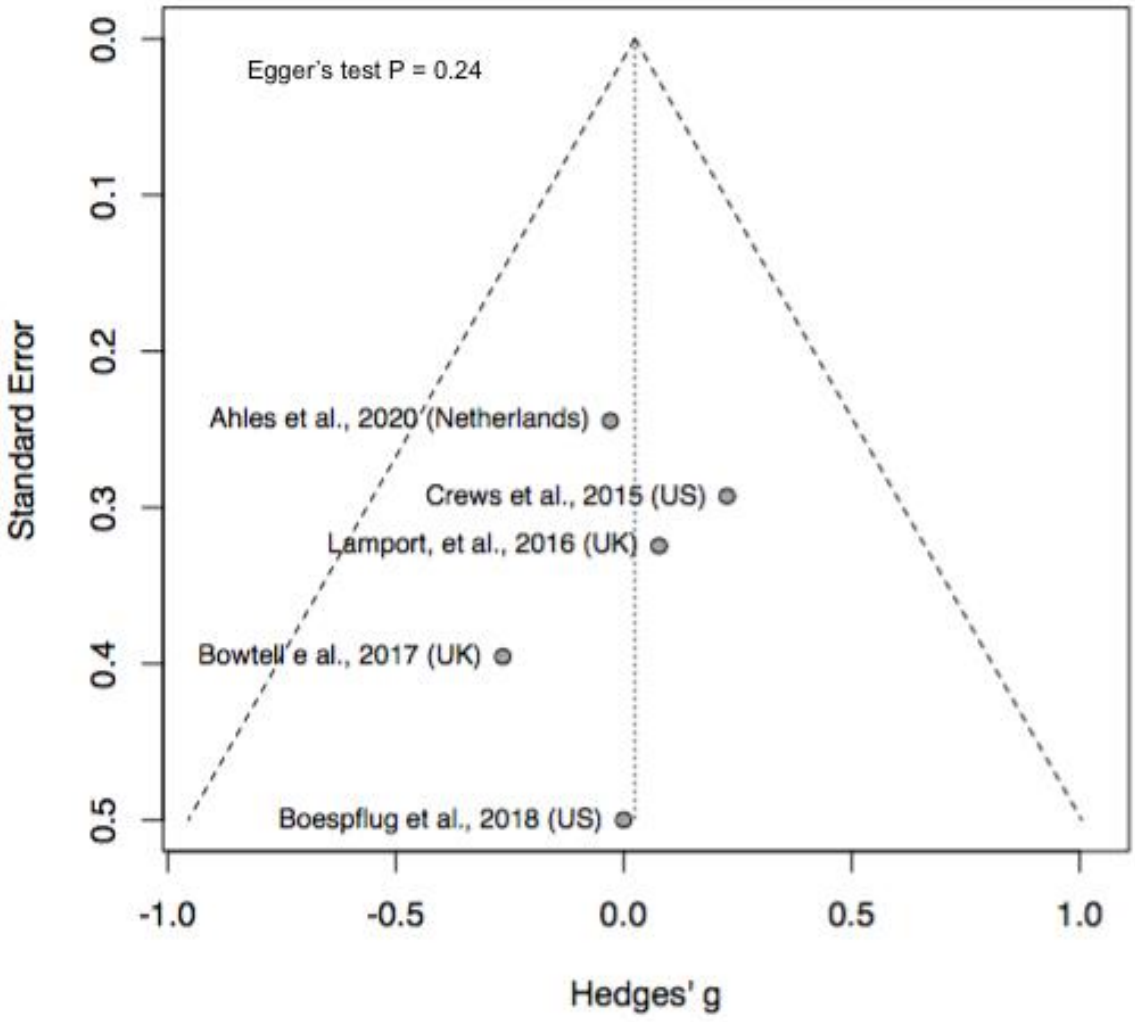
(Author/ year) country of origin	Jadad Score-Randomisation (1 or 0)	Jadad Score-blinding (1 or 0)	Jada score - withdrawals (1 or 0)	Jadad score extra point (1 or 0)	Jadad score extra point (1 or 0)	Total Jadad score (0 to 5)
Ahles et al. 2020 (Netherlands)	1	1	0	1	0	3
Kean et al. 2015 (UK)	1	1	1	1	1	5
Lamport, et al. 2016 (UK)	1	1	0	1	1	4
Bowtell et al. 2017 (UK)	1	1	1	0	1	4
Boespflug et al. 2018 (US)	1	1	0	0	0	2
Calapai, et al. 2017 (Italy)	1	1	0	1	0	3
Chai, S. C. et al. 2019 (US)	1	0	0	1	0	2
Crews et al. 2005 (US)	1	1	0	1	0	3
Kent, et al. 2017 (Australia)	1	1	0	1	1	4
Krikorian, et al. 2012 (US)	1	1	0	0	0	2

Krikorian, et al, 2010 (US)	1	1	1	0	0	3
Lee, et al. 2017 (US)	1	1	0	0	1	3
Miller, et al. 2017	1	1	0	1	1	4
Schrager, et al. 2015 (US)	1	1	1	0	0	3
Whyte, A. R. et al. (2018) UK	1	1 1	1	1	1	5

Appendix. 2.2 Forest plot of (a) Berry studies and (b) Cherry juice studies assessing executive function



Appendix. 2.3 Example funnel plot of berry studies assessing executive function



Appendix. 3.1 CRF

CASE REPORT FORM

Metabolic mechanisms underlying the responsiveness to blueberry interventions aimed at improving cognition and peripheral blood flow

Subject ID: |__|__|__|

Randomisation Number: |__|__|__|

COMPASS Number: |__|__|__|

Screening

Informed Consent

Date of screening/ signing of informed consent: |__|__|-|__|__|-|__|__| *(dd-mm-yy)*

Demographic Data

Date of birth |__|__|-|__|__|-|__|__| *(dd-mm-yy)*

Age at Enrolment: |__|__| years

If not between 18 and 60 years (inclusive) → ***exclusion***

Sex: ☐ Female ☐ Male ☐ Prefer not to say

Race:
- ☐ White
- ☐ Black
- ☐ Asian (Indian, Bangladeshi, Pakistani)
- ☐ Chinese
- ☐ Other, please specify:_____

Screening *continued*

Inclusion Criteria

Please check all following inclusion criteria: if one criterion is answered with “No”, the subject is not eligible for this study!		**Yes**	**No**
1.	Are you between the ages of 18 and 60 years (inclusive)?	☐	☐
2.	Willing to abstain throughout the trial from the intake of nutritional supplementation?	☐	☐
3.	Willing to abstain throughout the trial from the intake of blueberries or any blueberries containing and anthocyanin-rich products (other than those provided to you)?	☐	☐
4.	Have consumed blueberries before but don’t regularly consume blueberry or blueberry-contained products more than twice a week?	☐	☐

Exclusion Criteria

Please check all following exclusion criteria: if one criterion is answered with “Yes”, the subject is not eligible for this study! *“Do any of these apply to you?”*		**Yes**	**No**
1.	Food allergies **(*only exclude if allergic to berries*)**	☐	☐
2.	Have vascular disease, hypertension, or other cardiac abnormalities	☐	☐
3.	Have previously suffered any head injuries, history of seizures or other neurological disorders	☐	☐
4.	Have any metabolic disorders, malabsorption syndromes or gastrointestinal complications	☐	☐
5.	Irregular bowel function (less than 1 bowel movement per day)	☐	☐
6.	Have regularly used nutritional supplements or medications within the previous 3 months (defined as more than 3 consecutive days or 4 days in total)	☐	☐
7.	Learning difficulties and or dyslexia	☐	☐
8.	Visual impairment that cannot be corrected with glasses or contacts, including colour blindness	☐	☐

9.	Currently suffer from migraines (>1 per month)	☐	☐
10.	Smoking or the use of any nicotine replacement products e.g. vaping, gum, patches	☐	☐
11.	Pregnancy, seeking to become pregnant, or current lactation	☐	☐
12.	Current participation in other clinical or nutrition intervention studies	☐	☐

Screening *continued*

		Yes	**No**
13.	Not proficient in English equivalent to IELTS band 6 or above	☐	☐
14.	Have any known active infections	☐	☐
15.	Inability to complete all of the study assessments	☐	☐

Screening *continued*

Lifestyle habits	
Is the Subject vegetarian?	☐ Yes ☐ No
Is the Subject vegan?	☐ Yes ☐ No
How many portions of fruit and vegetable does the subject eat in average per day? |__|__| portion(s) per day *(Portion = one piece of fruit, a handful of vegetables or a glass of fresh fruit juice (each additional glass of juice does not count as extra))*	

Screening *continued*

Physical Examination

Physical examination after sitting in an upright position for 5 minutes. A first reading is taken but discarded. ***If the average reading is out of range, but the third is lower than the second reading, a fourth reading can be taken.*** *This reading alone will then be used as the final measurement. Readings should be taken > 1 minute intervals. If BP is >139-89, subject must be referred to GP.*

1st reading

Blood Pressure: Systolic |__ |__ |__| mm Hg

Diastolic |__ |__ |__| mm Hg

Heart Rate:

|__ |__ |__| BPM

2nd reading

Blood Pressure: Systolic |__ |__ |__| mm Hg

Diastolic |__ |__ |__| mm Hg

Heart Rate:

|__ |__ |__| BPM

3rd reading

Blood Pressure: Systolic |__ |__ |__| mm Hg

Diastolic |__ |__ |__| mm Hg

Heart Rate:

|__ |__ |__| BPM

4th reading

Blood Pressure: Systolic |__ |__ |__| mm Hg

Diastolic |__ |__ |__| mm Hg

Heart Rate:

|__ |__ |__| BPM

Average reading

Systolic |__ |__ |__| mm Hg

Diastolic |__ |__ |__| mm Hg

|__ |__ |__| BPM

If BP is ≥ 145/100 → ***exclusion; suggest to inform GP for treatment***

Height and Weight *If BMI is out of the range 18.5 –30.0* → ***exclusion***

Body weight: |__|__|.|__| kg

Body height: |__|__|__| cm

Body Mass Index |__|__|.|__|__| kg/m^2

Exclusion Criteria *continued:* Physiological Examination

		Yes	No
1.	Have high blood pressure (systolic over 145mm Hg or diastolic over 100 mm Hg)	☐	☐
2.	Have a Body Mass Index (BMI) outside of the range 18.5-30.0 kg/m^2	☐	☐

Screening *continued*

Is the subject eligible to be included in the study, fulfilling all inclusion criteria and none of the exclusion criteria? ☐ Yes ☐ No

I confirm that the information contained in the CRF is correct:

_____ Date: |__|__|-|__|__|-|__|__| (dd-mm-yy)

Participants signature

_____ Date: |__|__|-|__|__|-|__|__| (dd-mm-yy)

Researcher's signature

Prior to discharge of the subject

Hand out **1-day food diary**, **food frequency questionnaire** and **urine collection packs** (check urine kit is labelled correctly with Subject ID!) and give them the treatment if they are entering into the treatment phase next week! and ensure the participant understands they:

- Must collect urine samples prior to testing visits (within 24 hours of study session) (see urine instruction sheet for full instructions)
- Must record their dietary intake of the day prior next visit in 1-day food diary and bring it to testing visits.
- Must fast for 12 hours prior to testing visits 2-6.
- Should refrain from alcohol for 24 hours prior to their next study visit.
- Should not consume medication (except oral contraceptives) for 24 hours prior to the next visit (avoid oral antihistamines for 48 hrs; avoid non-prescription medications (e.g. paracetamol, ibuprofen etc.) and local (spray) antihistamines for 24 hrs).
- Should avoid all anthocyanin-rich berries (i.e. blueberries, raspberries, blackcurrant) and cherries or any foods containing them from this point until the study completion
- Will be required to give a urine sample (2nd urination of that day) when they arrive for their next testing visit)

Visit 2 BP measurement *continued*

Physical examination after sitting in an upright position for 5 minutes. A first reading is taken but discarded. ***If the average reading is out of range, but the third is lower than the second reading, a fourth reading can be taken.*** *This reading alone will then be used as the final measurement. Readings should be taken > 1 minute intervals. If BP is >139-89, subject must be referred to GP.*

1st reading

Blood Pressure: Systolic |__ |__ |__| mm Hg

Diastolic |__ |__ |__| mm Hg

Heart Rate:

|__ |__ |__| BPM

2nd reading

Blood Pressure: Systolic |__ |__ |__| mm Hg

Diastolic |__ |__ |__| mm Hg

Heart Rate:

|__ |__ |__| BPM

3rd reading

Blood Pressure: Systolic |__ |__ |__| mm Hg

Diastolic |__ |__ |__| mm Hg

Heart Rate:

|__ |__ |__| BPM

4th reading

Blood Pressure: Systolic |__ |__ |__| mm Hg

Diastolic |__ |__ |__| mm Hg

Heart Rate:

|__ |__ |__| BPM

Average reading

Systolic |__ |__ |__| mm Hg

Diastolic |__ |__ |__| mm Hg

|__ |__ |__| BPM

*If BP is ≥ 145/100 → **exclusion; suggest to inform GP for treatment***

Visit 3 BP measurement *continued*

Physical examination after sitting in an upright position for 5 minutes. A first reading is taken but discarded. ***If the average reading is out of range, but the third is lower than the second reading, a fourth reading can be taken.*** *This reading alone will then be used as the final measurement. Readings should be taken > 1 minute intervals. If BP is >139-89, subject must be referred to GP.*

1st reading

Blood Pressure: Systolic |__|__|__| mm Hg

Diastolic |__|__|__| mm Hg

Heart Rate: |__|__|__| BPM

2nd reading

Blood Pressure: Systolic |__|__|__| mm Hg

Diastolic |__|__|__| mm Hg

Heart Rate: |__|__|__| BPM

3rd reading

Blood Pressure: Systolic |__|__|__| mm Hg

Diastolic |__|__|__| mm Hg

Heart Rate: |__|__|__| BPM

4th reading

Blood Pressure: Systolic |__|__|__| mm Hg

Diastolic |__|__|__| mm Hg

Heart Rate: |__|__|__| BPM

Average reading

Systolic |__|__|__| mm Hg

Diastolic |__|__|__| mm Hg

|__|__|__| BPM

If BP is ≥ 145/100 → ***exclusion; suggest to inform GP for treatment***

Visit 4 BP measurement *continued*

Physical examination after sitting in an upright position for 5 minutes. A first reading is taken but discarded. ***If the average reading is out of range, but the third is lower than the second reading, a fourth reading can be taken.*** *This reading alone will then be used as the final measurement. Readings should be taken > 1 minute intervals. If BP is >139-89, subject must be referred to GP.*

1st reading

Blood Pressure: Systolic |__ |__ |__| mm Hg

Diastolic |__ |__ |__| mm Hg

Heart Rate:

|__ |__ |__| BPM

2nd reading

Blood Pressure: Systolic |__ |__ |__| mm Hg

Diastolic |__ |__ |__| mm Hg

Heart Rate:

|__ |__ |__| BPM

3rd reading

Blood Pressure: Systolic |__ |__ |__| mm Hg

Diastolic |__ |__ |__| mm Hg

Heart Rate:

|__ |__ |__| BPM

4th reading

Blood Pressure: Systolic |__ |__ |__| mm Hg

Diastolic |__ |__ |__| mm Hg

Heart Rate:

|__ |__ |__| BPM

Average reading

Systolic |__ |__ |__| mm Hg

Diastolic |__ |__ |__| mm Hg

|__ |__ |__| BPM

If BP is ≥ 145/100 → ***exclusion; suggest to inform GP for treatment***

Visit 5 BP measurement *continued*

Physical examination after sitting in an upright position for 5 minutes. A first reading is taken but discarded. ***If the average reading is out of range, but the third is lower than the second reading, a fourth reading can be taken.*** *This reading alone will then be used as the final measurement. Readings should be taken > 1 minute intervals. If BP is >139-89, subject must be referred to GP.*

1st reading

Blood Pressure: Systolic |__|__|__| mm Hg

Diastolic |__|__|__| mm Hg

Heart Rate: |__|__|__| BPM

2nd reading

Blood Pressure: Systolic |__|__|__| mm Hg

Diastolic |__|__|__| mm Hg

Heart Rate: |__|__|__| BPM

3rd reading

Blood Pressure: Systolic |__|__|__| mm Hg

Diastolic |__|__|__| mm Hg

Heart Rate: |__|__|__| BPM

4th reading

Blood Pressure: Systolic |__|__|__| mm Hg

Diastolic |__|__|__| mm Hg

Heart Rate: |__|__|__| BPM

Average reading

Systolic |__|__|__| mm Hg

Diastolic |__|__|__| mm Hg

|__|__|__| BPM

If BP is ≥ 145/100 → ***exclusion; suggest to inform GP for treatment***

Visit 6 BP measurement *continued*

Physical examination after sitting in an upright position for 5 minutes. A first reading is taken but discarded. ***If the average reading is out of range, but the third is lower than the second reading, a fourth reading can be taken.*** *This reading alone will then be used as the final measurement. Readings should be taken > 1 minute intervals. If BP is >139-89, subject must be referred to GP.*

1st reading

Blood Pressure: Systolic |__|__|__| mm Hg

Diastolic |__|__|__| mm Hg

Heart Rate: |__|__|__| BPM

2nd reading

Blood Pressure: Systolic |__|__|__| mm Hg

Diastolic |__|__|__| mm Hg

Heart Rate: |__|__|__| BPM

3rd reading

Blood Pressure: Systolic |__|__|__| mm Hg

Diastolic |__|__|__| mm Hg

Heart Rate: |__|__|__| BPM

4th reading

Blood Pressure: Systolic |__|__|__| mm Hg

Diastolic |__|__|__| mm Hg

Heart Rate: |__|__|__| BPM

Average reading

Systolic |__|__|__| mm Hg

Diastolic |__|__|__| mm Hg

|__|__|__| BPM

If BP is ≥ 145/100 → ***exclusion; suggest to inform GP for treatment***

Visit 7 BP measurement *continued*

Physical examination after sitting in an upright position for 5 minutes. A first reading is taken but discarded. ***If the average reading is out of range, but the third is lower than the second reading, a fourth reading can be taken.*** *This reading alone will then be used as the final measurement. Readings should be taken > 1 minute intervals. If BP is >139-89, subject must be referred to GP.*

1st reading

Blood Pressure: Systolic |__|__|__| mm Hg

Diastolic |__|__|__| mm Hg

Heart Rate: |__|__|__| BPM

2nd reading

Blood Pressure: Systolic |__|__|__| mm Hg

Diastolic |__|__|__| mm Hg

Heart Rate: |__|__|__| BPM

3rd reading

Blood Pressure: Systolic |__|__|__| mm Hg

Diastolic |__|__|__| mm Hg

Heart Rate: |__|__|__| BPM

4th reading

Blood Pressure: Systolic |__|__|__| mm Hg

Diastolic |__|__|__| mm Hg

Heart Rate: |__|__|__| BPM

Average reading

Systolic |__|__|__| mm Hg

Diastolic |__|__|__| mm Hg

|__|__|__| BPM

If BP is ≥ 145/100 → ***exclusion; suggest to inform GP for treatment***

Procedural Deviations

Date	Visit (e.g. T, TV1, Interim 1)	Assess-ment	Task	Deviation from procedure

Adverse Event Page 1 of 1

☐ **Tick if no Adverse Event reported**

Record No.	**Serious** 1=no 2=yes*	**Adverse Event**	**Start Date/ Stop Date** dd-mm-yy	**Severity** 1=mild 2=moderate 3=severe	**Relation-ship to IP possible?** 1=yes 2=no	**Action taken with IP?** 1=IP withdrawn 2= IP interrupted 3= Dose reduced 4=Dose not changed 5=Dose increased 6=Not applicable 7=Unkown	**Treatment of AE?** 1= None 2=Remedial Drug Therapy 3=Other (please comment below)	**Outcome of Event** 1= Recovered / resolved 2=Recovering / resolving 3= Recovered / resolved with sequale 4= Not recovered / not resolved 5=fatal 6= unknown
__|__|	__|	…… ……	__|__|-__|__|-__|__| __|__|-__|__|-__|__|	__|	__|	__|	__|	__|
__|__|	__|	…… ……	__|__|-__|__|-__|__| __|__|-__|__|-__|__|	__|	__|	__|	__|	__|
			__|__|-__|__|-__|__|					

____	__	....	____-____-____	__	__	__	__	__
____	__	....	____-____-____ ____-____-____	__	__	__	__	__

**Please fill in the complementary pages for serious adverse events and send it immediately to the sponsor.*

Record No __ __ Comment: _____

Record No __ __ Comment: _____

Study Completion

Date of Completion/early withdrawal: |__|__|-|__|__|-|__|__| *(dd-mm-yy)*

Did the subject complete the study as planned? ☐ Yes ☐ No

If No, please give primary reason for premature termination / discontinuation

☐ **Adverse event(s) (also specify on the Adverse Event form)**

☐ **Major protocol violation, including non-compliance**

☐ **Subject withdrew consent**

☐ **Lost to follow-up**

☐ **Administrative reasons**

☐ **Pregnancy**

☐ **Investigator opinion**

☐ **Sponsor request**

☐ **Other please specify**

☐ **Death** (please complete Serious Adverse Events form and record Adverse events leading to death on the Adverse Vents form)

Date of Death |__|__|-|__|__|-|__|__| *(dd-mm-yy)*

Principal Cause of death:_____

Please provide any relevant information related to the reason for premature discontinuation:

_____

_____

Was the randomisation code broken? ☐ Yes ☐ No

If yes, date of breaking: |__|__|-|__|__|-|__|__| *(dd-mm-yy)*

*Reason for breaking the code:*_____

Name of person who opened the envelope: _____

I have reviewed and found all data pertaining to this subject to be complete and accurate:

Principal Investigator's Signature _____

Date: |__|__|-|__|__|-|__|__| *(dd-mm-yy)*

Appendix. 3.2 1-day food diary

Northumbria University NEWCASTLE

Faculty of Health & Life Sciences

1 DAY FOOD DIARY

TITLE OF PROJECT: Metabolic mechanisms underlying the responsiveness to blueberry interventions aimed at improving cognition and peripheral blood flow

Principal Investigator: Yueyue Wang Email: yueyue.wang@northumbria.ac.uk

How to complete your 1 day food diary

I would like each participant to keep the diary for one day before each study visit – it should be typical of your dietary habits. It is important that every item of food/drink that is consumed is recorded and therefore it is a good idea to keep the diary with you at mealtimes and in-between meals. It will be easier and more accurate, if you fill in the diary as you go along rather than leaving it until the evening. Please indicate the time that the food/drink was consumed including the provided supplementation and note the quantity taken. A good description is required. Honesty is an essential part of the investigation so it is important that all foods and drinks are recorded!

An example of how to complete the diary is shown on the next page. A list of anthocyanin-rich foods you should avoid taking during the participation in this study is given in the Appendix..

The food diary templates that follow are for you to fill in accordingly. These can be accessed electronically or simply completed by hand written format. This will hopefully ensure the most convenient method is available.

Date: 7/11/11	**ID Number:**
Before Breakfast	
Time: 7.15 am	**Description of food/drink:** 1 mug coffee with semi skimmed milk and 1 tea spoon of white sugar.
Breakfast	
Time: 8.30am	**Description of food/drink:** Small glass of fresh orange juice (Tesco Value) 2 medium slices of wholemeal bread (Hovis) toasted, thickly spread with Utterly Butterfly, topped with one sliced banana.
Mid-Morning Snack	
Time: 10.00am **Time:** 11.30am	**Description of food/drink:** 1 Mars bar 1 packet of Hula Hoops (Original - Ready Salted)
Midday Meal	
Time: 1.00pm	**Description of food/drink:** 1 medium apple 1 baked potato with cheese, beans and side salad. 1 pint coca cola
Mid-Afternoon Snack	
Time:3.00pm	**Description of food/drink:** 1 cup of tea with semi skimmed milk and 2 teaspoons of white sugar. 1 chocolate digestive biscuit
Evening Meal	

Time: 6.00pm	**Description of food/drink:** Large bowl of pasta with pesto (green) Topped with a handful of grated cheddar cheese Pint of tap water 2 small strawberry Petis filous
Supper Snacks and During Night	
Time: 8.00pm **Time:** 1.30am	**Description of food/drink:** 2 double vodka, lime cordial and lemonade 1/2 bottle of rose wine (gallo) McDonalds Happy Meal, chicken nuggets with fizzy fanta.
Final Checklist – Any forgotten items?	
1 carton of Ribena	

DATE:	**ID Number:**
Before Breakfast	
Time:	**Description of food/drink**
Breakfast	
Time:	**Description of food/drink:**
Mid-Morning Snack	
Time:	**Description of food/drink:**
Midday Meal	

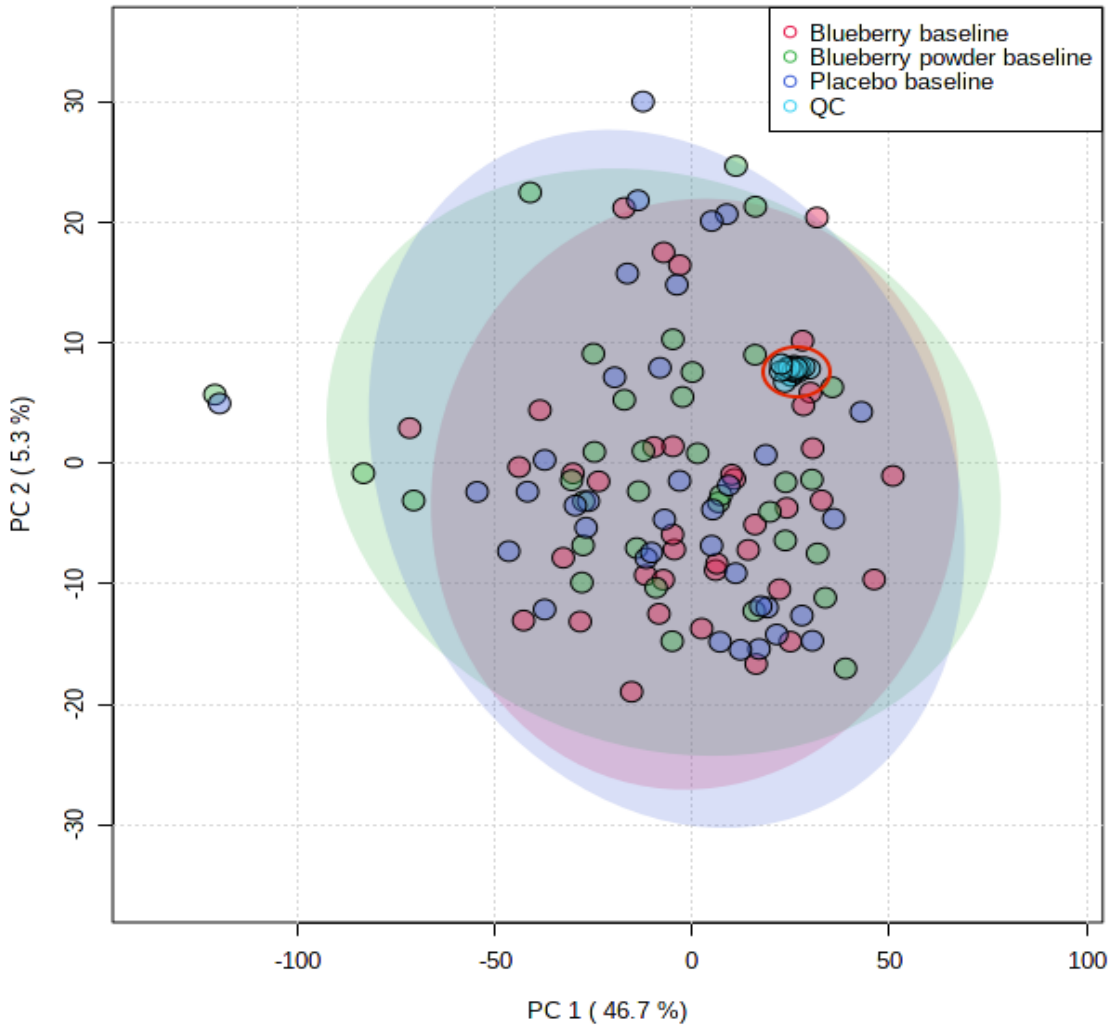
Time:	Description of food/drink:
Mid-Afternoon Snack	
Time:	**Description of food/drink:**
Evening Meal	
Time:	**Description of food/drink:**
Supper Snacks and During Night	
Time:	**Description of food/drink:**
Final Checklist – Any Forgotten Items?	

Kindly please note that following listed “purple foods” should be avoid during your participation in this study

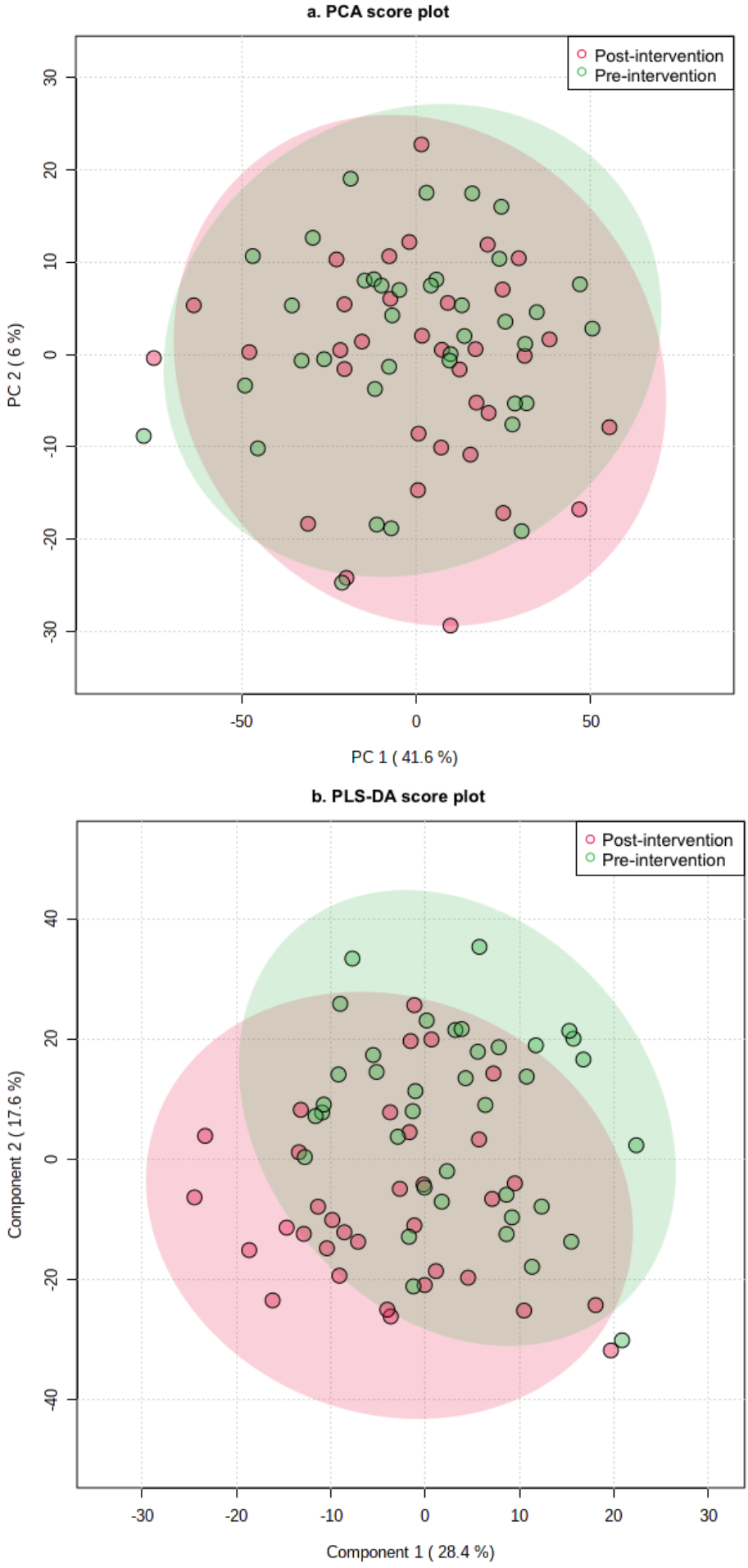
Berries
1. Blueberries 2. Blackberries 3. Blackcurrant 4. Concord grapes (dark/purple) 5. Cherries 6. Chokeberries 7. Raspberries 8. Strawberries
Other fruits
1. Plums 2. Prunes 3. Raisins

Veggies
1. Beetroot 2. Aubergine 3. Red cabbages 4. Red onions
Juices
All smoothie/juices blended from above foods
Alcohol
1. Red wine
Other foods
1. Pastry, cake or biscuits containing cherries and berries
2. Cherries/berries containing muesli and fruit flakes

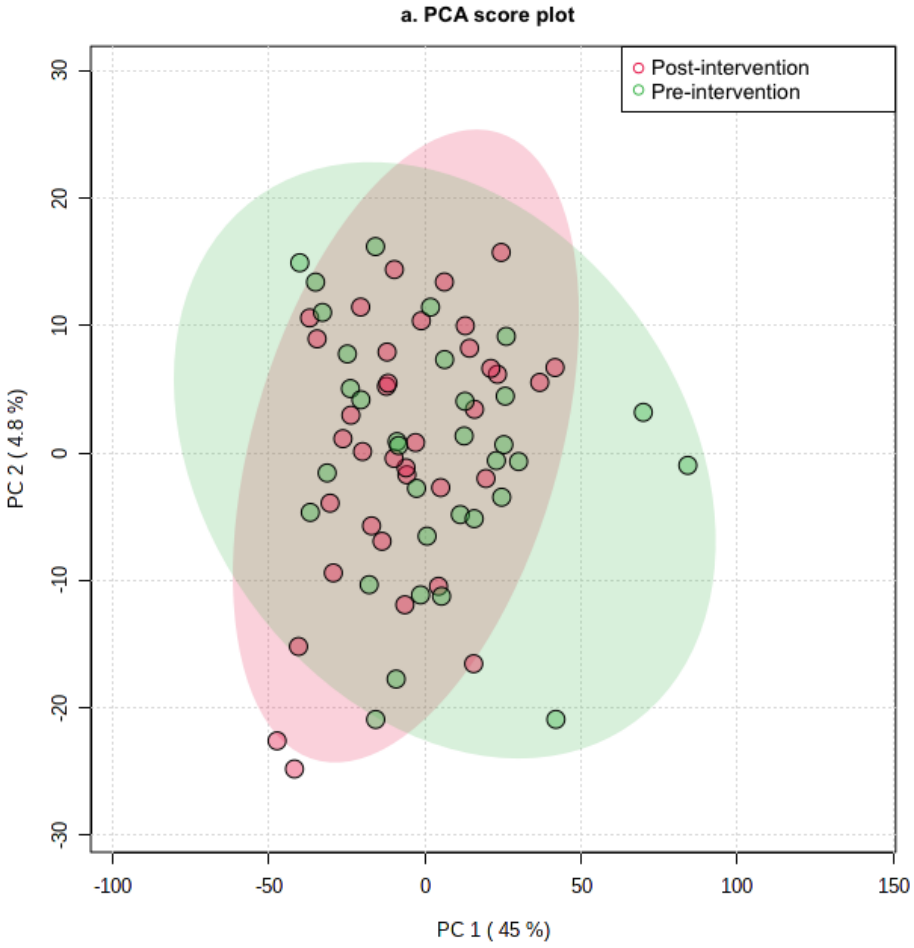
Appendix. 4.1 QC Sample and pre-intervention samples clustering in PCA

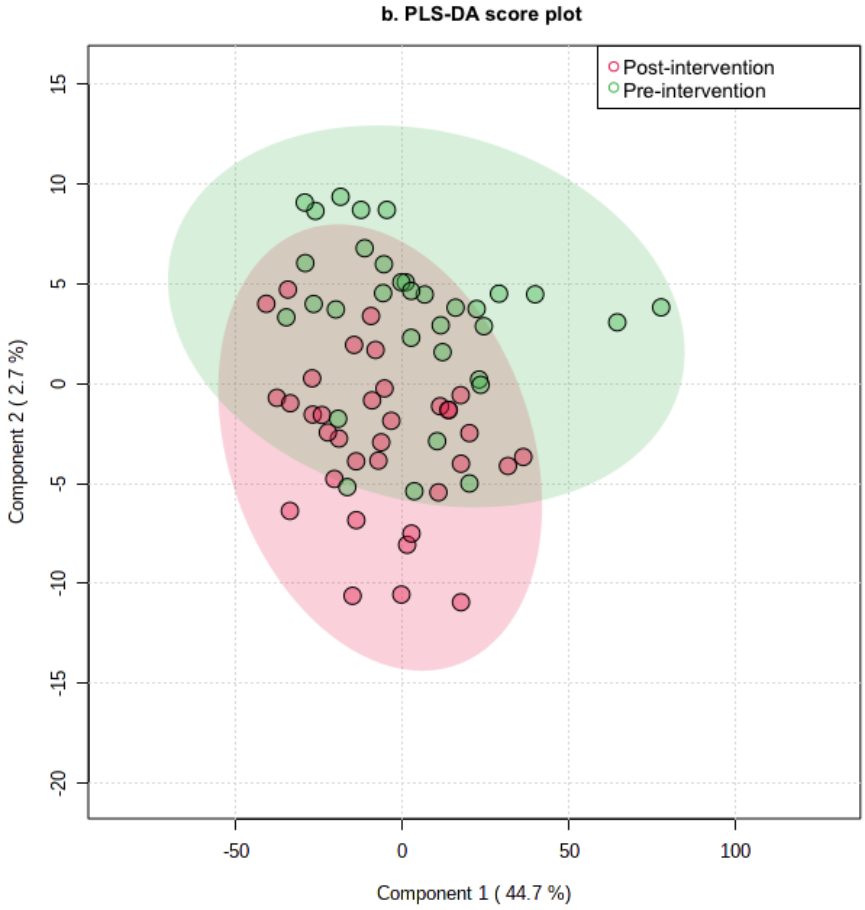


Appendix. 4.2 (a) PCA and (b) PLS-DA scores plot for pre- and post-blueberry intervention

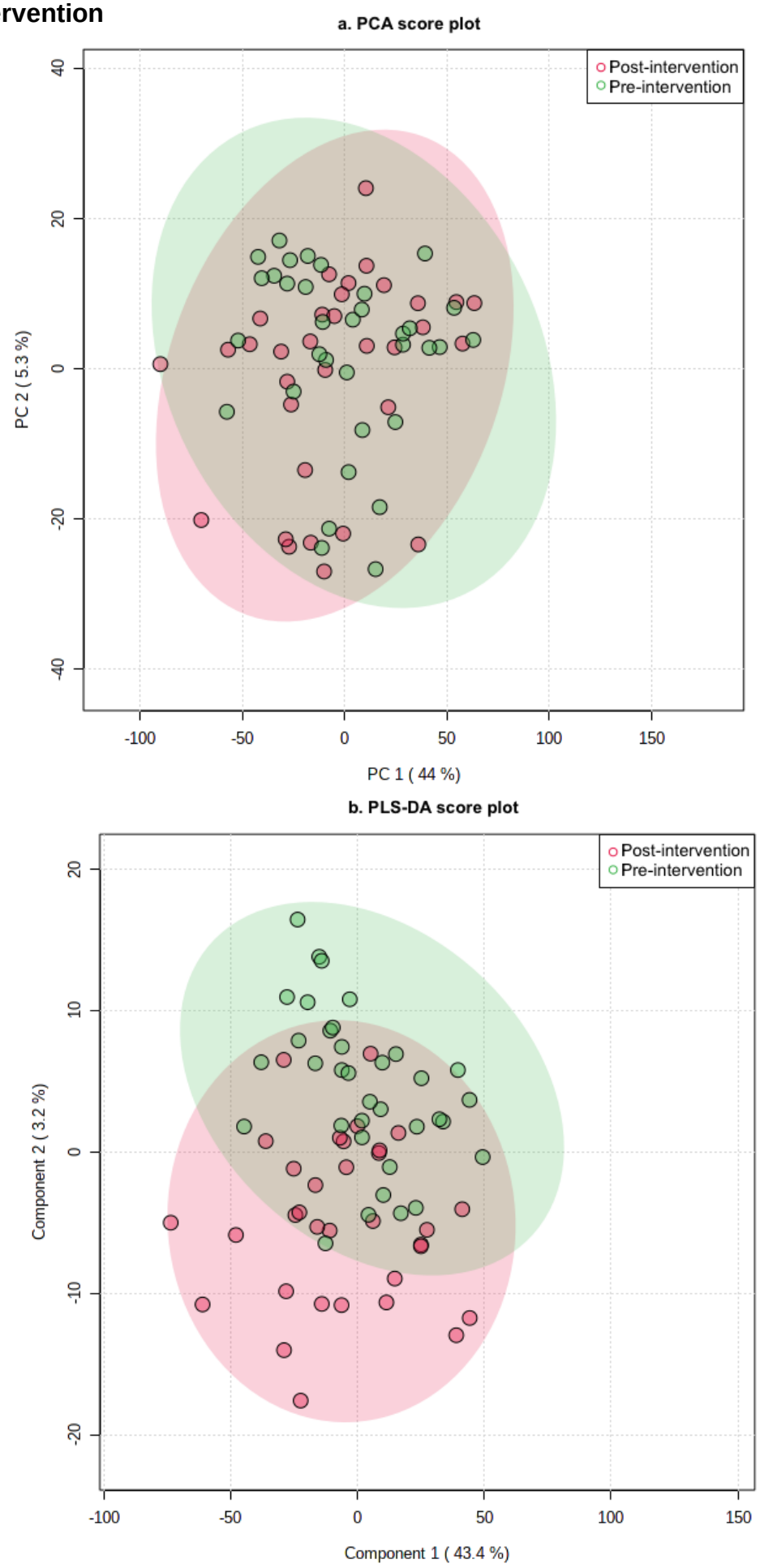


Appendix. 4.3 (a) PCA and (b) PLS-DA scores plot for pre- and post-blueberry powder intervention





Appendix. 4.4 (a) PCA and (b) PLS-DA scores plot for pre- and post-placebo intervention



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