Blueberry Anthocyanins: Assessing the Mediating Effect of a Novel Anthocyanin Metabotype on Acute Postprandial Cardiometabolic Health

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Abstract

Epidemiological evidence has associated anthocyanin intake, a subclass of flavonoids, with reduced cardiovascular disease risk; yet randomised controlled trial (RCT) data is equivocal. Interindividual variation in intervention response, mediated by differential metabolism, has been hypothesised as an underlying factor for inconsistent RCT findings. To date, however, the cardiometabolic consequences of anthocyanin metabolism profiles has not been confirmed by prospective volunteer recruitment to studies.

In this thesis, the flow-mediated dilation ultrasound technique was used to assess brachial artery endothelial function. Retrospective, exploratory analysis was performed, to establish the relevance of measuring an extended blood vessel parameters including time-to-peak, and low and high flow-mediated constriction. Subsequently, overweight older adults, prospectively recruited by quantification of four key colonic derived metabolites, were enrolled in a single-dose, crossover RCT. This study compared cardiometabolic responses for 48 hours, following blueberry or matched control intake alongside a basal metabolic rate and physical activity adjusted energy-dense meal.

Despite low study numbers restricting the capacity to test the study hypothesis (due to COVID-19 study abandonment), 'LOW' metabolisers had larger blood pressure reductions at 24 and 48 hours after blueberries. Whilst not anticipated, notably LOW metabolisers had habitually higher concentrations of key metabolites and arguably more 'healthful' diets i.e., higher wholegrains and tea; lower fat and beer intakes. When aligned with an existing background of healthier diet choices, the inclusion of blueberries appeared to reduce the burden of energy-dense meals. Additionally, assessments were made to identify whether absolute fat content and composite measures of health (QRISK3) were important design characteristics for future studies.

In summary, LOW metabolisers may uniquely experience vascular benefits from blueberry anthocyanins, and this may be influenced by their habitual diet and the capacity of their pre-existing gut microbiome to habitually produce key metabolites. Further research with adequately powered studies is required to confirm this.

Table of contents

Table of tables

Table of figures

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CHAPTER 1. Literature review on dietary anthocyanins and cardiometabolic health

1.1 Cardiovascular disease

The primary cause of death worldwide is cardiovascular disease (CVD), which accounts for approximately 17.7 million deaths per annum [1]. CVD is an umbrella term for diseases of the heart and blood vessels which include coronary heart disease, stroke, transient ischaemic attack, peripheral arterial disease and aortic disease [2]. Atherosclerosis is the underlying pathophysiology for cerebrovascular diseases and coronary artery disease which can progress to potentially devastating conditions such as myocardial infarction, stroke or acute coronary syndrome [3]. As shown in figure 1.1, atherosclerosis is a condition characterised by a build-up of hardened plaque in the arteries which over time, narrow the vessel and restrict blood flow [4]. Atherosclerosis is initiated by endothelial dysfunction which in turn is caused by a number of different factors [3]. The pathophysiology of CVD and the risk factors associated with CVD development are discussed in detail in section 1.1.1 and 1.1.2 respectively.

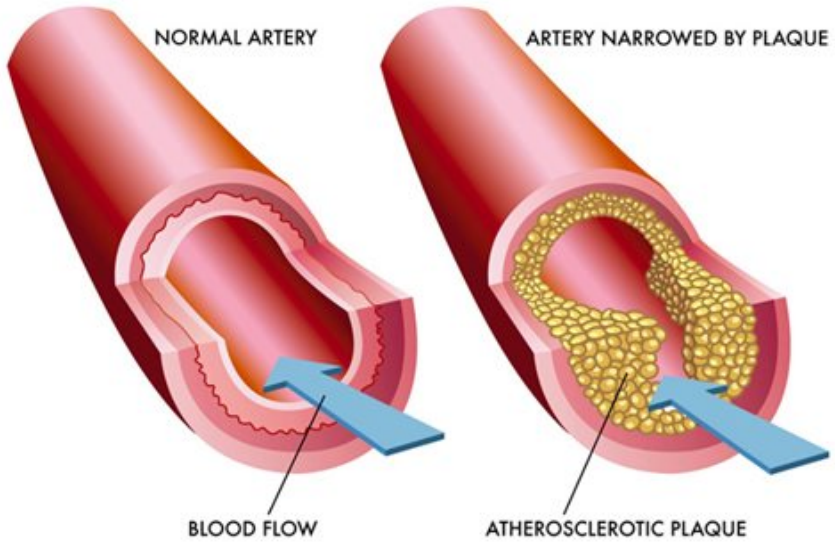


Figure 1.1: Cross-section of an artery showing the formation of an atherosclerotic plaque. Adapted from Heart Research Institute, 2018 – this image shows a normal artery (left) and an artery which has developed atherosclerosis (right); the artery is narrowed by the build-up of plaque.

1.1.1 The vascular endothelium and endothelial dysfunction

The endothelium is a single inner layer of endothelial cells (ECs), located next to smooth muscle cells in all blood vessels [5]. The functions of the endothelium include thrombosis and thrombolysis, coagulation, platelet and leukocyte interaction, regulation of vascular tone and growth, cell proliferation and angiogenesis. The definition of endothelial dysfunction encompasses the impairment of one or more of these functions [6] and is seen as a key step in the progression of CVD [7].

ECs are located next to the smooth muscle which helps to regulate blood flow through vasodilation and vasoconstriction when shear stress (the frictional force caused by increased blood flow) occurs. Nitric Oxide (NO), a signalling molecule, has a crucial role in vascular health and endothelial function; helping to regulate vascular tone. When the ECs transduce shear stress, calcium ion levels increase and NO is generated by activated endothelial NO synthase (eNOS). A figurative overview of this sequence is shown in figure 1.2. Shear stress can cause phosphorylation in the endothelium (through increased blood flow) which can also activate eNOS [5]. eNOS converts the amino acid L-arginine to NO, which then diffuses across the endothelium to the adjoining smooth muscle. In the smooth muscle, the enzyme soluble guanylyl cyclase (sGC) is activated by NO to increase the conversion of guanosine triphosphate (GTP) to cyclic guanine monophosphate (cGMP) [5]. An increase in cellular cGMP decreases smooth muscle tension, allowing the artery to vasodilate ion of the artery [8], [9].

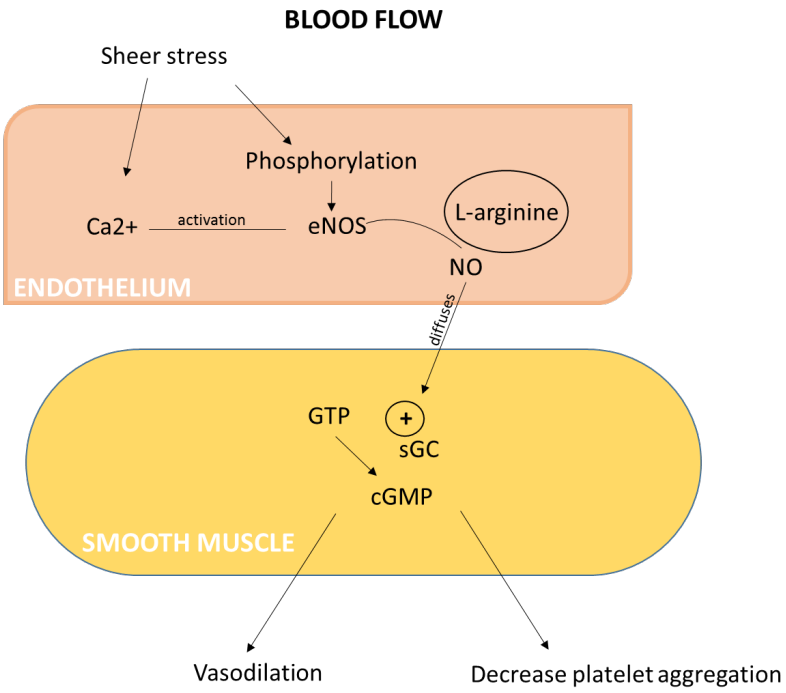


Figure 1.2: The role of nitric oxide (NO) in the pathogenesis of endothelial dysfunction. Shear stress causes Ca2+ to increase which activates endothelial nitric oxide synthase (eNOS). Shear stress also causes phosphorylation of eNOS. eNOS converts the amino acid L-arginine to NO which then diffuses from the endothelium to the smooth muscle, activating soluble guanylyl cyclase (sGC). This increases the conversion of guanosine triphosphate (GTP) to cyclic guanine monophosphate (cGMP). This process causes the smooth muscle to help with vasodilation.

1.1.2 Cardiovascular disease pathology and potentially modifiable risk factors

Endothelial dysfunction has been shown to be one of the major causes in the development of hypertension, a leading risk factor globally for CVD morbidity and mortality [10]. Although chronic elevation of blood pressure (BP) is preventable, and can be managed with medications, it is termed the 'silent killer' as it causes little or no symptoms and can go undetected for years, causing vascular damage. Even small rises of just 2mmHg in systolic BP can increase the risk of ischaemic heart disease by 7% [11].

One of the clinical predictors of hypertension is arterial stiffness [12] which is also positively associated with mortality and CVD events [13]. 'Arterial stiffness' refers to the reduction of arterial flexibility which can have consequential effects on blood flow, arterial width change and overall pressure of the artery [14]. Evidence suggests that not only can arterial stiffness lead to endothelial dysfunction, but that in turn endothelial dysfunction can lead to arterial stiffness creating a vicious circle of events [15].

The risk of developing CVD has been positively associated with elevated glucose, insulin, lipids, inflammatory markers and platelet activation. These are therefore key factors to consider when identifying CVD risk and determining the effectiveness of lifestyle interventions. Excessive glycaemic response to food has been linked to an increased risk of coronary heart disease [16]. Individuals with type 2 diabetes and pre-diabetes have higher fasting blood glucose levels and HbA1c, and a significantly higher risk of CVD than the general population [16]–[18]. Altered lipid profiles can increase the risk of atherosclerosis and thus CVD [19]. Examining lipids includes testing for high density lipoprotein cholesterol (HDLC), low density lipoprotein cholesterol (LDLC) and triglycerides. Inflammation is thought to contribute to atherogenesis, a key step in the development of atherosclerosis [20]. Raised inflammatory markers (especially C-reactive protein (CRP)) have been associated with a higher risk of coronary heart disease in both men and women [21]. As shown in figure 1.3, hyperglycaemia (leading to glucotoxicity), elevated lipid levels (leading to lipotoxicity) and inflammation share mechanisms leading to metabolic and cardiovascular diseases [22]. These are therefore key factors to consider and measure when identifying CVD risk.

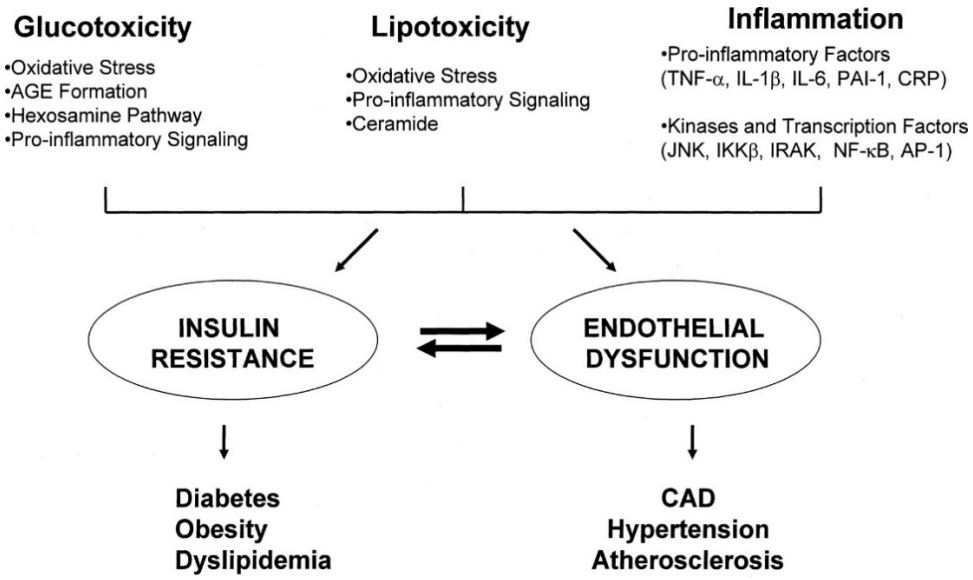


Figure 1.3: Interaction between glucose, lipids and inflammatory markers and disease. This figure shows the interaction between glucotoxicity, lipotoxicity and inflammation leading to insulin resistance and endothelial dysfunction (figure from Kim et al., 2006).

1.2 Current dietary approaches to cardiovascular disease prevention

In the UK the Eatwell guide is promoted as a tool to achieve a healthy and balanced diet, it incorporates many aspects of traditional healthy dietary patterns such as the Mediterranean diet, [23]. However, one of the three primary behavioural and dietary risk factors for death globally is reported to be a diet low in fruit [24].

Fruit and vegetables, one of the main constituents of the Eatwell guide and Mediterranean diet, are associated with an 8-13% reduction in relative risk (RR) of CVD for every 200g a day consumed (Aune *et al.*, 2017). This is due, in part, to compounds present in fruit and vegetables which may be protective such as polyphenols or nitrate. Flavonoids, the largest family of polyphenolic compounds found in fruit and vegetables, have been shown to be protective of CVD in large epidemiological studies [27]–[29]. In the UK only 27% of adults consume the recommended five portions of fruit and vegetables a day [30]. Similarly, in the USA, despite active encouragement from many different bodies to increase fruit and vegetable consumption the overall uptake has been poor [31]. For example, in the U.S.A 38% of the population report having less than one fruit a day and 23% report having less than one vegetable a day [32]. More tailored and specific advice on what fruits and vegetables to eat for a specific health benefit may help the public to implement changes to their diet.

1.2.1 The effect of energy-dense meals on cardiometabolic health

Energy-dense meals are frequently consumed in the UK with an estimated 22 million takeaways consumed by adults per week [33]. Evidence suggests that greater exposure to fast food takeaway outlets is positively associated with a higher BMI and greater risk of obesity [34]. The acute postprandial response to consuming these high-fat, energy-dense meals has been shown to cause adverse effects on cardiometabolic health; specifically inflammatory markers, blood lipids and vascular function [35]–[38].

Table 1.1 outlines studies examining the acute effects of high-fat, energy-dense meals on cardiometabolic health. As would be expected, one of the main consistent findings from

these studies is that, when compared to the control group, those having the energy-dense, high-fat meal had adversely affected lipid levels. As shown in figure 1.3, lipotoxicity contributes to insulin resistance, endothelial dysfunction and CVD. Therefore, the consequences of regular consumption of energy-dense meals, which precipitate these deleterious postprandial responses, are a potential longer-term health concern. This provides rationale to identify the protective effects of individual components of diet on metabolic markers.

Table 1.1: Studies examining the effect of energy-dense meals on cardiometabolic markers

Author	Population / age / sex	Energy-dense meal components	Energy (kcals)	Protein (g)	Carbohydrate (g)	Fat (g)	% Calories from fat	Cardiometabolic effects
[35]	16 participants, with Alzheimer's disease (*n* = 7, age 78 ± 9.3, BMI 25 ± 2kg/m^2) or age matched controls (*n* =9, age 76 ± 6.8, BMI 30.6 ± 9.3 kg/m^2)	1 plain bagel, 4 tsp cream cheese, 1 large egg, 2 tsp margarine, 1 cup of cantaloupe, 1 cup of whole milk	644	26	72	29	41	**Triglyceride:** Levels peaked at 3.5hrs, returned to baseline at 6hrs (both groups)
[38]	18 male and females, age 56 ± 3.2 years, BMI 35.3 ± 2.0 kg/m^2, (with T2DM for >5y but not on insulin)	2 scrambled eggs (no added fat), hash brown potatoes (70g), 2 buttermilk biscuits, butter (15g) and sausage patty (57g)	766	30	50	50	59	**Triglyceride:** Peaked at 4hrs, started to dip at 5 hours, had not returned to baseline at 6hours **Cholesterol:** Peaked at 6hrs, did not reach baseline **Glucose:** Peaked at 1 hr, returned to baseline by 4 hrs **Insulin:** Peaked at 2 hrs, returned to baseline by 6 hrs **HOMA-IR:** Peaked at 1-2 hrs, returned to baseline by 6 hrs
[37]	Male and females (BMI 31.7kg/m^2, age 49, & 2 traits of metabolic syndrome)	Grilled cheese sandwich (with either vegan cheese or cheddar cheese and wholemeal bread) & a blended beverage	1,002	28	104	57	50	**Triglycerides:** Not reached peak by 6hrs **Cholesterol:** Peaked at 1hr, not returned to baseline at 6hrs **Inflammatory markers:** IL-6, IL-8, CRP changed significantly over the 6hrs post-meal
[36]	Males (BMI 27.6kg/m^2, age 46yrs, labelled as good general health)	Unknown - took acai smoothie supplement with high-fat meal	626	9	57	50	71	**FMD:** Peaked at 2 hours, almost at baseline at 4hrs, went back up at 6hrs.

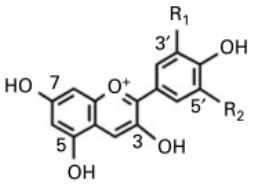
[39]	Males (BMI 27kg/m^2 for intervention group + 26.4kg/m^2 for control, mean age for both groups 51yrs) admitted for MI	Pasta, boiled chicken breast, green peas and mayonnaise	1,000	~32.5	~71	65	60	**Triglycerides:** Peaked at 3 hours - had not returned to baseline at 6hrs
[40]	Male and females, age 56±6yrs, BMI 39.5±6.5 kg/m^2, with T2DM and elevated waist circumference (diabetes for >5yrs not on insulin)	2 scrambled eggs, 2 tsp butter, hash brown potatoes, 2 buttermilk biscuits and a sausage patty	974	31	55	70	65	**Triglycerides:** Not reached peak at 4hrs
[41]	Hyperlipidaemic male and females, age 50.9±15, BMI 29.2±2.3kg/m^2	Bagel, cream cheese, whole milk, egg, margarine, cantaloupe	961	37	134	31	29	**Triglycerides:** Peaked at 4 hours - not at baseline at 6 hours

Table examines different study's approach to testing a fixed high-fat, energy-dense meal and testing various cardiometabolic effects include serum lipids, lipoproteins, glucose, FMD and insulin. Abbreviations: Type 2 diabetes (T2DM), body mass index (BMI), myocardial infarction (MI), Alzheimer's disease (AD), flow-mediated dilation (FMD), interleukin 8 (IL-8), interleukin 6 (IL-6), C-reactive protein (CRP) and homeostatic model assessment of insulin resistance (HOMA-IR).

1.3 Flavonoids and anthocyanins

Flavonoids are polyphenolic constituents found in many fruit, vegetables and herbs [42]. Flavonoids, which are secondary metabolites of plants, can be divided into six main sub-classes; flavanols, flavones, flavanones, isoflavones, flavan-3-ols and anthocyanins, which are differentiated based on the oxidation or unsaturation of the C ring and its carbon [42], [43]. Habitual intakes of several flavonoid sub-classes have been associated with a wide range of health benefits including vascular health, glycaemic control and cognitive function [28], [44]–[46]. In particular, there is mounting evidence from both epidemiological studies and randomised control trials (RCT) which highlight the potential benefits of anthocyanins, a subclass of flavonoids, specifically for cardiometabolic health.

Anthocyanins are considered to be non-essential nutrients responsible for the purple, blue and red colours in plant based foods [42]. Their colours differ depending on their chemical structure and pH (see figure 1.4) [43]. Anthocyanins are present in a range of fruits and vegetables, such as berries, aubergines and red grapes (see table 1.2; which shows particularly abundant sources of anthocyanins). Notably, not all red, blue or purple plant foods contain anthocyanins; for example, beetroot has a deep purple colour but contains no anthocyanin, its pigment is due to betalains [47].



Anthocyanidin	R_1	R_2	Colour
Pelargonidin	H	H	Orange-red
Cyanidin	OH	H	Red
Delphinidin	OH	OH	Pink
Peonidin	OCH_3	H	Bluish purple
Petunidin	OCH_3	OH	Purple
Malvidin	OCH_3	OCH_3	Redish purple

Figure 1.4: Anthocyanin structure and list of sub-compounds (taken from [48]).

Anthocyanins are conjugated anthocyanidins and can be divided into six main compounds; cyanidin, malvidin, delphinidin, peonidin, petunidin and pelargonidin (see figure 1.4) [48]. Unlike other flavonoid subclasses, anthocyanins are predominantly found and consumed in their glycosylated form [49], are linked to a sugar molecule and differ in structure depending on their sub-compounds [48].

Table 1.2: A list of rich sources of anthocyanins.

Food	**Mean - Phenol Explorer (mg/100g FW)** [50]	**Mean - USDA (mg/100g)** [51]
Highbush Blueberry	134.0	163.3
Sweet Cherry	171.4	32.0
Sour Cherry	54.4	33.4
Cranberry	49.9	104.0
Elderberry	1,316.7	485.3
Lingonberries	60.2	40.2
Red Grape	/	48.0
Strawberry	73.0	27.0

Values taken from both Phenol explorer (http://phenol-explorer.eu/) and the USDA database [51], [52] as a comparison of how flavonoid content of foods can vary greatly between databases.

1.4 Potential vascular benefits of anthocyanin consumption

1.4.1 Epidemiological evidence

Summarising the epidemiological data presented (see table 1.3), there is evidence to suggest that higher intakes of anthocyanins are protective of developing coronary heart disease and improve risk markers associated with CVD development. These data are predominantly from studies performed in the USA, with one from the UK, one from Finland and one from Denmark (table 1.3). Increased consumption of anthocyanins has been associated with a reduced relative risk (RR) of CVD (RR 0.91, $p<0.03$) and total mortality (RR 0.9, $p<0.01$) [27]. Specifically, higher anthocyanin intakes are associated with a reduced risk of coronary heart disease in both men and women of varying ages; with reported hazard ratio (HR) and RR of coronary heart disease ranged from 0.68-0.88 [27]–[29], [53]. The wide range in RR and HR may be due to differences in sex, menopausal status and age. In terms of the intermediate markers of CVD risk, the increased consumption of anthocyanins has been associated with reduced BP in both men (RR 0.87, 95% CI) and women (RR 0.93, CI 95%) [45]. Similarly, a higher consumption was associated with decreased arterial stiffness [54]; measured by pulse wave velocity (PWV) and augmentation index (AIx).

Anthocyanin consumption, however, has not been universally associated with a reduced risk of CVD and in particular stroke. McCullough *et al.*, (2012) and Mursu *et al.*, (2008) found no association between anthocyanin intake and CVD mortality. Specifically for stroke, anthocyanins have previously been thought not to be as protective [27], [29], [55], [56]. However recently a study found median intakes of 36mg/day of anthocyanins was associated with a reduced risk of ischaemic stroke in men and women (RR 0.85 (0.79-0.93) CI (95%)) [57]. The reason for the differences in findings for stroke and coronary heart disease is not clearly understood. The pathophysiology and risk factors for both diseases are very similar making it difficult to determine with any certainty why the differences are seen within epidemiological data. One possible theory is how anthocyanins are absorbed and cross the blood-brain barrier (BBB) [29]. Other flavonoid subclasses, such as flavanones, (which evidence suggests are protective of stroke) have shown to have a higher cell uptake than anthocyanins at the BBB [58].

Interestingly, the results for anthocyanins and CVD are seen across very low daily intakes of anthocyanins. For example, Cassidy *et al*., (2013) saw a 32% reduction in coronary heart disease development with mean anthocyanin intakes of 25.1mg (equivalent to two large strawberries) a day. However, these intakes varied across studies - Cassidy *et al.*, (2011) observed in a pooled analysis that intakes of more than one serving of blueberries a week (equal to approximately 107mg of anthocyanins), were associated with a 10% reduction in hypertension rate. The same author observed that > 3 portions of anthocyanin rich food per week, was associated with a decreased risk of developing myocardial infarction in women [53]. The reason for the variation in an effective anthocyanin dose is likely to be multi-faceted. As population-based studies use food frequency questionnaires (FFQ) it can be difficult to quantify the exact intake required for vascular effects. This leads to uncertainty regarding the effective doses of anthocyanins and whether different levels are needed for different populations. Well-designed, controlled studies have the advantage of testing the efficacy of a single intervention. RCTs offer an opportunity to explore hypotheses that epidemiological data have highlighted.

Table 1.3: Epidemiological associations between anthocyanins and cardiometabolic health

Author	Intake of anthocyanins (mg/day)	Outcome Measured	Population / sex / age / BMI / country	Results
[28]	Mean 24.7mg/day (quintile with highest consumption)	Incidence of CHD	30,239 men and women 45 years USA	**Coronary Heart Disease:** HR 0.71 (95% CI, 0.52, 0.98, p value 0.04)
A. [54]	8.4-23.6mg/day (mean / SD -> 17.7-14.9, values for interquartile range)	Blood pressure, augmentation index	1,898 twin women, 18-75 yrs., mean BMI 25.3kg/m^2, UK	**Central SBP:** -3.04 $\pm$ 1.41 (p value 0.02) **Mean arterial pressure**: -2.31 $\pm$ 1.16mm Hg (p value 0.04) **Peripheral DBP:** -1.85 $\pm$ 0.99mm Hg (p Value 0.06) **Central DBP:** -1.86 $\pm$ 1.01 mm Hg (p value 0.05)
B. [54]	Interquartile range 8.4-23.6mg/day (mean / SD -> 17.7-14.9, values for interquartile range)	Pulse wave velocity	728 (taken from larger cohort, those with the highest intakes of anthocyanins)	**Pulse wave velocity:** -0.4 $\pm$ 0.22m/s (p value 0.04)
A. [45]	Mean inter quartile range 14mg/day (5.4-17.8 SD), mean of quintile with highest consumption 18mg/d	Incident of hypertension (14yr follow-up)	81,242 women aged 25-42yrs (mean age 36), BMI 24.3 + 4.3kg/m^2, USA	**Hypertension:** 0.87 RR (0.81-0.92), CI 95%, P value <0.00
B. [45]	Mean inter quartile range 12.5mg/day (4.6-15.9 SD), mean of quintile with highest consumption 16.2mg/d	Incident of hypertension (14yr follow-up)	46,672 women aged 30-55yrs (mean age 55), BMI 24.8 +5.0 kg/m^2, USA	**Hypertension:** 0.93 RR (0.88-0.98), CI 95%, P value 0.02
C. [45]	Mean inter quartile range 15.2mg/day (5.8-19.3 SD), mean of quintile with highest consumption 21.9mg/d	Incident of hypertension (14yr follow-up)	23,043 men aged 40-75yrs (mean age 56), BMI 25.2 $\pm$ 3.0kg/m^2, USA	**Hypertension:** 0.99 RR (0.90-1.09), CI 95%, P value 0.82
[27]	0.2 median (range 0.01-1040mg/day) anthocyanidins	Incident of CHD, stroke, CVD and mortality (16yr follow-up)	34,489 postmenopausal women, aged 55-years, USA	**Coronary Heart Disease:** RR 0.88 95% CI (0.78, 0.99), P value 0.03 **Stroke:** RR 1.01 95% CI (0.83, 1.24) p value 0.896 **Cardiovascular Disease:** RR 0.91 95% CI (0.83, 0.99) p value 0.03 **Total mortality:** RR 0.9 95% CI (0.86, 0.95) p value <0.00
A. [55]	Interquartile range median 3.8-22.2mg/day, median of quintile with highest consumption 22.2mg/d	Incident of CVD mortality (7yr follow-up)	98,469 men (mean age 70yrs) and postmenopausal women (mean age 69yrs), USA	**Cardiovascular Disease Mortality:** **Men:** RR 0.91 (0.77, 1.06) p value 0.3 **Women:** RR 0.82 (0.69, 0.99) p value 0.06 **Men & women:** combined – 0.86 RR (0.76, 0.97) p value 0.04
B. [55]	Interquartile range median 3.8-22.2mg/day, median of	Incident of IHD mortality (7yr follow-up)	98,469 men (mean age 70yrs) and postmenopausal	**Ischaemic Heart Disease:** **Men:** RR 0.81 (0.65, 1.00) p value 0.2 **Women:** RR 0.81 (0.60, 1.07) p value 0.2

	quintile with highest consumption 22.2mg/d		women (mean age 69yrs), USA	Men and women – RR 0.79 (0.67, 0.94) p value 0.06
C. [55]	Interquartile range median 3.8-22.2mg/day, median of quintile with highest consumption 22.2mg/d	Incident of stroke	98,469 men (mean age 70yrs) and postmenopausal women (mean age 69yrs), USA	**Incident of Stroke:** **Men:** RR 0.84 (0.59, 1.20) p value 0.2 **Women:** RR 1.05 (0.76, 1.46) p value 0.6 **Men and women:** RR 0.95 (0.75, 1.20) p value 0.7
A. [29]	Median of quintile with highest consumption 26.3mg/day	Incident of coronary artery disease (24yr follow-up)	43,880 men, aged 39-77years (median age 53yrs), USA	**Total myocardial infarction cases:** HR 0.97 95% CI (0.87, 1.07) p trend 0.44 **Nonfatal MI cases**: HR 0.87 95% CI (0.75, 1.00) p value = 0.04 p-trend = 0.098 **Fatal MI cases:** HR1.10 95% CI (0.94, 1.28) p trend 0.56
B. [29]	Median of quintile with highest consumption 26.3mg/day	Incident of stroke (24yr follow-up)	43,880 men, aged 39-77years (median age 53yrs), USA	**Total stroke cases:** HR 1.00 95% CI (0.85, 1.17), p value 0.71 **Ischemic stroke**: HR 0.93 95% CI (0.75, 1.15) p value 0.51 Haemorrhagic stroke – HR 1.06 (0.69, 1.61) p value 0.93
[53]	2-35mg/day (mean of 25.1mg/d for quintile with highest consumption)	Incident of CHD (MI risk) 18yr follow-up	93,600 women, aged 25-42yrs (median age 48.9yrs), USA	**Coronary Heart Disease:** HR 0.68 (0.49, 0.96) p value 0.047
A. [56]	6.2mg/day	Incident of ischemic stroke (15.2yr follow-up)	1,950 men aged 42-60yrs, Finland	**Ischemic Heart Disease:** RR 0.88 95% CI (0.47, 1.62) p value 0.81
B. [56]	6.2mg/day	Incident of CVD mortality (15.2yr follow-up)	1,950 men aged 42-60yrs, Finland	**Cardiovascular Disease Mortality:** RR 0.99 95% CI (0.62, 1.85) p value 0.19
[57]	Median intakes 36mg/day, (range 24-53mg/day)	Incident of ischaemic stroke (21yr follow-up)	55,169 mean and women, aged 52-60years, Denmark	**Ischaemic stroke:** RR 0.85 (0.79-0.93) CI (95%) p <0.05

Comparing lowest intake of anthocyanins with the highest intake of anthocyanins.
Abbreviations: coronary heart disease (CHD), cardiovascular disease (CVD), MI (myocardial infarction), USA (united states of America), SBP (systolic blood pressure), DBP (diastolic blood pressure), IHD (ischaemic heart disease).

1.4.2 Human randomised controlled trials

This section specifically examines the potential effect of anthocyanins on a number of cardiometabolic health markers in randomised controlled trials (RCTs). Specific focus is given to endothelial function, blood pressure, arterial stiffness and serum lipids and lipoproteins to identify current research gaps.

1.4.2.1 Endothelial Function

To date, a number of RCTs have examined the effects of a single dose of anthocyanins (or anthocyanin rich foods) (table 1.4) and repeated doses of anthocyanins (table 1.5) on the cardiometabolic health of different populations. Endothelial function is frequently assessed non-invasively by either flow-mediated dilation (FMD) (described in more detail in section 1.7.1) or reactive hyperaemia index (RHI). Six acute, single-dose studies measured endothelial function up to 24 hours after consumption (table 1.4). In all instances, except one [59], anthocyanin intake improved endothelial function, as measured by either FMD or RHI [60]–[64].

In a study of 10 healthy men, a significant increase in FMD was observed (compared with the placebo treatment) 1-2 hours (2.4 $\pm$ 0.5%) and 6 hours (1.2 $\pm$ 0.6%) after consuming a blueberry drink (containing 310mg of anthocyanins; equivalent to 190g of fresh blueberries). In addition, levels of NADPH oxidase (a precursor for hypertension and atherosclerosis, precipitated through endothelial dysfunction – see section 1.1.1) were also reduced following blueberry intake and coincided with the reported increases in FMD [64]. In this study, which incorporated a dose-response element (testing doses of anthocyanins between 129-724 mg), it was observed that higher doses of anthocyanins (517mg and 724mg of anthocyanins) did not improve FMD responses above those seen at 310mg of anthocyanins [64]. These data may demonstrate a potential plateau of FMD effects, above an intake threshold of anthocyanins. Similarly, a study of 12 hypercholesterolemic participants significantly improved FMD ($p < 0.05$) after purified anthocyanin intake (320mg) compared with the control group [62]. Notably this increase in FMD was correlated with an increase in the vasodilator cGMP ($p<0.05$) [62].

The impact of processing (through cooking) anthocyanins on endothelial function has been examined in a study providing a blueberry drink (339mg anthocyanins), a blueberry baked bun (196mg anthocyanins) and a control baked bun to 10 healthy male volunteers (27 $\pm$ 1yrs, BMI 25 $\pm$ 0.8kg/m^2) [63]. In this study it was found that both the processed anthocyanins (included in the baked products), and non-processed anthocyanins (included in the blueberry drink) increased FMD at 1 and 2 hours ($p < 0.05$) after consumption compared with the control. However the non-processed blueberry drink further increased FMD compared to the processed anthocyanins and control at 6 hours ($p < 0.05$) [63]. A further study on healthy male volunteers saw improvements in FMD at 1, 2, 4, 6 and 8 hours following cranberry juice (providing between 6.8mg and 23.2mg of anthocyanins depending on the dose provided – lower than doses provided in previously mentioned interventions) intake with maximal effects at 4 hours from the polyphenol dose containing 23.2mg of anthocyanins [61]. Notably this study is different from those previous RCTs mentioned as the study intervention, cranberry juice, contained a large variety of other polyphenolic compounds making it more difficult to attribute the effects to anthocyanins specifically [61]. Del Bo' *et al.*, 2013 found no improvement in RHI at 24 hours in their cross-over designed study in 10 healthy males. Interestingly they found that despite their cross-over design there was high interindividual variation in the percentage changes in RHI which could have affected results (Del Bo' *et al.*, 2013). Interindividual variability in response to anthocyanins will be explored further in section 1.6.

RCTs examining endothelial function in response to repeated doses of anthocyanins (or anthocyanin rich foods) have had variable responses (table 1.5). Similar to the single dose intervention studies in table 1.4, endothelial function is measured by both FMD and RHI. Zhu et al., 2011 examined 320mg purified anthocyanins in 150 men and women after 12 weeks and found significant imporvements in FMD and cGMP ($p<0.05$). Two studies examining repeated doses in men and women with metabolic syndrome found improvements in endothelial function measured by FMD and RHI [65], [66]. Despite these improvements from chronic anthocyanin intakes, similar to the acute RCT interventions, not all studies found improvements on endothelial function. Riso *et al.*, 2013 found that after 6 weeks of 400mg anthocyanins daily in 18 healthy men, there was no significant effect on RHI.

1.4.2.2 Blood pressure

Similar to endothelial function, the data from short- and long-term intervention studies examining the effect of anthocyanins on blood pressure are inconsistent (table 1.4 and 1.5). For example, a study looking at the effects of 1 x 300ml serving of cherry juice (containing 207mg of anthocyanins) in both younger (mean age 21.8 $\pm$ 0.97) and older adults (mean age 77.5 $\pm$ 6.2) observed a statistically significant mean decrease of 5.5mmHg in systolic BP and 5.5mmHg in diastolic BP [68]. Perhaps surprisingly, given the deleterious effects of smoking on vascular function, young male smokers who had a single 300g serving of fresh blueberries (348.3mg of anthocyanins) had significantly decreased systolic BP, compared with the control [60]. Conversely, studies in healthy men have tended to show no benefit of anthocyanins on BP [60], [61], [64], [67], [69]; in these studies the participants had normal BP levels at baseline and, as a consequence, may be less likely to see vascular benefits (table 1.4 and 1.5).

Interestingly, Kent *et al.*, (2016) (who reported an effect on BP) found no effect on postprandial BP when the 300ml cherry drink was given as 3 x 100ml servings (100ml per hour (each with 69mg of anthocyanins), for 3 hours). These data suggest that, in acute postprandial RCTs, a minimum dose may be needed to elicit acute vascular effects. In comparison, population-based data suggest improvements in BP at habitual doses of as little as 12.5mg a day (section 1.4.1). Although a long-term study has yet to confirm this, it is plausible that long term improvements can be attained and sustained from smaller, and potentially more infrequent intakes over a prolonged period of time.

A number of studies have reported on the impact of repeated doses of anthocyanins on BP – generally on resting BP observed in a clinical setting. Across three of these studies, reductions in BP have been observed and in populations which varied in both age and health status. The first examined post-menopausal women with pre and stage-1 hypertension (American Heart Association, (2018) describes pre -hypertension as 120-129/80mmHg and stage-1 hypertension as 130-139/80-89mmHg) for 8 weeks supplementing daily with either 22g of freeze dried blueberries (470mg of anthocyanins) or a placebo powder [71]. Reductions in BP were seen at 8 weeks (not 4 weeks) in this group with participants'

diastolic BP moving into the normotensive range (recognised by American Heart Association, (2018) as <120/80mmHg) and systolic BP into the pre-hypertension stage. The study authors also reported significant improvements in brachial to ankle PWV ($p<0.01$) with a mean reduction of 6.5%. The second study was conducted in men and women with metabolic syndrome supplementing with 50mg of freeze dried blueberry powder (742mg of anthocyanins) split into two doses (8 hours apart) for a total of 8 weeks [72]. 742mg of anthocyanins is equivalent to over 500g of blueberries in one day, which is unrealistic for most people to consume. A mean change of 7.8mmHg in systolic BP and 2.5mmHg in diastolic BP was seen at the end of the study, similar results to those seen in Johnson *et al.*, 2015. Interestingly, two further studies on a similar population, men and women with metabolic syndrome supplementing 374mg anthocyanins daily for 6 months [65] and 290.3mg anthocyanins daily for 6 weeks [66] observed no improvements in BP.

The inconsistent findings from several studies (table 1.4 and 1.5) on BP following anthocyanin consumption are contrary to the epidemiological data on reduced incidence of hypertension ([45], [54]. A potential disadvantage of the RCTs discussed is how BP is measured, and this could influence the results. Most of these studies have chosen to measure BP at rest, this is known to have its limitations such as a lack of measurements and potential to show white-coat hypertension [73]. The gold standard measurement of BP is ambulatory blood pressure monitoring (ABPM) as it captures an entire 24-hour period during normal activities and environments for the participant. However, two studies used 24 hour blood pressure monitoring after consumption of anthocyanins also found no improvements [66], [69].

1.4.2.3 Arterial stiffness

Although there is promising epidemiological data for long term anthocyanin intake being associated with improved arterial stiffness [54] the evidence from RCT's is equivocal. Pulse wave velocity (PWV) and augmentation index (AIx) are both non-invasive measures of arterial stiffness (discussed in further detail in section 1.7.2). Table 1.4 examines three studies which have measured arterial stiffness and found no improvements after a single dose of anthocyanins [60], [61], [64]. Similarly Riso et al., 2013, who also examined the effect of daily anthocyanins (400mg) for 6 weeks on arterial stiffness (Aix) in healthy men,

found no improvements. This is comparable to the lack of effect seen on BP in healthy men after acute anthocyanin consumption (section 1.4.2.2).

Conversely, some longer-term, repeated dose, studies (table 1.5) have found significant improvements in arterial stiffness after anthocyanin consumption. A study in 14 healthy men and women who consumed 288mg anthocyanins for 14 days (from the purple majesty potato) found improvements in carotid to femoral PWV compared with the control group [74]. Interestingly 48 post-menopausal women, with pre or stage 1 hypertension, had improvement in brachial to ankle, but not carotid to femoral, PWV after 8 weeks of daily anthocyanins (469.8mg) compared with the control [71]. A much smaller dose of anthocyanins (50mg from blood orange juice) given to men and women (age 52.2±13.6yrs, BMI 29±5.1) daily for 4 weeks found no improvement in carotid to femoral or brachial to ankle PWV [75]. It should be noted that Hollands *et al.*, 2018 found no improvements on any cardiometabolic outcomes measured (table 1.5). This may be due to the low dose of anthocyanins provided which was much smaller than used in studies that observed benefits [75].

1.4.2.4 Cardiometabolic and inflammatory markers in blood

There are short term, repeated dose interventions researching the impact of anthocyanins on a number of metabolic risk factors given their contribution to atherosclerosis and ultimately risk of CVD [5]. One such metabolic risk factor is inflammation, which negatively impacts endothelial function, an important predictor of CVD [76]. Epidemiological data suggests higher habitual intakes of anthocyanins were associated with improvements in CRP, a marker of inflammation [77]. However, short term repeated dose interventions have not found anthocyanins to be associated with any changes seen in inflammatory markers [71], [72], [78]. This may be because of study duration (all studies were less than 8 weeks) or because maintained changes in CRP are more likely to be observed in those with habitual anthocyanin intakes.

In addition to inflammatory markers, serum lipid and lipoprotein levels are markers of CVD risk. Lipids and lipoprotein levels have been assessed in both acute and chronic anthocyanin RCTs (table 1.4 and 1.5). Increased LDLC levels and reduced HDLC levels can promote

atherosclerosis [3] and thus are important to examine in relation to CVD risk. After 8 weeks supplementation with freeze dried blueberries in obese men and women had decreases in oxidative LDL (-28%, p=0.01) [72]. However, in this same study triglycerides, LDLC and HDLC were all unchanged after 8 weeks. Conversely, HDLC increased (p = 0.03) in men and women given 320mg anthocyanins daily for 12 weeks when compared with the control [62]. Similarly in another study, after 6 months supplementation of blueberry anthocyanins (374mg) in men and women with metabolic syndrome, HDLC levels had increased compared with control (p=0.03) [65]. However, despite these data no other studies in table 1.4 and 1.5 measuring HDLC reported improvements following anthocyanin intake [66], [72], [74], [75]. In fact, no changes were reported in glucose, triglycerides or LDLC following acute or chronic anthocyanin supplementation in any of the studies in table 1.4 and 1.5.

1.4.2.5 Limitations of anthocyanin randomised controlled trials

Notably in many of the single dose interventions described above (detailed in table 1.4) included a variety of potentially bioactive compounds, including anthocyanins, and thus establishing the relative contribution of each is problematic. For example, cranberry juice contains a variety of potentially bioactive compounds (including more flavonols than anthocyanins), and there is evidence that flavonols influence vascular function; with changes in both NO and BP [79]. In a study by Del Bo' *et al.*, (2014) changes in RHI were seen in young male smokers but it is difficult to interpret these findings and apply them to the general population. Smokers may have increased vascular dysfunction as a result of smoking and ROS [80].

Czank *et al.*, (2013) has shown anthocyanin metabolites to still be circulating in the body 48 hours after initial consumption. One of the limitations of the studies shown in table 1.4 is that most only examine the vascular affects up to 8 hours, some examining the effects for much less. Rodriguez-Mateos *et al.*, (2013) found that for some doses (310mg and 517mg of anthocyanins) FMD was still significantly different from the control at 6 hours as were the serum metabolites (the last time point for outcomes measured). It would therefore, have been informative to examine the vascular effects between 8 and 48 hours and whether they had returned to similar levels seen in the control – thus far no RCTs have done this. As noted in section 1.4.2.1, Del Bo' *et al.*, 2013 found high interindividual variability in RHI response in

their study looking at anthocyanin consumption over 24 hours. Czank *et al.*, (2013) suggested that the wide interindividual response may mediate vascular outcomes. It is therefore also possible that differences in response to anthocyanins may be due to differential metabolism profiles. Section 1.6 describes in detail the bioavailability and metabolism of anthocyanins.

Reductions of 10 mmHg of systolic BP are associated with reduced risk of major cardiovascular events which are likely to only be achieved via pharmacological intervention [82]. Although none of the studies detailed in tables 1.4 and 1.5 discussed reported changes of this magnitude, their results are similar to diet and lifestyles interventions aimed at lowering BP such as physical activity or a healthy diet [83], [84]. Longer term trials are required to establish whether these and/or further reductions can be sustained. Where possible, these studies should assess BP using ambulatory techniques, which may provide a more robust overview of BP in response to anthocyanins. Using freeze-dried fruit offers the least loss of nutrients [71] and means researchers can test a batch for the exact anthocyanin content. Many different factors affect the anthocyanin content of fresh fruit such as growing conditions or storage [51]. This may mean participants in a fresh fruit intervention may not be consuming the same amount of anthocyanins and this should be considered when planning future research.

The studies described show a wide diversity in response to anthocyanin interventions. Emerging literature suggests that the variation in the way individuals metabolise anthocyanins may also mediate vascular outcomes. This concept is described in detail in section 1.6.

Table 1.4: Single dose, postprandial, randomised control trials examining anthocyanins and markers of cardiometabolic health.

Author	Duration (hours)	Population / age / sex	Dose	Results
[64]	6	10 healthy men (aged 18-40yrs)	Doses of 129mg, 258mg, 310mg, 517mg or 724mg of anthocyanins in freeze-dried blueberry powder mixed with 500ml water or control.	**FMD:** 310mg: Significantly increased from control and baseline at 1, 2 and 6hrs 517mg: Significantly increased from control at 1, 2 and 6hrs and from baseline at 1 and 2hrs 724mg: Significantly increased from control at 1 and 2hrs and from baseline at 1hr **NADPH oxidase:** 310mg: Significantly lower than the control at 1-2, 4 and 6hrs ND at 2, 4 or 6 hours for PWV, AIx, DVP, PSBP, PDBP, CDBP, CSBP or heart rate. **PWV:** ND **Aix:** ND
[61]	8	10 healthy men (aged 18-35yrs) – cross-over intervention	Doses of 6.8mg, 16.2mg, 23.2mg, 26.3mg or 32.3mg of anthocyanins from a cranberry juice drink or a matched control.	**FMD:** 6.8mg: Significantly increased from control at 2hrs 16.2mg: Significantly increased from control at 1, 2, 6, 8hrs 23.2mg: Significantly increased from control at 1, 2, 4, 6hrs 26.3mg: Significantly increased from control at 1, 2, 4, 6, 8hrs 32.2mg: Significantly increased from control at 1, 2, 4, 6, 8hrs ND for **BP, PWV** or **AIx**
[68]	6	13 young (18-35yrs) and older (>55yrs) adults – cross-over intervention	Doses of either 1 x 207mg of anthocyanins or 3 x 69mg of anthocyanins each dose separated by 1 hour. Doses given in the form of a cherry juice drink or a matched control.	**SBP:** 3 x 69mg: ND 207mg: Significant decrease of 5.5mmHg between 0-2hrs **DBP:** 3 x 69mg: ND 207mg: Significant decrease of 5.5mmHg between 0-2hrs **HR:** 3 x 69mg: ND 207mg: Significant decrease of 4.8 beats per minute between 0-2hrs
[60]	2	16 young adult male smokers (age 23.6±0.7) – cross-over intervention	300g of fresh blueberries or carbohydrate matched solution mixed with water	**RHI:** Significantly increased for blueberry treatment -25.2% compared with -6.6% at baseline **SBP:** Significant reduction seen when compared with the control **DBP:** ND **HR:** ND **Aix:** ND
[62]	4	12 hypercholesterolemic men and women (40-	320mg purified anthocyanins or matched control capsule	**FMD:** Significantly increased from the control at 1 hrs **cGMP:** Significant reduction from the control at 1 and 2 hrs

		65yrs) – cross-over intervention		
[63]	6	10 healthy mean (age 27±1yrs, BMI 25±0.8kg/m^2)	339mg anthocyanins (blueberry drink) or 196mg anthocyanins (in a baked bun) or control baked bun (matched to macronutrient of the anthocyanins in the baked bun)	**FMD:** 339mg drink: Significantly increased from control at 1, 2 and 6 hrs 196mg baked bun: Significantly increased from control at 1 and 2 hrs **BP:** ND **HR:** ND
[59]	24	10 healthy male volunteers (age 20.8±1.6yrs, BMI 22.5±2.1 kg/m^2)	116.1mg anthocyanins	**RHI:** ND **SBP:** ND **DBP:** ND **HR:** ND **Nitric Oxide:** ND

Abbreviations: ND (no difference), FMD (flow-mediated dilation), PWV (pulse wave velocity, AIx (augmentation index), SBP (systolic blood pressure), DBP (diastolic blood pressure), HR (heart rate), ABPM (ambulatory blood pressure monitor), RHI (reactive hyperaemia index), NADPH (nicotinamide adenine dinucleotide phosphate), cGMP (cyclic guanine monophosphate), BMI (body mass index) and BP (blood pressure).

Table 1.5: Repeated dose, randomised control trials examining anthocyanins and markers of cardiometabolic health.

Author	Duration	Population / age / sex	Dose	Results
[62]	12 weeks	150 hypercholesterolemic men and women (40-65yrs)	320mg purified anthocyanins	**FMD:** Significantly increased at 12 weeks when compared with the control **cGMP:** Significantly increased at 12 weeks when compared with the control **BP:** ND **HDLC:** Significantly increased at 12 weeks when compared with the control **LDLC:** ND **Triglycerides:** ND **Glucose:** ND
[72]	8 weeks	48 men and women with metabolic syndrome (age 50.0±3.0, BMI >30kg/m^2)	742mg anthocyanins (blueberry)	**SBP:** Significantly decreased after 8 weeks compared with the control **DBP:** Significantly decreased after 8 weeks compared with the control **Glucose:** ND **Triglycerides:** ND **HDLC:** ND **LDLC:** ND
[67]	6 weeks	18 men (age 47.8 ± 9.7yrs, BMI 24.8 ± 2.6kg/m^2)	400mg anthocyanins (blueberry)	**RHI:** ND **AIx:** ND **SBP:** ND **DBP:** ND **Nitric oxide:** ND **Triglycerides:** ND **Glucose:** ND **LDLC:** ND **HDLC:** ND
[65]	6 months	115 men and women with metabolic syndrome (age 62.8±7.1, BMI 31.2±3.0kg/m^2)	374mg anthocyanins (blueberry)	**FMD:** Significantly increased after 6 months compared with the control **SBP:** ND **DBP:** ND **LDLC:** ND **HDLC:** Significantly increased compared with the control
[66]	6 weeks	44 men and women with metabolic syndrome (>50 yrs.)	290.3mg anthocyanins (blueberry)	**RHI:** Significantly increased at 6 weeks when compared with the control **SBP:** ND **DBP:** ND **24hr SBP (ABPM):** ND **24hr DBP (ABPM):** ND **Glucose:** ND

				Triglycerides: ND **LDLC:** ND **HDLC:** ND
[71]	8 weeks	48 post-menopausal women with pre and stage 1 hypertension (45-65yrs)	469.8mg anthocyanins (blueberry)	**SBP:** Significantly decreased after 8 weeks compared with the control **DBP:** Significantly decreased after 8 weeks compared with the control **cfPWV:** ND **baPWV:** Significantly decreased after 8 weeks compared with the control **Nitric Oxide:** Significantly increased after 8 weeks compared with the control
[75]	4 weeks	41 men and women (age 52.2±13.6yrs, BMI 29±5.1)	50mg (blood orange juice)	**SBP:** ND **DBP:** ND **cfPWV:** ND **baPWV:** ND **HDLC:** ND **LDLC:** ND **Triglycerides:** ND **Nitric Oxide:** ND **Glucose:** ND
[74]	14 days	14 healthy men and women (aged 25-55yrs)	288mg anthocyanins (purple majesty potato)	**cfPWV:** Significantly decreased after 14 days compared with the control **SBP:** ND **DBP:** ND **LDLC:** ND **HDLC:** ND **Triglycerides:** ND **Glucose:** ND
[69]	4 weeks	27 healthy men (age 41±3yrs, BMI 27±3)	80mg purified anthocyanins	**SBP (supine, sitting & 24hrs):** ND **DBP (supine, sitting & 24hrs):** ND

Abbreviations: ND (no difference), FMD (flow-mediated dilation), PWV (pulse wave velocity), cfPWV (carotid femoral pulse wave velocity), baPWV (brachial ankle pulse wave velocity), AIx (augmentation index), SBP (systolic blood pressure), DBP (diastolic blood pressure), HR (heart rate), ABPM (ambulatory blood pressure monitor), RHI (reactive hyperaemia index), cGMP (cyclic guanine monophosphate), BMI (body mass index) HDLC (high density lipoprotein cholesterol) and LDLC (low density lipoprotein cholesterol).

1.5 Potential mechanisms of action of anthocyanins: Evidence from *In vitro* studies

The associated clinical benefits of anthocyanins on cardiometabolic health markers from epidemiological studies and RCTs have been outlined in section 1.4. Human intervention studies show improvements in endothelial function, blood pressure, arterial stiffness and serum CVD markers following acute and chronic anthocyanin supplementation. To understand the mechanism of action of anthocyanins further examination of *in vitro* studies is warranted.

Generated by the activation of Ca2+ channels (see figure 1.2), NO plays a key role in endothelial function. Evidence suggests dietary intake of anthocyanins are associated with increased NO correlating with clinical improvements in vascular endpoints such as blood pressure and arterial stiffness (table 1.5) [71]. Additionally, NO diffuses into the smooth muscle cells helping the conversion of cGMP (figure 1.2). Improvements in cGMP, correlating with increases in FMD have been observed in both short and longer term human interventions [62].

In vitro work supports associations between anthocyanins and NO. Firstly, it has been suggested that this may be through the phosphatidylinositol-3 PI3/protein kinase B (Akt/PKB) pathway, via activation of endothelial nitric oxide synthase (eNOS) (figure 1.5). In an *in vitro* study, anthocyanin rich black currant fruit concentrates were used to treat human umbilical vein endothelial cells (HUVECs) and were found to increase the phosphorylation of Akt and eNOS ($p < 0.05$) [85]. As vitamin C, a vitamin abundant in many anthocyanin rich fruits such as blackcurrants and blueberries, is also known to activate the PI3/Akt pathway the researchers also treated the HUVECs with the vitamin C removed from the blackcurrant juice. They found the pathway was still activated, regardless of vitamin C content, with difference between the two black currant juice treatments [86].

Alongside inhibition of eNOS, anthocyanins have potential anti-inflammatory actions. The nuclear factor *k*B (NF- *k*B) transcriptor is important in regulating inflammation and the

immune response [87]. Activation of IKK (an enzyme complex – potentially activated through stress agents, cytokines etc.) can cause activation of NF- *k*B (figure 1.5) and this in turn can activate a series of target genes which increase the production of inflammatory cytokines, chemokines and adhesion molecules [88]. An *in vitro* study treating bovine arterial endothelial cells with malvidin-3-glucoside (an anthocyanin) found that eNOS and NO were upregulated. However they also found an anti-inflammatory response by inhibition of the NF- *k*B activation [89]. Similarly, in a study determining the protective effects of cyanidin-3-glucoside (a type of anthocyanin) in inflammatory diseases, inhibition of the NF-*k*B activation was found in intestinal epithelial cells treated with cyanidin-3-glucoside [90]. Ultimately, though the exact mechanism of action of anthocyanins on the vascular function is still to be established, it is possible that it is the activation, or inhibition, of more than one pathway. Before conclusions are definitive, findings from *in vitro* studies need to be further explored in human intervention studies in order to continue to advance anthocyanin research.

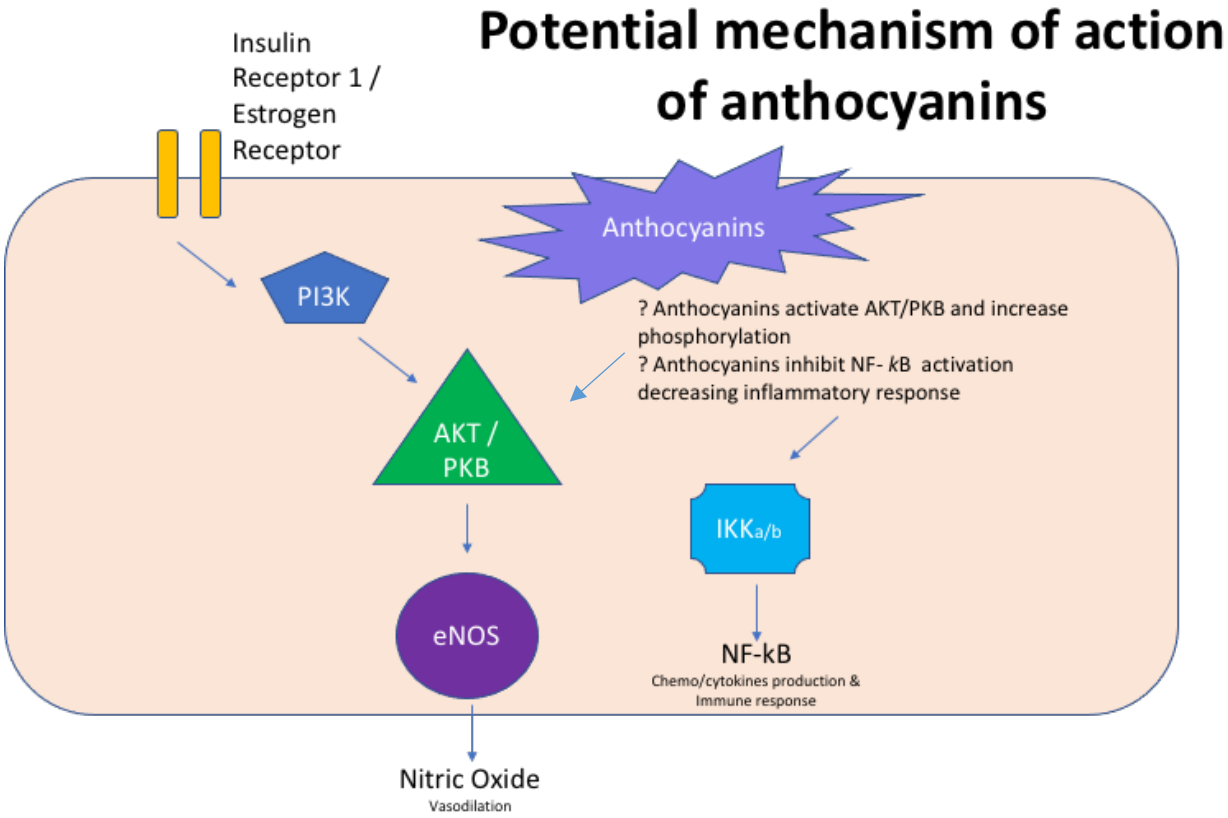


Figure 1.5: The potential mechanism of action for anthocyanins in the endothelial cell.
Abbreviations: Phosphatidylinositol-3 (PI3K), protein kinase B (AKT/PKB), endothelial nitric oxide synthase (eNOS), enzyme complex (IKKa/b) and nuclear factor *k*B (NF-kB)

1.6 Metabolism and bioavailability of anthocyanins

As shown in section 1.4.2, anthocyanin rich foods have shown promising cardiovascular and metabolic effects; however, the results are inconsistent. This variability has not been adequately explored to date. Wide interindividual variability in anthocyanin metabolism has been reported as a key potential factor in explaining the bioactivity of anthocyanins [91] and it is plausible that differences in the absorption, distribution, metabolism and elimination (ADME) may mediate cardiometabolic responses. For example, Rodriguez-Mateos *et al.*, (2013) found peaks in FMD correlated with peak concentrations of key anthocyanin metabolites including benzoic and vanillic acid after 310mg of anthocyanins. These data are associative, rather than causal and the next step to advance the field is to determine whether metabotypes / metabolic profiles for specific polyphenols mediate physiological responses. This would lead towards the concept of 'stratified nutrition' which would optimise the health benefits of tailored nutrient intake on an individual basis [91], [92]. Within the flavonoid literature there is already evidence that metabotypes exist, , i.e. for ellagitannins (a polyphenol) [93], and also isoflavones [94]. These data demonstrate that grouping individuals by similarity in metabolism profiles can help to untangle the variability often seen when reporting the overall population based effects of flavonoid intake [93], [94]. The concept of metabotypes is based on the hypothesis that individual variability in response is mediated by individual or clusters of metabolites and that there are commonalities in the concentration of these compounds in various groups of the population [95].

Flavonoids undergo 'phase 1' and 'phase 2' metabolism when ingested. Anthocyanins are absorbed differently to other flavonoids and have been found in their unchanged glycoside form in both the urine and plasma [96]. Most other flavonoid classes have their glycosides cleaved off before absorption [91]. There is evidence that anthocyanins (as well as other compounds also found in anthocyanin rich foods, such as tannins) may be poorly absorbed in the small intestine suggesting they are metabolised further in the colon where the gut microbiome may be involved [48]. Feeding studies prior to 2013 showed that anthocyanins, despite the wide variety of studies suggesting their beneficial effects, have one of the lowest bioavailability of all flavonoid subclasses [97]. This led to questions about whether the

cardiovascular benefits observed with anthocyanins could be attributed to anthocyanins themselves given they have such low circulating concentrations. However, in 2013 a carbon labelled ^{13}C tracer study, conducted at UEA, changed opinion on the absorption, distribution, metabolism and elimination of anthocyanins. The study, which isotopically labelled 500mg of cyanidin-3-glucoside (C3G), included 8 healthy male participants who provided blood, urine, breath and faeces over a 48-hour period (following ingestion). The study found that the bioavailability of anthocyanins was much higher than previously thought (12.3 $\pm$ 1.3%) and found that metabolites were still circulating in the body up to 48 hours after ingestion [81]. This suggests that microbially-derived metabolites could be a contributor to the cardiovascular benefits. However, it is important to note this study did not assess any cardiovascular outcomes and so research in this area is needed to confirm this theory. Czank *et al.*, (2013) found that the parent compound ^{13}C-C3G was only found in the serum for up to 6 hours but its metabolites were present for up to 48 hours in concentrations sometimes higher than the parent compound (see figure 1.6). Metabolite concentrations in the urine peaked between 6 and 24 hours. These data highlight the need for further acute studies examining the acute vascular effects at 24 and 48 hours when metabolites are still circulating. If vascular benefits are apparent at 24 or 48 hours after consumption, it potentially supports epidemiological data which suggest only three portions of anthocyanin rich food a week may be needed to achieve cardiovascular benefits [53]. In the same study, a wide variation in the concentrations of key metabolites was also observed across the participants over the 48 hour period (shown by large standard error of the mean for many metabolites); these data suggest that people may metabolise at different rates or different quantities [81], [98].

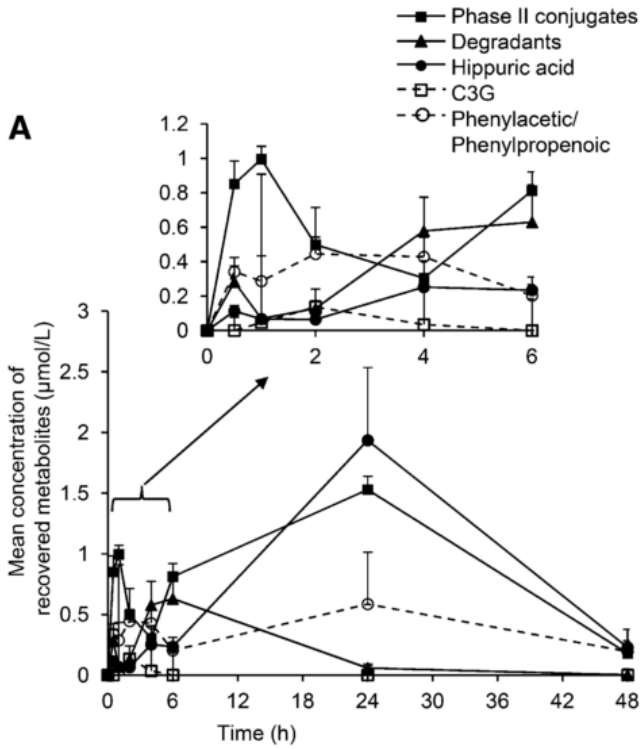


Figure 1.6: Taken form Czank *et al.*, (2013) this figure shows mean concentration of metabolites in serum following 500mg of ^{13}C-C3G over 48 hours.

Although the data outlined above suggest interesting associations between metabolism and function, to date, no trial has prospectively recruited on the basis of anthocyanin metabolism profile [91]. One suggested way of partitioning anthocyanin metabolism could be the rate at which people metabolise anthocyanins or the type of metabolites produced at specific timepoints; this may provide a surrogate marker of not only transit time, but also interaction with microbial species in the gut. Whether that's slowly (meaning metabolites are circulating in the body for longer, possibly rendering more benefits) or quickly, or whether an individual has higher or lower overall concentration of metabolites which may also affect the benefits an individual receives. This would also potentially support the earlier recommendation by Cassidy *et al.*, 2013, that three portions of anthocyanin rich foods a week are protective of myocardial infarction perhaps because the cardiometabolic effects of anthocyanins last longer than 24 hours.

1.7 Established markers of cardiovascular risk used in acute anthocyanin randomised controlled trials

1.7.1 Flow-mediated dilation

Endothelial dysfunction is an established marker of elevated CVD risk [10]. The gold-standard, non-invasive assessment of endothelial function is widely recognised as FMD, which measures the brachial artery's responsive vasodilation, comparing it to baseline, after 5 minutes of occlusion with a blood pressure cuff [99]. The FMD procedure assesses the blood vessels capacity to respond to stress or physical stimuli by dilating and adjusting to allow blood to flow [100]. The creation of reactive hyperaemia (via brachial artery occlusion), followed by a shear stress response (post-occlusion) is thought to induce nitric oxide (NO) production. This results in endothelial cell membrane release of calcium activated potassium channels which activate eNOS thus increasing the amount of NO produced [100]. In meta-analysis of 14 studies, it has been determined that each 1% increase in percentage peak FMD equates to a 10-13% decreased risk of CVD, and as such is clinically meaningful [76].

Though peak FMD is a well-established marker of endothelial function, additional parameters attainable from the FMD assessment have the potential to compliment and offer a more extensive view of endothelial health. These include low flow-mediated constriction (LFMC) and high flow-mediated constriction (HFMC) which consider the vasoconstriction of the artery when reactive hyperaemia is applied and directly thereafter. Time to peak (TTP) is an additional measurement which reports the responsiveness of the artery by the time taken to reach peak FMD.

1.7.2 Pulse wave velocity and augmentation index

Arteries are responsible for carrying blood at high pressures around the body, stiffness of the arterial wall is positively associated with CVD [15]. In healthy men and women, arterial stiffness, as measured by PWV, has been shown to be an independent risk factor of coronary heart disease and stroke [101]. As shown in section 1.4, arterial stiffness is frequently measured using both PWV (otherwise described as a measure of aortic distensibility) and AIx (otherwise described as a measure of systemic arterial stiffness). PWV

is the measure of the velocity of the arterial pressure wave and is widely considered the gold standard technique to measure arterial stiffness [102]. Validated measures include carotid to femoral PWV and brachial to ankle PWV however a recent meta-analysis found no significant difference in either measure of PWV [103]. Measurements can be performed using a validated non-invasive device (such as Vicorder) which gives an automatic calculation of PWV.

AIx is also a measure of arterial stiffness and has been associated with CVD risk [104]. It is an expression of the augmentation of the aortic pressure wave shown in figure 1.7. BP is taken alongside this measurement to help establish the pulse pressure (PP, difference between diastolic BP and systolic BP). A decrease in either PWV or AIx would signify decreased arterial stiffness and thus decreased CVD risk [105].

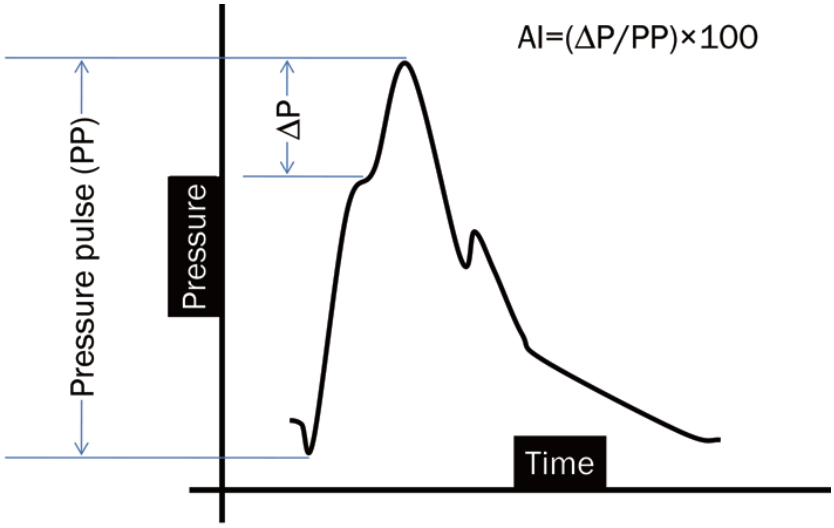


Figure 1.7: Shows the calculation of AIx, from the augmentation pressure (ΔP) divided by the pulse pressure (PP) x 100 (expressed as a percentage) (adapted from Shirwany and Zou, 2010).

1.7.3 Blood pressure

Hypertension is seen as a leading preventable and modifiable risk factor for CVD in the UK [106]. The NHS defines optimal BP (systolic BP/ diastolic BP) as between 90/60mmHg and 120/80mmHg with hypertension defined as being >140/90mmHg [107]. People suffering with hypertension have been shown to have significantly lower life expectancy (5.1 years for men and 4.9 years for women) and higher incidence of CVD, myocardial infarction and

stroke [108]. BP is frequently measured in clinical studies at rest using an automated sphygmomanometer.

Ambulatory blood pressure monitoring (ABPM) is a technique to assess intermittent BP for 24 hours or more whilst maintaining a normal routine. Recent research on a cohort of 63,910 men and women has shown data from ABPM monitoring to be a better predictor of cardiovascular mortality than causal BP measurements [109]. The National Institute for Health and Clinical Excellence (NICE) guidelines recommend that all individuals with a resting high BP reading (>140/90mmHg) in general practice should be offered ABPM to confirm a diagnosis of hypertension [110]. ABPM measurements offer a more comprehensive assessment of an individual's BP and minimise risk of 'white coat' hypertension and 'masked' hypertension which normal BP monitoring may fail to pick-up [111]. Only two RCTs discussed in section 1.4.2.2 measured BP with ABPM [66], [69].

1.8 Summary and concluding remarks

The following are the key limitations and gaps in the literature:

1. Acute dose response anthocyanin trials have only observed changes in cardiometabolic function for up to 8 hours; despite the literature showing that metabolites circulate for at least 48 hours after intake.
2. There is a need to study the impact of differential metabolism on vascular function and metabolic markers; prospectively recruiting participants on the basis of different metabolic profiles would facilitate this.
3. Most existing studies have frequently used one off assessments of BP and have not considered longer term more robust measurements of BP, such as ambulatory, which would provide a more comprehensive assessment.
4. No anthocyanin acute studies to date have adequately controlled the background diet to improve the clarity of metabolism profiles; providing participants with an extended period of set meals (low in flavonoids) would reduce the level of metabolism attributable to the background diet.

5. No existing anthocyanin RCTs measuring FMD have considered measuring alternate analysis methods such as LFMC, HFMC and TTP which may compliment percentage peak FMD

In summary the population-based data suggest that anthocyanins may be protective of CVD and coronary heart disease in particular. This is somewhat supported by RCT data but with substantial variation in outcomes. Although it is difficult to draw definitive conclusions as to why variation in health outcomes may have occurred, a key possibility is the difference in the absorption, distribution, metabolism and elimination of anthocyanins. The focus of this thesis is that understanding the impact of interindividual variability in metabolism may illuminate why different people experience variable cardiometabolic effects following the same intervention [112]. By understanding the impact of metabolism, potentially by establishing consistent anthocyanin profiles, and subsequently measuring cardiovascular outcomes, we will be better positioned to develop dietary strategies to help all individuals benefit from dietary intervention.

1.9 PhD Hypothesis

It is hypothesised that a single dose of blueberries (containing 364mg anthocyanin) will have a sustained effect on vascular and metabolic function (over 48 hours, with an energy-dense meal) and that this effect will be influenced by an individual's metabotype. This will be achieved through addressing several of the objectives below.

Specific PhD objectives:

1. To assess the efficacy of using novel parameters of FMD analysis to understand their relative importance to cardiometabolic health
2. To retrospectively apply these novel analysis parameters of FMD to a completed blueberry intervention in older adults with metabolic syndrome
3. To investigate whether a single dose of blueberry anthocyanins improves vascular and cardiometabolic function over 48 hours
4. To examine if anthocyanin metabolism profiles mediate cardiometabolic function

5. To investigate if metabolism profile affects glucose and lipid responses after an energy-dense meal (tested in a parallel manner, by comparing the response to placebo and energy-dense meal, across the two groups of metabolisers

6. To explore the potential consequences of differences in CVD risk profiles and individualised test meals based on energy requirements when designing an RCT

CHAPTER 2: Application of novel analysis parameters for flow-mediated dilation: A retrospective re-assessment of data from a completed blueberry trial in adults with metabolic syndrome

2.1 Aims and objectives of the chapter

This chapter addresses two key aims:

1) To determine correlation(s) between participant characteristics and medication use, and the flow mediated dilation (FMD) parameters low flow-mediated constriction (LFMC), high flow-mediated constriction (HFMC) and time to peak (TTP) dilation.

2) To assess the acute postprandial effect of blueberry anthocyanins on these extended FMD parameters.

To achieve these aims, a previously collected dataset was used – from a human intervention randomised controlled trial, in predominantly obese adults with metabolic syndrome.

2.2 Hypotheses tested in the chapter

This chapter addresses two hypotheses:

1) Pronounced vasoconstriction occurs in low and high flow periods of the FMD procedure (i.e. LFMC and HFMC) and a longer duration to peak dilation, will correlate with poorer vascular function.

2) Acute blueberry anthocyanin consumption will reduce vasoconstriction in LFMC and HFMC and shorten TTP.

2.3 Understanding Flow-mediated Dilation

2.3.1 Endothelial Function

The endothelium, made up of endothelial cells (EC), is the inner layer (intima) of a blood vessel [5] which has a variety of physiological functions, including; thrombosis and thrombolysis, coagulation, platelet and leukocyte interaction, regulation of vascular tone and growth, cell proliferation and angiogenesis. Conversely, endothelial dysfunction is characterised by the impairment of one or more of these functions [6]. Regarding the location of ECs within the vascular structure; they are found directly next to the smooth muscle and are constantly exposed to shear stress generated by blood flow (figure 2.1). The technical definition of shear stress is shear force applied parallel to a cross-section. When we describe shear stress in relation to the endothelium, it is referring to the frictional 'drag' or force caused by a change in blood flow in the lumen [113]. Shear stress is the stimulus which activates the various functions of the endothelium, including molecules which induce vasodilation [114].

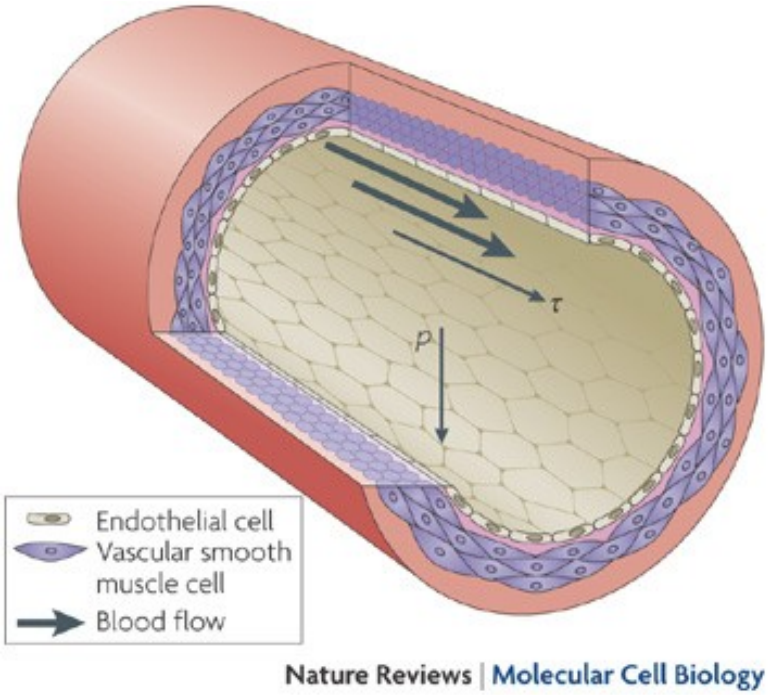


Figure 2.1: A cross section of the endothelium showing endothelial cells lining the inner layer and located next to vascular smooth muscle cells. Shear stress (*τ)* is in the direction of blood flow and can affect arterial pressure (p) on the endothelial cells causing the stretching, or vasodilation, or the arterial wall. Image taken from [115].

Force on the ECs activates calcium (Ca^{2+}) channels, stimulating nitric oxide synthase (NOS) activity and increasing nitric oxide (NO) levels [116]. NO, a signalling molecule produced in the endothelial cells, has a crucial role in vascular health and endothelial function; helping to regulate vascular tone. NO diffuses from the endothelium to the smooth muscle where it activates the enzyme soluble guanylyl cyclase (sGC) which facilitates the conversion of guanosine triphosphate (GTP) to cyclic guanine monophosphate (cGMP) [5]. In response to elevated cGMP, smooth muscle tension decreases, allowing relaxation and vasodilation of the artery.

There are several different ways shear stress can be applied physiologically; red blood cell aggregation is linked with higher blood pressure [117], [118] requiring the circulatory system to work harder to pump blood around the body. Similarly a high salt diet can cause water retention, increasing blood volume and therefore blood pressure on the endothelial wall [119]. Psychosocial stress, for example, releases catecholamine (a neurotransmitter), which can cause vasoconstriction [120]. The body's ability to acutely induce a level of arterial vasoconstriction is part of a healthy vascular system, which indicates elasticity in the endothelial walls. However, the development of hypertension has been linked to sustained force being applied on the arterial wall, which increases the risk of CVD [121].

Endothelial dysfunction is an established marker of elevated CVD risk [10] which can be associated with smoking, increased lipid levels, reactive oxygen species, high blood glucose levels or inactivity [6]. As might be assumed, considering the previously described interaction between vasoactive biochemicals, a dysfunctional endothelium produces less NO, which further compounds the capacity for arterial dilation in response to shear stress. This may, in part, accelerate the progression of atherosclerosis and hypertension [6]. The negative effect of endothelial dysfunction on reactive oxygen species production is also of particular importance and can be derived from an uncoupled nitric oxide synthase (NOS) but is most predominantly from nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and can contribute to hypertension and atherosclerosis. However in turn these conditions may also contribute to excess reactive oxygen species causing a chain reaction [122].

2.3.2 Flow-mediated Dilation and percentage peak dilation

FMD generally refers to a non-invasive procedure using ultrasound to detect brachial vasodilation after a short period of distal ischaemia [123]. The technique, first introduced in 1992 where it was performed on the superficial femoral artery, has developed and progressed our knowledge of endothelial function and health [124]. Notably many studies went on to preferentially choose to study the brachial rather than femoral artery due to its larger diameter (>5.0mm), those with smaller diameters (<5.0mm) tended to pose more difficulties in measurement making vasodilation more difficult to interpret [100]. Studies investigating the FMD further found that shear stress (by induced ischaemia) activated calcium channels, that flow helped to increase NO and that drugs such as antagonists to NO decreased vasodilation in FMD [123]. The gold-standard, non-invasive assessment of endothelial function is now widely recognised as FMD, referring specifically to the degree of vasodilation (known and displayed as percentage peak dilation) that occurs when the pressure, or shear stress is released [99]. A meta-analysis of 14 studies, estimated that each 1% increase in peak dilation during FMD equates to a 10-13% decreased risk of CVD, and as such is clinically meaningful [76]. Although the FMD is not used as a diagnostic tool, its prognostic potential for CVD mean it is widely used in clinical studies [123].

The FMD procedure is an ultrasound technique that measures the comparative brachial artery diameter prior to, and following 5 minutes of occlusion with a blood pressure cuff [99]. When referring to percentage 'peak FMD', it assesses the blood vessels maximum capacity to dilate (measured over a number of sequential diameter assessments), in response to a controlled stressor, which induces an increase in blood flow [100]. The stressor is created by brachial artery occlusion, which induces reactive hyperaemia, followed by a shear stress response (post-occlusion), which is thought to induce NO production. This manipulation of blood flow results in endothelial cell membrane release of calcium activated potassium channels, which activate endothelial nitric oxide synthase (eNOS) thus increasing the amount of NO produced [100]. FMD can be influenced by external factors such as the participants diet, exercise, caffeine / alcohol consumption and medication intake [123]. Though most of the established literature has reported the use of FMD, and the prognostic capabilities of assessing percentage peak FMD, a number of emerging FMD parameter analyses, such as low flow-mediated constriction (LFMC), high

flow-mediated constriction (HFMC) and time to peak dilation (TTP) have been identified as having potential to improve clinical assessment. This chapter explores these novel assessment endpoints in more detail.

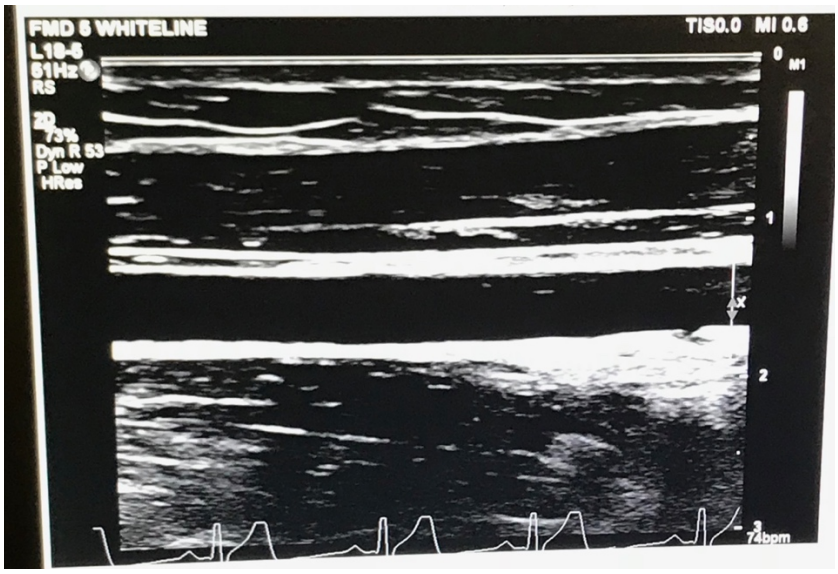


Figure 2.2: The brachial artery at rest by ultrasound technique.

In healthy individuals, in response to the 5-minute occlusion period during FMD measurements, shear stress is induced, and the artery responds by dilating, the maximum dilation it achieves being described as the 'peak' (see figure 2.3). This is usually expressed as a proportion of the expansion above the vessel diameter at rest (i.e., determined during a 'baseline' run-in period of measurement; see figure 2.3).

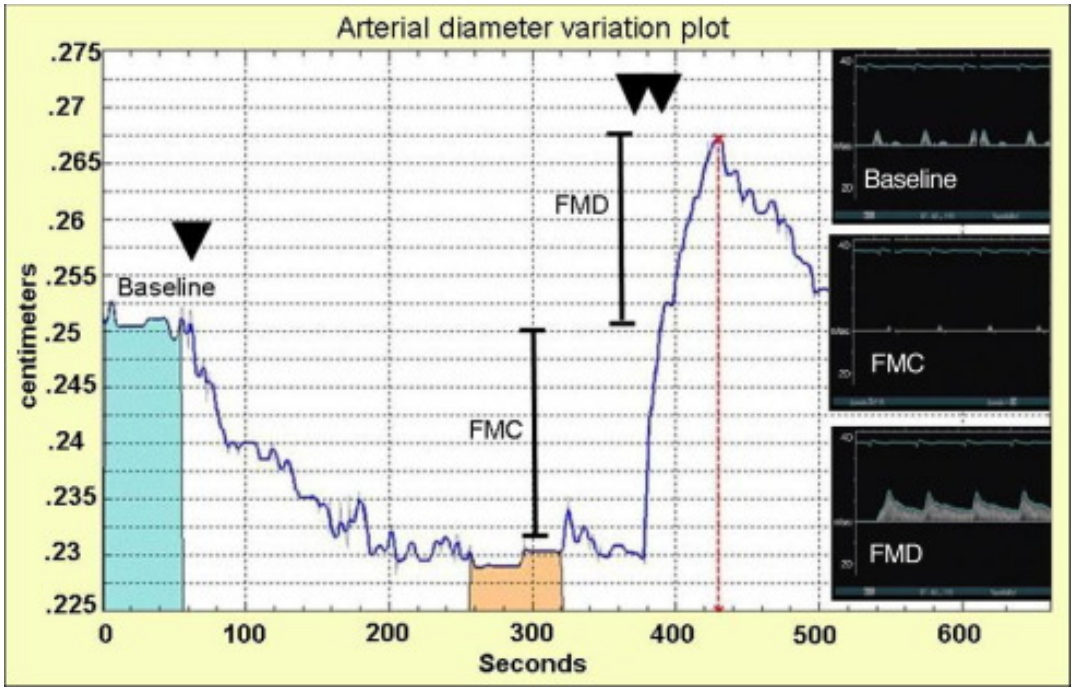


Figure 2.3: The stages of the flow-mediated dilation assessment of the radial artery, displayed as change vessel diameter. It identifies 3 different stages or changes in diameter; baseline, FMC (flow-mediated constriction) and FMD (flow-mediated dilation) (figure from Gori et al., 2008).

Peak dilation is measured by first calculating the mean diameter of the artery at baseline (first minute of assessment; performed at rest) and then calculating the average of the 'peak' diameter of the artery after occlusion using the equation below.

$$\text{Peak FMD \%} = \frac{\text{Average diameter during Peak FMD} - \text{Average diameter at baseline}}{\text{Average diameter at baseline}} \times 100$$

Approaches to calculating peak FMD have differed in the literature. Some investigators use a specified time point or range of time and calculate change in diameter from baseline [100]. This approach is limited because other factors, such as age, impact on the time taken for an individual to reach their maximal dilation in FMD [126], [127]. Thus, researchers choosing a specified time point to calculate FMD may produce results which are not reflective of their population. Calculating peak FMD by maximum dilation has been shown to reduce intra-individual variability and pre-defined time points may also underestimate the individuals peak FMD [128]. More recently there has been a move to measuring peak FMD as an average of the frames where diameter was highest post occlusion [128]–[130].

2.3.3 Low flow-mediated constriction

When the blood flow in the endothelium is reduced, in response to shear stress or an applied stimulus, a period of 'low-flow' is experienced (see figure 2.3, expressed as FMC). During this phase, the vessel constricts beyond its baseline diameter, and is described as low flow-mediated constriction (LFMC) [131], [132].

The following details how LFMC is calculated:

$$\text{LFMC \%} = \frac{\text{Average diameter during the last 30s of occlusion} - \text{Average diameter at baseline}}{\text{Average diameter at baseline}} \times 100$$

A cut-off of 0 is frequently used, thereby if the mean LFMC falls below the mean baseline, the participant is considered to be experiencing LFMC [133].

LFMC can offer a measure of vascular tone and compliment percentage peak FMD to give a more in-depth view of endothelial health [134], [135]. Measuring, assessing and interpreting vasoconstriction seen in LFMC is far more complex than this. The vascular system is a constant balance of different systems, pathways and molecules. As pressure in the arterial system increases so does flow and shear stress (figure 2.1). The artery's ability to respond to this pressure timely and accurately by either vasodilation or vasoconstriction is called the 'myogenic response' [136]. It is known that the myogenic response decreases with age, meaning less vasodilation and less vasoconstriction occurs in response to application and release of pressure [137]. It is also mediated somewhat by changes in blood pressure as the application of shear stress and pressure occurs.

This ignores the complexities of the vascular system and may be an oversimplification of vasoconstriction. For example in heart failure, vasoconstriction at rest occurs, which may be due to alterations in the NO/cGMP pathway which reduces vasodilation [136]. However, in heart failure the angiotensin I/II pathway is activated; angiotensin II is a known

vasoconstrictor and therapy for heart failure often includes drugs that inhibit this pathway [138]. Similarly in patients with idiopathic pulmonary hypertension, vasoconstriction is observed and those with portal hypertension tend to have a decreased sensitivity to vasoconstrictors [136]. These findings demonstrate that endothelial dysfunction can occur from vascular disease, manifesting in differential responses; each of which disrupt the myogenic response to pressure observed in healthy individuals. Increased LFMC has been seen in patients early after percutaneous coronary intervention ($p = 0.02$) [135]. However, after convalescence, when LFMC was measured again, LFMC had improved (increased) ($p = 0.02$). During this time vasoconstrictor endotehlin-1 was measured which did not change significantly through the study despite alterations in LFMC [135]. There is evidence for the importance of vasoconstriction but understanding the role of LFMC, a relatively new and understudied FMD analysis technique, is limited.

Interpretation of LFMC, a snapshot of the brachial artery's ability to vasoconstrict, is challenging. Many studies have shown a more pronounced LFMC (further vasoconstriction) correlates with a weakened ability to vasodilate after ischaemia (reduced percentage peak FMD) [134], [135]. In the study reporting increased LFMC after percutaneous coronary intervention there was a decreased percentage peak FMD ($p < 0.01$) and improvements in LFMC correlated with improvements in FMD [135]. At present, LFMC is best considered as a complimentary analysis technique to percentage peak FMD, a well-studied measurement of endothelial health [139].

2.3.4 High flow-mediated dilation

Another understudied metric is high flow-mediated constriction (HFMC). HFMC measures the time after occlusion (release of a cuff) during the FMD assessment when the artery goes from a period of low blood flow to high blood flow. HFMC has been previously investigated on the radial artery but as the radial artery is small and hard to identify using ultrasound FMD it is not often used in practice. FMD is more commonly applied to the brachial artery [76].

At present, data on the clinical relevance of HFMC is limited and different methodologies are used by different researchers; Dobrosielski *et al.*,2006 measured HFMC as the 10 frames (for a maximum of 5 seconds) with the smallest diameter after cuff release while Ostrem *et al.*, 2017 measured HFMC as the 10 second average of the diameter 3 seconds after cuff release. Accepting these methodological differences, it has been shown that over 2/3rd of adults (*n* = 246; mean age 36 years) experienced HFMC [140], with other researchers confirming more HFMC in younger men, compared with healthy older men (p = 0.02) [141]. In the study by Ostrem *et al.*, 2017, HFMC was not associated with biochemical markers of CVD (blood pressure, glucose, triglycerides, HDLC and LDLC) but it was significantly associated with increased body mass (p = 0.01), fat mass (p = 0.01) and BMI (p = 0.02). Conversely, the younger men in the Dobrosielski *et al.*, study, who had a higher HFMC, also had a higher peak FMD (p<0.01) than the older cohort. Further investigation into HFMC when measuring FMD would be useful to understand its relevance to endothelial function.

In order to best capture the brachial artery's diameter change from low flow to high flow the analysis technique used in this thesis will slightly differentiate from Dobrosielski *et al.*, 2006 and Ostrem *et al.*, 2017 but using elements of how each calculated HFMC. In the analysis that follows in this thesis, the mean diameter of the images 10 seconds post cuff release was used to calculate HFMC to enable more data to be included. The equation below describes how percentage HFMC will be calculated. A score of < 0.1%, has been categorised as being indicative of HFMC.

$$\text{HFMC \%} = \frac{\text{Average diameter during H-FMC} - \text{Average diameter at baseline}}{\text{Average diameter at baseline}} \times 100$$

Currently, given limited research and mixed results in young *versus* old, it remains unclear whether HFMC is a risk factor for endothelial dysfunction. However, some evidence thus far suggests HFMC may be linked with risk factors associated with CVD (such as BMI and body fat) and warrants further investigation.

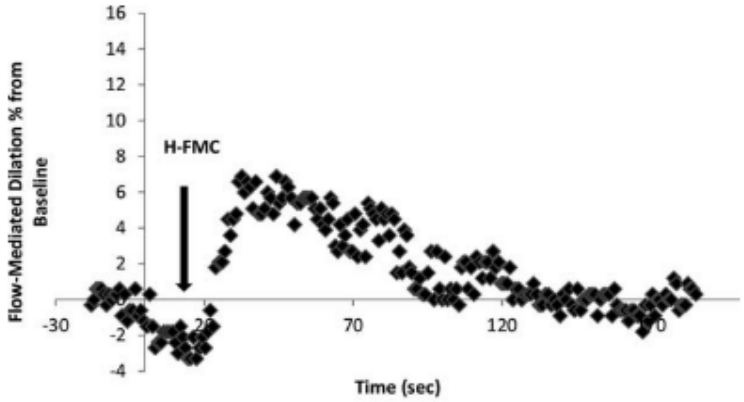


Figure 2.4: Identifies high-flow-mediated constriction (HFMC) by percentage change from baseline during the flow-mediated assessent (Figure taken from Ostrem et al., 2017).

2.3.5 Time to peak dilation

The duration taken to reach peak arterial dilation, i.e., ‘‘time to peak’, may be of importance because of its association with poor health. Time to peak (TTP) measures the duration between the release of occlusive pressure induced by a blood pressure cuff, and the timing of the maximal endothelial dilation (‘peak’ FMD). It has been reported that time to peak is longer in those individuals with increased CVD risk, including those with metabolic syndrome [142], sedentary older adults [126] and children with type 1 diabetes [143], when compared with healthy matched controls. It has also been shown that those with a TTP at >50 seconds had a higher Framingham risk score and consequently a greater risk of CVD development [144]. Though TTP is infrequently assessed, these data demonstrate that both the magnitude (of peak FMD) and timing of peak FMD (TTP) may have clinical significance.

2.4 Methods: Using flow-mediated dilation analysis methods to provide further understanding of endothelial function – retrospective reassessment

2.4.1 Introduction

In this chapter, the efficacy of assessing additional FMD analysis parameters was explored retrospectively in a dataset provided to Caoimhe Flynn (CF) for FMD method development (some of dataset previously published by Curtis *et al.*, 2019). The rationale for this was to determine the clinical relevance of the variables LFMC, HFMC and TTP (which had not been

assessed in this dataset) and examine if they are affected by an acute anthocyanin intervention in order to inform the development of a future intervention. To date, only percentage peak FMD has been reported from the dataset CF was provided access to [65] and the re-assessment of new datapoints was central to CF's method development chapter; to be then used in a subsequent follow-up RCT.

Prior to presenting the study description and results for the new parameters CF assessed, it is of critical importance to confirm that only the calculation of percentage peak FMD was planned in the previous study; the justification being that this measure (percentage peak FMD) is validated and considered a relevant marker of CVD risk [76]. In this previous study, the ultrasound sonographers received no instruction to maintain high quality images during additional timepoints (i.e., during the occlusion, or immediately before / after blood pressure cuff release) and thus image quality during these times (which are critical to LFMC and HFMC) was highly variable. Subsequently, the quality of the ultrasound imaging, at these critical times, dictated the data CF could include in this proof-of-concept assessment. Data from 19 participants were selected for these analyses based on quality of the FMD images.

The additional assessments (LFMC, HFMC and TTP) were analysed firstly to describe any correlations between the new assessment parameters and the baseline participant characteristics of the study population and secondly to characterise the acute impact of freeze-dried blueberries (equivalent to 1 U.S cup; i.e., 150g fresh weight) on acute postprandial endothelial health in participants with metabolic syndrome. In addition, participants were given a high-fat meal with their blueberry anthocyanin intervention which is known to have deleterious effects on the vascular system [35], [36] but adds a 'real world' element to test the intervention given that they are regularly consumed. Results were compared against a placebo group. The aim was to better understand what methods should be used when analysing the acute postprandial FMD images in the study population of the follow-up study (described in Chapter 3); a study which fed freeze-dried blueberries, again within a high-fat meal, to healthy, overweight, 50-80 year old adults.

2.4.2 Summary of study design and data collection (the CIRCLES study) providing CF data

A retrospective, method development analysis was performed on data from a previously completed randomised, parallel designed, placebo controlled, dietary intervention study; known as the 'CIRCLES' study. The aim of CIRCLES was to determine whether 6-month daily intake of freeze-dried anthocyanin rich blueberries was associated with beneficial effects on insulin resistance, vascular health and cardiovascular disease (CVD) risk in adults, aged 50-75 years, with metabolic syndrome. At 0-months (baseline), an acute postprandial assessment was conducted in a sub-set of the study participants in the full dose and placebo groups (*n* = 45 in total). After baseline assessments were performed, an energy-dense, high-fat meal was consumed accompanied by either 26g blueberry intervention material or 26g placebo powder. The resultant effect on cardiometabolic health was assessed for a 24-hour period.

The focus of this current retrospective, method development analysis, was to assess acute postprandial responses at the 0-month (baseline) timepoint to investigate if emerging parameters of the FMD analysis could provide additional understanding of whether anthocyanin affected endothelial function in older adults with metabolic syndrome.

2.4.2.1 Study Population

Data from 27 men and women (postmenopausal, at least 1 year since last menstruation) aged 50-75 years with a body mass index (BMI) of ≥ 25kg/m^2 and ≥3 or more characteristics of metabolic syndrome (as defined by Alberti *et al.*, 2009) were provided for this acute postprandial assessment. Participants were all non-smokers (never having smoked or ceasing smoking ≥ 6 months prior to screening), with no GP diagnosis of diabetes or CVD and not prescribed hypoglycaemic therapies. Participants were recruited via a variety of different methods including radio adverts, GP practices, newspaper adverts, retired police force / NHS workers and the local hospital.

2.4.2.2 Dietary restrictions

21 days prior to attending the acute assessment visit, participants were asked to avoid blueberries and limit foods and supplements rich in anthocyanins (such as raspberries,

strawberries etc.) to once a week as intake could confound the study results. Tea and coffee (major sources in the diet of flavonoids) were restricted to 4 cups a day and intake of alcohol and oily fish were restricted to dietary guideline levels (two portions a week) [146], [147], dark chocolate (two, 5 chunk portions a week) and red wine (125ml per week). 24 hours prior to the assessment days, nitrate and nitrite rich foods (such as spinach and beetroot) were also restricted as these can impact on vascular assessments. A checklist was completed on the study visit day to assess adherence. Participants were instructed to maintain their habitual exercise during the study but limit vigorous exercise at least 48 hours before their study visit.

2.4.2.3 Study Design

On the day of the assessment participants had all vascular measurements (including flow-mediated dilation, measures of arterial stiffness, blood pressure and serum lipids and lipoproteins) taken prior to being given the intervention material (at 0 hours - baseline measurement) and for the 24 hours after. They were given 26g of either freeze dried blueberry powder (364mg of anthocyanins) or placebo powder (caloric controlled, matched for sugar, flavour and colour) with a high-fat, energy-dense challenge (a 500ml drink containing 60g of fat from palm oil - rich in saturated fat, 75g glucose and 20g protein). The drink was mixed in an opaque vessel to ensure participants were blinded. Participation in the acute postprandial study was offered to all in the 6-month intervention, participants self-selected to partake based on their individual availability.

2.4.2.4 Vascular Function Measurements

The measurements of vascular function performed in the postprandial study were; FMD (as a measure of endothelial function), pulse wave velocity (PWV) (as a measure of aortic stiffness), augmentation index (AIx) (as a measure of systemic arterial stiffness) and office blood pressure assessments. FMD, PWV, AIx and blood pressure were assessed at 0, 180mins, 360mins and 24 hours after the test meal. Blood pressure measurements were taken 3 times (with a 3-minute break in between each measurement using an automated sphygmomanometer) following 15 minutes of supine rest. Carotid to femoral PWV was used to assess aortic distensibility with calculations completed by Vicorder software (Smart

Medical, UK). AIx was used to measure systemic arterial stiffness 3-6 assessments were taken by Vicorder software (Smart Medical, UK). Blood samples were also taken to assess biomarkers relevant to health and the postprandial response (e.g., lipid profile, inflammatory markers, insulin and glucose) samples were taken at 30mins, 60mins, 90mins, 120mins, 180mins, 360mins and at 24hours. All blood samples were analysed by the local hospital laboratory (Norfolk and Norwich Hospital).

Data on blood pressure, BMI, fat mass, arterial stiffness, total lipid and lipoprotein profile was used in the retrospective analysis. This data was chosen to provide the opportunity to assess whether the novel FMD parameters being analysed were associated with differential cardiometabolic health or response to blueberry intake.

2.4.2.5 'CIRCLES' study flow-mediated dilation procedure

Two experienced research scientists (who attended an FMD training course at Kings College London, Department of Clinical Pharmacology, Cardiovascular Division, King's College London, St Thomas' Hospital, London, UK; under the supervision of Ben Yu Jiang (an international expert in vascular sonography) conducted all FMD measurements throughout the study. Participants were asked to fast for at least 10 hours prior to arrival at the research facility and to limit exercise (48 hours before) and caffeine (24 hours before). FMD was performed in a quiet, dimly lit, temperature-controlled room (between 21-24°C) with the blinds shut to prevent any outside distraction and direct sunlight. Participants were in a supine position on a hospital bed for at least 15 minutes prior to the procedure. Researchers limited communication with participants in an effort to minimise distractions that may affect measurements being taken. A blood pressure cuff was placed on the participant's right forearm. Ultrasound assessments were performed on a Philips iE33 machine, with the cardiology extension package; a 11-3MHz linear transducer was used for image acquisition. Once a clear image of the brachial artery was located, the probe was held in place on the participant's arm with a stand. Image capture was trigged by the participant's heartbeat via a 3-lead ECG and Vascular imager software (Vascular Imager software; Medical Imaging Applications LLC, Coralville, USA). The procedure lasts 11 minutes; 1-minute capture at baseline (rest), 5 minutes occlusion with the blood pressure cuff inflated at 220 mmHg and 5 minutes post occlusion (cuff deflation).

2.4.2.6 Initial analysis of percentage peak flow-mediated dilation

Initial analysis of the image sequences for Peak FMD was completed independently by 2 scientific researchers blinded to intervention treatment group. A region of interest was identified and automated edge-detection software used (Brachial Analyzer v5; Medical Imaging Applications LLC, Coralville, Iowa) to minimise human error. A mean baseline diameter was calculated as an average of all viable baseline frames. Peak FMD was calculated as percentage; $(\text{diameter}_{max} - \text{diameter}_{baseline}) / \text{diameter}_{baseline} \times 100$.

2.5 Retrospective analysis of new flow-mediated dilation parameters by Caoimhe Flynn

2.5.1 Overview

To calculate TTP, LFMC and HFMC an independent researcher (CF), blinded to treatment, assessed all image sequences (*n* = 27, see figure 2.5). In order to be able to compare these analysis techniques to the participants percentage peak FMD, the region of interest determined by the 2 initial independent researchers remained the same. Region of interest in FMD refers to the section of the artery, captured by ultrasound, that was chosen as an area of viable quality for FMD analysis.

Prior to statistical analysis a captured FMD sequence required analysis using Brachial Analyzer analysis software. As the region of interest, baseline resting arterial diameter and percentage peak FMD had already been analysed the focus of this current study was to measure arterial diameter for HFMC and LFMC and assess TTP. As has been described in section 2.2.1, when researchers were conducting FMD during the study the protocol did not specify maintaining good ultrasound image quality during occlusion (LFMC) and for the first 10 seconds after occlusion release (HFMC) which meant that many of the captured FMD sequences were not viable for use in this exploratory extended analysis. As a first step CF re-ran and watched each participant's baseline, 0-hour, 11 minute FMD sequence to assess if LFMC and HFMC timings were of sufficient quality. Those deemed of reasonable standard (showing at least some of the arterial vessel on ultrasound images) went through to a

quality assessment (QA) further outlined in section 2.3.2., *n* = 8 were excluded due to their baseline FMD sequence not meeting the strict QA criteria.

Following QA, previously omitted image frames during the periods of LFMC and HFMC, in the FMD sequence, were re-imported by CF and the arterial diameter at these times confirmed using Brachial Analyzer. Following the analysis (of LFMC, HFMC) each re-imported image was manually checked to ensure the software had detected the edge on the arterial wall. If the software had failed to do so, it was manually corrected (using an automated 'detect' function). Following this, all diameters during the FMD assessment were exported to a Microsoft Excel document where the LFMC, HFMC and TTP calculations could be made (using equations described in section 2.1).

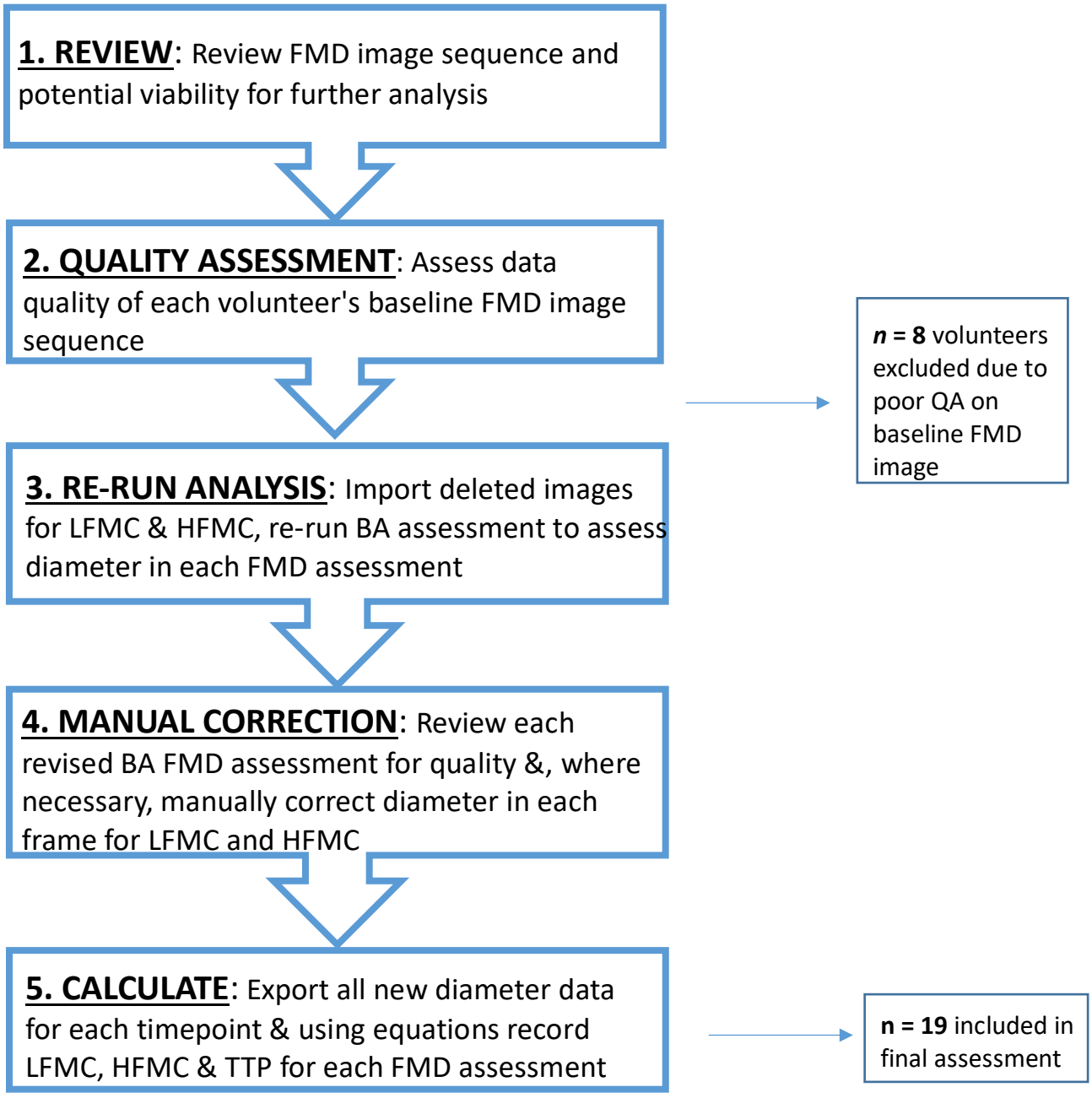


Figure 2.5: The process of analysis for low flow-mediated constriction, high flow-mediated constriction and time to peak by Caoimhe Flynn (CF). Abbreviations: FMD (flow-mediated dilation), LFMC (low flow-mediated constriction), HFMC (high flow-mediated constriction), BA (Brachial Analyzer), TTP (time to peak), QA (quality assessment)

2.5.2 Quality Assessment

As a single researcher (CF), to ensure data quality, a stringent quality assessment (QA) method was implemented *a priori*. The QA was designed for this study to ensure a standard was met for each FMD sequence included and to avoid single researcher bias. The QA designed was filled in for each assessment in Microsoft Excel (see table 2.1) and included information on first glance whether the peak FMD was recorded, if the vessel line was automatically detected (and if not, could it be detected manually) and if >70% of frames for the given parameter being measured were included. The QA also included a rating of image quality on a scale of 1-5, 1 being the analysis was not possible and 5 being the images were of excellent quality. A score of 1 for image quality warranted immediate exclusion from analysis. All scores above this were taken into account with the other parts of the QA. For example, in order to have at least >70% of frames included a reasonable image quality would be likely score at least 3 or above. Note initially area under the curve (AUC) was a potential parameter being assessed hence why it is included in table 2.1 AUC analysis was not possible due to too few FMD image sequences being deemed of viable quality to include in the analysis.

Volunteer ID	Time point	First glance: Is image quality deemed adequate?	Is peak FMD recorded to determine time to peak? *If not this outcome should be discarded*	Image quality rating (Scale 1-5; 1 being analysis not possible, 5 being image of excellent quality) *It marked 1, consider immediate exclusion of analysis for*				Is the vessel line automatically detectable? *If not the line should be attempted manually*				Can the vessel line be detected manually (using auto-detect function)? *If not - exclude from analysis*				>70% of frames included in analysis? *If not - exclude from analysis*			
				L-FMC	H-FMC	AUC	Baseline Diameter	L-FMC	H-FMC	AUC	Baseline Diameter	L-FMC	H-FMC	AUC	Baseline Diameter	L-FMC	H-FMC	AUC	Baseline Diameter

Table 2.1: Flow-mediated dilation data quality. Table 1 describes the data quality assessment method for the acute FMD image files being analysed.

2.5.3 Statistical Analysis

Exploratory associations between baseline FMD parameters and CVD markers were assessed by Pearson's correlation. Effects of dietary intervention, blueberry anthocyanin *versus* control matched placebo, on the new FMD parameters were analysed using an independent samples T – test. The statistical analysis was performed using SPSS software.

A sample size of 19 participants met the inclusion criteria set out by the QA (section 2.3.2) and were included in this retrospective exploratory analysis of FMD. A total of *n* = 8 additional volunteers who had baseline FMD data deemed inadequate for further analysis based on the QA were excluded. Of the 19 participants deemed eligible for analysis *n* = 2 had missing data for cholesterol, HDLC, LDLC and triglyceride, and for PWV *n* = 1 had missing data. The QA resulted in *n* = 18 HFMC data, *n* = 17 for LFMC data and *n* = 19 for TTP data deemed of suitable quality for this retrospective analysis.

Due to the exploratory nature of this method development chapter and the analysis of new parameters being unknown during the time of capturing the FMD images, the following were applied:

a) As quality is the main inclusion criteria, a formal power calculation was not performed
b) Statistical tests were not adjusted for multiple variables due to limited participant numbers
c) An effect was considered significant when the p value was <0.05.

The researcher remained blinded during the retrospective image and data analysis. Image data quality control methods excluded those time points that were deemed inadequate.

2.6 Results

2.6.1 Descriptive characteristics

Table 2.2: Baseline study population characteristics n = 19

		N	Mean	Min	Max	SD
Age (years)		19	62.00	50.00	75.00	6.70
Sex (male)		13	68.00%			
BMI (kg/m^2)		19	30.89	26.49	34.51	2.79
Metabolic syndrome score						
	3	12	63.00%			
	4	6	32.00%			
	5	1	5.00%			
SBP (mmHg)		19	139.42	116.00	168.00	14.14
DBP (mmHg)		19	84.50	69.50	97.50	8.78
Statin use (yes)		7	37.00%	1.00	0.35	0.49
BP medication (yes)		9	47.00%			
FMD (%)		19	2.71	0.00	7.83	2.36
HFMC (%)		18	-1.01	-4.99	2.43	1.60
LFMC (%)		17	-0.69	-4.42	2.47	1.74
TTP (seconds)		19	00:01:02	00:00:31	00:02:33	0.00
AIx (%)		19	37.36	23.31	59.04	9.21
PWV (m/s)		18	11.00	8.62	14.20	1.71
Glucose (mmol/l)		19	4.17	3.67	5.41	0.41
Total cholesterol (mmol/l)		17	5.37	3.70	7.90	1.16
HDLC (mmol/l)		17	1.30	0.84	2.22	0.34
LDLC (mmol/l)		17	3.34	2.10	5.70	1.10
Triglyceride (mmol/l)		17	1.69	0.67	2.91	0.74

Abbreviations: SD (standard deviation), BMI (body mass index), SBP (systolic blood pressure), DBP (diastolic blood pressure), FMD (flow-mediated dilation), HFMC (high flow-mediated dilation), LFMC (low flow-mediated dilation), TTP (time to peak), AIx (augmentation index), PWV (pulse wave velocity), HDLC (high density lipoprotein cholesterol), LDLC (low density lipoprotein cholesterol).

The 19 study participants included 13 men (68%) and 6 women (32%) all diagnosed with metabolic syndrome of a diagnostic score between 3-5 as described in Curtis *et al.*, 2019 (table 2.2). Correlations between descriptive information and baseline FMD analysis techniques, LFMC, HFMC and TTP, were analysed. Statistically significant ($p < 0.01$) Pearson's correlation was observed between fasted baseline LFMC and HFMC

measurements (table 2.3, figure 2.6). There were no other significant associations between HFMC, LFMC, TTP and any of the baseline characteristics shown in table 2.2.

Table 2.3: Baseline correlations between low flow-mediated constriction and high flow-mediated constriction

Variable		Mean	SD	Minimum	Maximum	1	2
1. LFMC 0hr		-0.69	1.74	-4.42	2.47		
	Pearson Correlation						0.87
	Sig. (2-tailed)						0.00
	N						14
2. HFMC 0hr		-1.01	1.59	-4.99	2.43		
	Pearson Correlation					0.87	
	Sig. (2-tailed)					0.00	
	N					14	

LFMC and HFMC were analysed from fasted flow-mediated dilation assessments from 14 participants at baseline (prior to blueberry treatment or placebo dose). Correlation between the 2 variables was assessed using Pearson's correlation coefficient test. Results show a significant correlations where P=< 0.01. Abbreviations: LFMC (low flow-mediated dilation), HFMC (high flow-mediated dilation), SD (standard deviation).

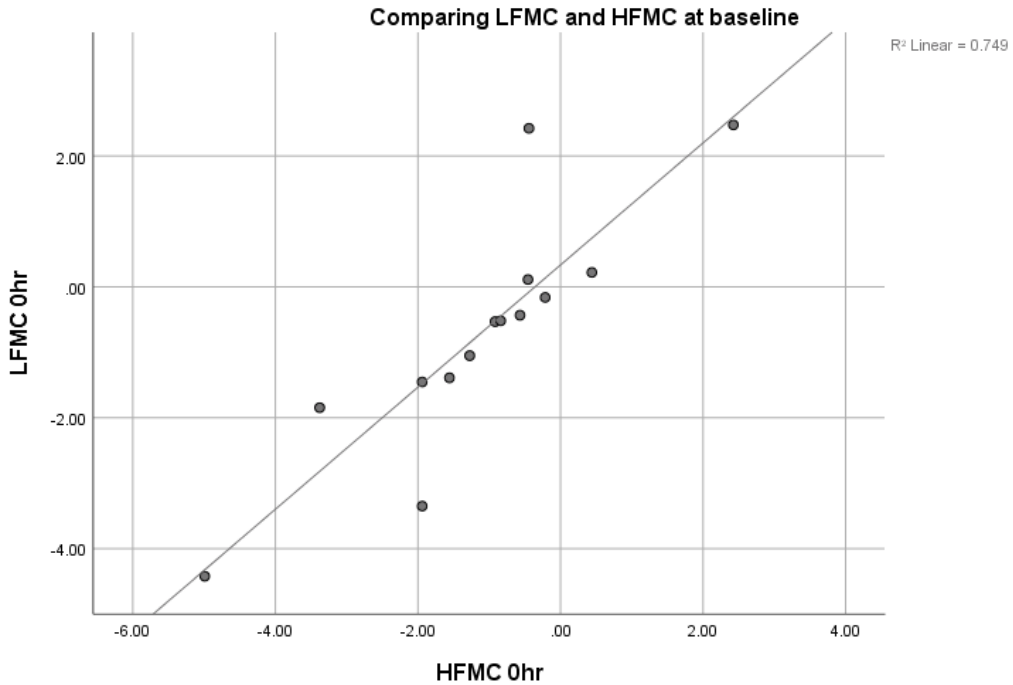


Figure 2.6: Scatter plot comparing low flow-mediated constriction and high flow-mediated constriction at baseline. There was a strong positivte correlation between the two variable with R^2=0.75. Abbreviations: LFMC (low flow-mediated dilation), HFMC (high flow-mediated dilation).

2.6.2 Analysis of flow-mediated dilation parameters by sex

The baseline participant characteristics and LFMC, HFMC and TTP were subsequently analysed to determine the impact of sex, which showed that HDLC concentration and TTP was significantly different between sexes (p=0.04 and p=0.03 respectively). As shown in table 2.5, males had lower HDLC and a faster TTP than females. There were no differences in baseline HFMC and LFMC by sex (see table 2.4).

Table 2.4: The baseline characteristics of the different sexes (*n* = 19)

	Female					Male					
	n	**Mean**	**Min**	**Max**	**SD**	**n**	**Mean**	**Min**	**Max**	**SD**	**P value**
Age (y)	6	63.83	57.00	69.00	5.12	13	61.23	50.00	75.00	7.35	0.45
BMI (kg/m^2)	6	31.24	28.20	34.51	2.16	13	30.73	26.49	37.37	3.11	0.73
Metabolic Syndrome Score											
3	4	66.60%	/	/	/	8	62.03%	/	/	/	
4	2	33.30%	/	/	/	4	30.77%	/	/	/	
5	0	0.00%	/	/	/	1	7.69%	/	/	/	
SBP (mmHg)	6	138.83	116.00	168.00	19.85	13	139.69	127.00	166.00	11.64	0.91
DBP (mmHg)	6	78.67	69.50	90.50	7.66	13	87.19	71.00	97.50	8.14	0.45
Statin Use (Yes)	6	33.3% (2)	/	/	/	13	53.8% (7)	/	/	/	0.73
BP Medication (Yes)	6	33.3% (2)	/	/	/	13	53.8% (7)	/	/	/	0.32
FMD (%)	6	3.88	0.00	7.83	2.81	13	2.17	0.38	7.81	2.01	0.15
Aix (%)	6	44.58	37.56	59.04	8.01	13	34.02	23.31	47.84	7.89	0.15
PWV (m/s)	5	10.64	9.58	12.03	0.92	13	11.13	8.62	14.20	1.69	0.55
Glucose (mmol/l)	6	3.96	3.67	4.20	0.22	13	4.27	3.74	5.41	0.46	0.14
Cholesterol (mmol/l)	6	5.83	4.40	7.90	1.32	11	5.15	3.70	7.00	1.05	0.26
HDLC (mmol/l)	6	1.52	1.19	2.22	0.40	11	1.16	0.84	1.61	0.24	0.04
LDLC (mmol/l)	6	3.63	2.50	5.70	1.30	11	3.17	2.10	4.80	1.00	0.43
Triglyceride (mmol/l)	6	1.57	0.67	2.82	0.78	11	1.76	0.74	2.91	0.74	0.64

Abbreviations: SD (standard deviation), BMI (body mass index), SBP (systolic blood pressure), DBP (diastolic blood pressure), BP (blood pressure), FMD (flow-mediated dilation), PWV (pulse wave velocity), AIx (augmentation index), SBP (systolic blood pressure), DBP (diastolic blood pressure), HDLC (high density lipoprotein cholesterol) and LDLC (low density lipoprotein cholesterol).
Statistical differences between the females and males was established using a T-test, for all nominal data a Chi square test was performed. A P value of < 0.05 was considered statistically significant.

Table 2.5: Comparing differences between low flow-mediated constriction, high flow-mediated constriction and time to peak at baseline by sex

		n	Mean	SD	p value
TTP (HH:MM:SS)	Female	6	00:01:25	00:00:47	0.03
	Male	13	00:00:50	00:00:23	
LFMC (%)	Female	5	-0.52	2.09	0.81
	Male	11	-0.76	1.67	
HFMC (%)	Female	5	-0.47	1.69	0.39
	Male	12	-1.23	1.57	

Differences in LFMC, HFMC and TTP at baseline by sex were assed using an independent samples T test grouping by sex (equal variances assumed). A p value of <0.05 was considered statistically significant. Abbreviations: TTP (time to peak), LFMC (low flow-mediated dilation), HFMC (high flow-mediated dilation) and SD (standard deviation).

2.6.3 Analysis of flow-mediated dilation parameters by statin use

In sub-analysis by statin (shown in Table 2.6), it was shown that participants using statins had significantly lower concentrations of total cholesterol (p = 0.01), LDLC (p = 0.01) and triglycerides (p = 0.02), and greater HDLC levels (p = 0.04). Similarly, HFMC differed by statin user categorisation, with non-users of statins having a lower baseline HFMC (p = 0.03) (table 2.7).

Table 2.6: The characteristics of statin users and non-users of statins (*n* = 19)

	No statin					Statin					
	N	**Mean**	**Min**	**Max**	**SD**	**N**	**Mean**	**Min**	**Max**	**SD**	**p value**
Age (y)	11	61.55	52.00	69.00	5.35	7	62.86	50.00	75.00	9.25	0.71
Sex (male)	7		/	/	/	5		/	/	/	0.73
BMI (kg/m^2)	11	30.04	26.49	33.21	2.25	7	32.53	28.31	37.37	3.12	0.07
Metabolic Syndrome Score											
3	8	72.73%	/	/	/	3	42.86%	/	/	/	
4	3	27.27%	/	/	/	3	42.86%	/	/	/	
5	0	0.00%	/	/	/	1	14.28%	/	/	/	
SBP (mmHg)	11	137.59	117.00	168.00	13.28	7	140.07	116.00	166.00	16.10	0.73
DBP (mmHg)	11	85.68	72.00	97.50	7.59	7	82.57	69.50	97.00	11.32	0.49
BP Medication (Yes)	11	36.36% (4)	/	/	/	7	71.43% (5)	/	/	/	0.15
FMD (%)	11	2.88	0.00	7.81	2.45	7	2.73	0.38	7.83	2.44	0.90
Aix (%)	11	37.96	24.82	48.17	7.86	7	38.25	23.31	59.04	11.05	0.95
PWV (m/s)	11	10.90	8.62	13.37	1.42	6	10.64	9.25	12.20	1.18	0.71
Glucose (mmol/l)	11	4.16	3.67	5.41	0.55	7	4.18	3.90	4.36	0.16	0.92
Cholesterol (mmol/l)	11	5.91	4.60	7.90	1.04	6	4.43	3.70	5.60	0.68	0.01
HDLC (mmol/l)	11	1.17	0.84	1.40	0.17	6	1.51	0.86	2.22	0.46	0.04
LDLC (mmol/l)	11	3.83	2.10	5.70	1.07	6	2.43	2.10	2.90	0.27	0.01
Triglyceride (mmol/l)	11	1.99	1.06	2.91	0.71	6	1.14	0.67	1.65	0.41	0.02

Statistical differences between the females and males was established using a T-test, for all nominal data a Chi square test was performed. A p value of < 0.05 was considered statistically significant. Abbreviations: SD (standard deviation), BMI (body mass index), SBP (systolic blood pressure), DBP (diastolic blood pressure), BP (blood pressure), FMD (flow-mediated dilation), PWV (pulse wave velocity), AIx (augmentation index), SBP (systolic blood pressure), DBP (diastolic blood pressure), HDLC (high density lipoprotein cholesterol) and LDLC (low density lipoprotein cholesterol).

Table 2.7: Comparing differences between low flow-mediated constriction, high flow-mediated constriction and time to peak at baseline by statin use

		n	**Mean / %**	**SD**	**p value**
TTP (HH:MM:SS)	No Statins	11	00:01:07	00:00:40	0.59
	Statin Use	7	00:00:57	00:00:30	
LFMC (%)	No Statins	9	-1.29	1.33	0.12
	Statin Use	6	0.2	2.16	
HFMC (%)	No Statins	9	-1.7922	1.48479	0.03
	Statin Use	7	-0.0202	1.31838	

Differences in LFMC, HFMC and TTP at baseline by statin use were assed using an independent samples T test grouping by sex (equal variances assumed). Results show a significant difference between groups where p= <0.05 (p = 0.03). Abbreviations: TTP (time to peak), LFMC (low flow-mediated dilation), HFMC (high flow-mediated dilation) and SD (standard deviation).

2.6.4 Analysis blueberry anthocyanins on flow-mediated dilation parameters

Of the 19 participants, *n* = 10 were randomised to the blueberry treatment material and *n* = 9 were randomised to the placebo. Participants reported compliance to the dietary restrictions asked of them prior to and during the acute intervention. There were no significant differences between the baseline descriptive characteristics (table 2.8) or LFMC, HFMC and TTP results at 3, 6 or 24 hours when between group analysis was performed (table 2.9).

Table 2.8: The baseline characteristics of the blueberry and the placebo group (*n* = 19)

	Blueberry					Placebo					
	N	**Mean**	**Min**	**Max**	**SD**	**N**	**Mean**	**Min**	**Max**	**SD**	**p value**
Age (y)	10	62.00	52.00	69.00	5.16	9	62.11	50.00	75.00	8.42	0.97
Sex (male)	8	80.00%	/	/	/	5	55.56%	/	/	/	0.25
BMI (kg/m^2)	10	30.68	26.49	37.37	3.50	9	31.12	28.31	34.51	1.92	0.74
Metabolic Syndrome Score											
3	6	60.00%	/	/	/	6	66.60%	/	/	/	/
4	3	30.00%	/	/	/	3	33.30%	/	/	/	/
5	1	10.00%	/	/	/	0	0.00%	/	/	/	/
SBP (mmHg)	10	140.30	117.00	166.00	14.31	9	138.44	116.00	168.00	14.76	0.78
DBP (mmHg)	10	87.70	72.00	97.50	8.31	9	80.94	69.50	95.00	8.28	0.09
Statin Use (Yes)	10	20.00% (2)	/	/	/	5	55.56%	/	/	/	0.15
BP Medication (Yes)	10	50.00% (5)	/	/	/	4	44.44%	/	/	/	0.64
FMD (%)	10	2.22	0.00	7.81	2.59	9	3.24	0.92	7.83	2.09	0.36
LFMC (%)	9	-0.64	-1.85	0.22	0.72	7	-0.75	-4.42	2.47	2.62	0.91
HFMC (%)	8	-1.08	-3.38	0.44	1.17	9	-0.94	-4.99	2.43	1.96	0.87
TTP (HH:MM:SS)	10	0:01:04	0:00:31	0:02:15	0:00:35	9	0:00:58	0:00:32	0:02:33	0:00:38	0.72
Aix (%)	10	36.62	24.50	47.84	7.92	9	38.18	23.31	59.04	10.89	0.72
PWV (m/s)	10	11.39	8.62	14.20	1.73	8	10.50	9.25	12.03	1.06	0.22
Glucose (mmol/l)	10	4.18	3.74	5.41	0.46	9	4.15	3.67	4.99	0.39	0.88
Cholesterol (mmol/l)	8	5.49	3.70	7.90	1.31	9	5.30	4.00	7.00	1.08	0.75
HDLC (mmol/l)	8	1.13	0.84	1.40	0.22	9	1.43	1.00	2.22	0.38	0.08
LDLC (mmol/l)	8	3.45	2.10	5.70	1.26	9	3.23	2.30	4.80	1.01	0.70
Triglyceride (mmol/l)	8	1.96	1.12	2.91	0.68	9	1.46	0.67	2.82	0.74	0.17

Statistical differences between the females and males was established using a T-test, for all nominal data a Chi square test was performed. A P value of < 0.05 was considered statistically significant. Abbreviations: SD (standard deviation), BMI (body mass index), SBP (systolic blood pressure), DBP (diastolic blood pressure), BP (blood pressure), FMD (flow-mediated dilation), PWV (pulse wave velocity), AIx (augmentation index), SBP (systolic blood pressure), DBP (diastolic blood pressure), HDLC (high density lipoprotein cholesterol) and LDLC (low density lipoprotein cholesterol).

Table 2.9: Postprandial effect of blueberry treatment *versus* placebo on low flow-mediated constriction, high flow-mediated constriction and time to peak

		BL			3hr					6hr				24hr				
		N	Mean	SD	N	Mean	SD	Δ BL	P Value	N	Mean	SD	Δ BL	P Value	N	Mean	SD	Δ BL	P Value
LFMC	Blueberry	9	-0.64	0.72	7	-0.55	1.48	-0.13	0.36	6	-1.41	1.54	-0.68	0.75	5	-1.90	2.46	-1.42	0.83
	Placebo	7	-0.75	2.62	5	-2.39	3.72	-2.31		2	-3.96	2.88	-1.06		2	-0.24	2.65	-0.78	
HFMC	Blueberry	8	-1.08	1.17	8	-1.30	1.27	0.11	0.94	7	-2.11	1.24	-1.15	0.56	7	-2.74	2.35	-1.78	0.38
	Placebo	9	-0.94	1.96	8	-2.53	2.93	-2.10		6	-1.48	1.93	-0.61		4	-0.47	1.67	-0.38	
TTP (seconds)	Blueberry	10	64.40	35.40	10	59.50	27.20	-4.90	0.94	9	51.11	20.10	-15.11	0.27	9	56.89	17.90	-9.33	0.53
	Placebo	9	58.33	38.30	9	55.11	18.50	-3.22		9	70.00	41.40	11.67		8	60.88	27.30	4.25	

TTP, LFMC and HFMC were analysed from fasted flow-mediated dilation assessments from eligible quality assessed participants at baseline (prior to blueberry treatment or placebo dose), 3hrs, 6hrs and 24hrs (after blueberry treatment or placebo). The mean change LFMC, HFMC and TTP from baseline of blueberry treatment and placebo was compared using and independent samples T test grouping by treatment given (equal variances assumed). Results show no significant difference (p=<0.05) between groups at any time point.
Abbreviations: LFMC (low flow-mediated dilation), HFMC (high flow-mediated dilation), TTP (time to peak), BL (baseline) and SD (standard deviation).

Data from the chronic study (FMD results 6 months after daily blueberry treatment or placebo) was analysed but only a very small number of assessments were deemed to be of sufficient quality (*n* = 7 for LFMC and *n* = 10 for HFMC). No significant difference in HFMC, LFMC or TTP was found between the blueberry treatment group or placebo group at 6 months (data not shown).

2.7 Discussion

A single dose of blueberry anthocyanins did not acutely affect LFMC, HFMC or TTP. There were significant associations between LFMC and HFMC (table 2.3, figure 2.6) which has not been reported before. To our knowledge, there has not been an analysis of LFMC and HFMC in older adults with metabolic syndrome. A greater degree of LFMC (vasoconstriction) has been associated with a delayed time to peak [134]. It is possible that, as figure 2.6 shows, the greater constriction found in LFMC results in a greater amount of constriction in HFMC because the vessel takes longer to recover from the low-flow state. It should be noted despite the delayed TTP being associated with increased vasoconstriction for Irace *et al.*, 2016, we did not find any association with TTP in this population.

Interestingly, we did not find a significant associations between LFMC and percentage peak FMD, as previously reported [134], [135]. The relationship between peak FMD and LFMC has been reported to show that a larger degree of vasodilation is associated with less vasoconstriction in [134], [135]. In these studies, it has been suggested that LFMC can be a complimentary assessment to FMD, which gives a broader overall picture of endothelial health. In our population, likely poorer overall endothelial health associated metabolic syndrome [148] may explain why our findings differ from healthier populations [134]. Impaired myogenic response of arteries in those with metabolic disease has been reported before with causes including elevated blood glucose levels and potentially obesity, though the evidence for the latter seems less conclusive [149]. Future research should continue to look at how LFMC, HFMC, TTP and peak FMD may associate with one another in different populations to better understand differences in the relationship between these parameters.

When comparing the baseline LFMC, HFMC and TTP by sex in table 2.5, we found that females took longer to reach their peak FMD than their male counterparts (p = 0.03). The group had no significant characteristic differences apart from females having a higher level of HDLC (p = 0.04) (table 2.5). Sex differences in response to FMD have been previously reported which can make mixed-sex clinical studies difficult to interpret (if not statistically adjusted), in particular if women are pre-menopausal. In a small study of *n* = 10 women and *n* = 10 men, women were found to have a more pronounced shear stress mediated vasoconstriction and vasodilation than the age matched men [150]. Similarly another study found that continuous (30 minutes) blood pressure cuff occlusion in men, but not women, blunted percentage peak FMD (p <0.00); showing pre-menopausal women may have some comparative advantage in terms of vasodilatory capacity [151]. An underlying component of this is thought to be oestrogen [152]; this may also be associated with the previously observed lower risk of CVD in women during the premenopausal phase (against age-matched men) [153]. It has also been shown that the CVD risk accelerates in women after the menopause (when oestrogen production is reduced) with the CVD risk comparable with men [153], [154]. In the retrospective analysis of the CIRCLES study, the female study population were on average 63.83 years (SD 5.12); thus, our data is likely representative of women who are post-menopausal.

In the analysis which categorised participants by statin user status (i.e., YES / NO), there were no differences between statin users and non-statin users for percentage peak FMD (table 2.6). This finding was surprising, as percentage peak FMD has previously been reported to be improved following statin therapy in those with type 2 diabetes, metabolic syndrome and in healthy men [155]–[159] and has been shown to convey both acute and long-term benefits on endothelial function [160]. We found that HFMC was significantly less pronounced in statin users (p = 0.03; table 2.7) which may indicate some favourable vascular compliance. As the literature on HFMC is very limited, the importance of this finding is unknown. Ostrem *et al.*, 2017 reported that HFMC was more pronounced (constrictive) in those with increased body mass, fat mass and BMI. Therefore, it remains plausible that a more constrictive HFMC score is indicative of increased health risk (associated with body mass). As such, the exploratory analysis on statins adds to the existing

limited literature on HFMC indicating it may be a complimentary measure of endothelial health alongside peak FMD.

To our knowledge, no other researchers have reported on the acute effects of blueberry anthocyanins on LFMC, HFMC and TTP in older adults with metabolic syndrome. Our data showed no significant difference of the blueberry intervention when compared with the control group at 3, 6 or 24 hours (table 2.9). Although not statistically significant it was notable that the blueberry group shortened the TTP at each time point after baseline, indicating a faster mobilisation of the dilation response. At 24 hours, the blueberry group had not returned to baseline TTP, whereas the placebo group were above their baseline at 6 hours and 24 hours. As stated previously (see section 2.1.5), delayed TTP is linked with associated with poorer health outcomes. Blueberry anthocyanins have been shown to improve percentage peak FMD acutely previously [161]. Although the previous researchers found no acute improvements in FMD in this population (paper currently under review), and no improvements in LFMC, HFMC and TTP were observed in this analysis, the direction of travel towards a prompter TTP in the blueberry group is potentially worthy of follow-up, in an adequately powered future RCT.

This analysis comes with its own limitations considering its exploratory and retrospective nature. No formal power calculation was performed which means that our data is hypothesis generating. When this FMD data was originally captured maintenance of image clarity during occlusion in order to be able to assess LFMC and HFMC was not specified in the protocol. This meant a large proportion of the participant FMD sequences (n = 8) were of insufficient quality. Additionally, a single researcher (CF) assessed the retrospective data independently which could lead to bias. Future research should consider two independent researchers to analyse the data and should aim to assess descriptive data of LFMC, HFMC and TTP in a healthy population.

In conclusion, no differences in HFMC, LFMC and TTP were seen in the blueberry anthocyanin intervention. There were significant associations between LFMC and HFMC, which has not been previously reported. HFMC was less constrictive in statin users, which may indicate favourable endothelial health. Further studies, with adequate power an

maintained image clarity during FMD, are required to determine more definitive health effects of these FMD analysis parameters.

CHAPTER 3. A double blinded, randomised controlled trial providing a single dose of blueberry anthocyanins in overweight older adults: Protocol and methods

3.1 Introduction to the AMP study

Regular consumption of blueberries, rich in anthocyanins, are associated with protection against CVD in large epidemiological studies [45], [54], [77], [162], [163]. Within RCTs, the data regarding the cardioprotective properties of blueberry anthocyanins are equivocal. This is possibly due to interindividual variation in response to anthocyanins and the potential of different metabolic profiles [91]. Using previous data from our group Curtis et al, 2019 [65], a novel blueberry anthocyanin metabotype for blueberry anthocyanins was identified that we hypothesised was associated with improved cardiometabolic health.

Using 24-hour urinary metabolite data from the chronic element of the 6-month CIRCLES study [65], a mathematical modelling assessment was made by Laura Haag (LH, PhD student) to predict an anthocyanin metabolism profile associated with improved cardiometabolic health. This profile, which comprised of hippuric acid, 3-hydroxyhippuric acid, 4-hydroxy-3-methoxyphenylacetic acid, and 3,5-dihydroxyphenylpropionic acid metabolites, was hypothesised to be characteristic of greater colonic metabolism of anthocyanins. Collectively, increased levels of these metabolites were positively associated with a greater percentage peak FMD (PhD award outstanding, data not published currently). The metabolite sample analysis and data analysis are not part of the current thesis, as this was the sole work of LH. Chapters 3, 4, 5 and 6 of this thesis reports on participants in the AMP study, who were prospectively recruited on the basis of an initial screening assessment to determine their metabolism profile – where volunteers were categorised as having profiles reflecting either HIGH or LOW metabolism (Phase 1 of the study).

A double blinded, randomised controlled cross-over study was conducted to determine whether blueberry anthocyanin metabolism profiles (HIGH / LOW) were associated with

acute differential cardiometabolic responses to a single dose of freeze-dried blueberries (Phase 2 of the study). To simulate real-world dietary practices, the blueberry and placebo powders were served with a milk-based drink, as part of an energy-dense meal. A single-dose assessment was chosen, to enable the testing of cardiometabolic effects over a 48-hour period, with rigorous dietary control of intake prior to and throughout the assessments. Dietary intakes, including the energy-dense meal, were adjusted on the basis of estimated basal metabolic rate (BMR) and habitual exercise levels. Overweight older adults were chosen for inclusion as they are at greater risk of CVD than their younger counterparts and would benefit more from a cardioprotective dietary intervention [2]. As prospective recruitment on the basis of anthocyanin metabolism profile has not been tested previously, this assessments provides the initial step in determining whether longer term studies would benefit from metabolism profile specific interventions. The principal objective of this research approach was to determine whether pre-existing anthocyanin metabolism profiles had a bearing on 1) postprandial responses to a high-fat meal +/- blueberry inclusion, 2) cardiometabolic function over a 48-hour time period when it was anticipated that anthocyanin metabolites would remain in circulation in some participants.

At study inception, a target of 315 healthy men and women (power calculation described in section 3.6.1) was calculated. Participants were recruited from within a 25-mile radius of the clinical research facility, through media advertisements and GP practices. A total of *n* = 15 GP practices were recruited to host the study. In broad terms, study participants were aged 50-80 years old with a BMI ≥25kg/m^2 and judged to be otherwise 'healthy' according to inclusion / exclusion criteria detailed in tables 3.1 and 3.2. Research nurses and the study clinical advisor (GP; Dr Jane Ewing) at the NHS clinical research facility (Quadram Institute, Norwich) determined study eligibility – based on inclusion / exclusion criteria, and clinical judgement (Dr Ewing) when required on a case-by-case basis.

3.1.1 Rationale for Phase 1 design

Previous studies have reported interindividual variation in response to flavonoids, often with both responders and non-responders to studies looking at health outcomes, including cardiometabolic health [93], [94]. To address this, the concept of dietary metabolic profiling,

or metabotyping, has been put forward and explored by several research groups in order to give targeted dietary advice for different health conditions [93], [164]–[166]. Laura Haag (LH) set out to create a novel metabotype for blueberry anthocyanins targeting cardiometabolic health outcomes, specifically improvements in endothelial function measured by FMD. A previous chronic intervention within our research group [65] demonstrated a significant increase in percentage peak FMD following 6 months of blueberry intake. Further analysis of urine samples from this intervention by LH showed metabolites present following blueberry intake. As part of LH's retrospective analysis (documented in her unpublished thesis), a factor analysis modelling approach identified a clustering of predominantly gut-microbial derived metabolites which in combination were associated with an improvement in FMD following chronic (6-month) daily blueberry intake. The anthocyanin 'metabotypes' were identified in the sampled population, which were categorised as being characteristic of LOW (15% population), MEDIUM (70% population) and HIGH (15% population) metabolism of blueberry anthocyanins. These analyses and interpretations are not part of this thesis, however, to summarise the main findings;

- A LOW metaboliser was characterised by having the lowest extreme of the microbial-derived metabolites in the 48 hours after blueberry intake – the reasoning was that such an individual may readily metabolise and excrete blueberry anthocyanins prior to microbial interrogation; thus, a limited amount of anthocyanin substrate was metabolised in the large intestine to produce the microbial-derived metabolites in the FMD associated profile.
- HIGH metabolisers had high levels of microbial-derived metabolites in the 48 hours after blueberry intake. The reasoning was that a HIGH individual may retain the metabolites in circulation for longer undergoing enterohepatic cycling producing microbial derived metabolites in the FMD associated profile.
- MEDIUM metabolisers had neither high nor low levels of microbial-derived metabolites in the 48 hours after blueberry intake are defined as those with metabolites concentrations in the middle, at neither extreme.

3.1.2 Rationale for phase 2 design

Previous studies have suggested that a variation in response to flavonoids may be driven by the gut and metabolites produced [81], [92], [93]. There have been associations between circulating anthocyanin metabolites and vascular response, specifically improved endothelial function measured by FMD [167]. To our knowledge, no previous studies have prospectively recruited participants on the basis of anthocyanin metabolism profiles. As such the focus of the present study, to determine vascular responses to blueberry intake in participants at the extremes of anthocyanin metabolism (HIGH or LOW metabotypes) was considered both novel and likely to improve understanding of how flavonoid metabolism may mediate health effects. A trial was designed to test the acute effects of blueberry anthocyanins on cardiometabolic function and to understand differences by metabotype. In addition, this study was designed to uniquely test vascular responses to the blueberry intervention over 48 hours – which is longer than previous studies [81]. In previous research at the University of East Anglia, where an anthocyanin treatment was labelled with a carbon-13 isotope (^{13}C), labelled metabolites were found to be circulating in urine, blood, breath and faeces between 1.5 and 48 hours after consumption [81]. Despite this, few studies have assessed effects of single anthocyanin intake for more than 6 hours postprandially. In the study by Czank *et al.*, urinary concentrations of metabolites were highest between 6 and 24 hours after anthocyanin consumption suggesting possible enterohepatic cycling. On the basis of these kinetic data, the current research study co-ordinated it's cardiometabolic health testing from 90 minutes to 6 hours on day 1, at 24 hours and at 48 hours – which was considered likely *a priori* to overlap with the anticipated prolonged circulation of anthocyanin metabolites.

A cross-over design was chosen, as this allows equal recruitment of participants in both treatment arms (where individuals act as their own control). The age group of 50-80 years and inclusion of overweight or obese (BMI ≥25 kg/m^2) participants was appropriate because of the increased CVD risk in this group [168]. Current dietary guidance encourages intake of fruit and vegetables for numerous benefits including cardio protective [23]. The intake of anthocyanins is proposed to be a key contributor for CVD protection [29], [45], [53].

An energy-dense meal was incorporated to investigate the negative impact of high-fat, energy-dense meals on cardiovascular health, and to establish whether blueberry anthocyanins may attenuate these responses. It has been shown previously that energy-dense meals increase triglyceride levels and reduce endothelial function acutely [35]–[38]. Despite this, energy-dense meals are frequently consumed in westernised societies, including by those with compromised health. The energy-dense meal provided was 50% of daily energy intake (considered appropriate for a combined meal of breakfast and lunch) and 40% of calories from fat (further detail in section 3.4.6). This level of calories and fat is similar to meals served at well-known popular fast-food chains throughout the UK.

3.2 The consequences of the COVID -19 pandemic and subsequent lockdowns on study conduct

In March 2020, a total of 119 people had completed the first part of the study (Phase 1 – data included for PhD thesis of LH) and metabotype profiling had identified potentially 50 participants for the second phase of intervention inclusion (Phase 2 – data included for thesis of CF). Phase 2 of the intervention study was ongoing, with *n* = 8 having completed both arms of the cross-over study, *n* = 14 having completed their first intervention (either placebo, or blueberry). Another *n* = 6 participants were recruited and had their first scheduled appointment after the March 2020 lockdown. We expected *n* = 28 participants to have completed both arms of the study by the first week of June.

On 18th of March 2020, which coincided with the impending first national COVID-19 national lockdown, guidance was given to immediately halt human research within the NHS clinical research facility, and a notification (with sponsors approval) to pause this research study was submitted to the research ethics committee. All study assessments were cancelled with immediate effect and due to capacity issues within the clinical research facility, approval to re-start the study was not provided until mid-September 2020. At this stage, a number of participants expressed reservations about attending a hospital environment (for a voluntary appointment) and withdrew their consent to participate. In addition, key research team members were isolated overseas (having returned to Australia for family reasons at the start

of the pandemic) or had completed their period of study registration (LH; PhD student responsible for metabolite analysis and biological sample handling). In January 2021, after the implementation of the third National lockdown, the research study was abandoned prematurely – predominantly due to uncertainties in the longevity of the metaboliser profiles, participant withdrawal and staffing shortages.

Due to these unforeseen circumstances associated with the COVID-19 pandemic, the structure and focus of this thesis was substantially amended – 1) an incomplete dataset was used for this thesis, 2) As well as analysing the available data in the original 'cross-over' manner, a secondary analysis has been performed with intervention treatment (blueberry *versus* placebo) assessed as a *post hoc* 'parallel group' study (which was not anticipated / planned at study inception) (see consort diagram, figure 3.1 and 3.2).

The remaining three results chapters (Chapters 4, 5 and 6) will explore and analyse the data collected while noting that the intention for the data (collected for a cross-over design analysis) was only possible for 8 participants that completed both intervention arms before study cessation. Participants who completed only 1 treatment arm (blueberry or placebo) (i.e. n = 22 in total) were analysed in a parallel design. The presented data cannot be conclusive as it does not meet the required power calculation numbers, with further research required to confirm any notable observations.

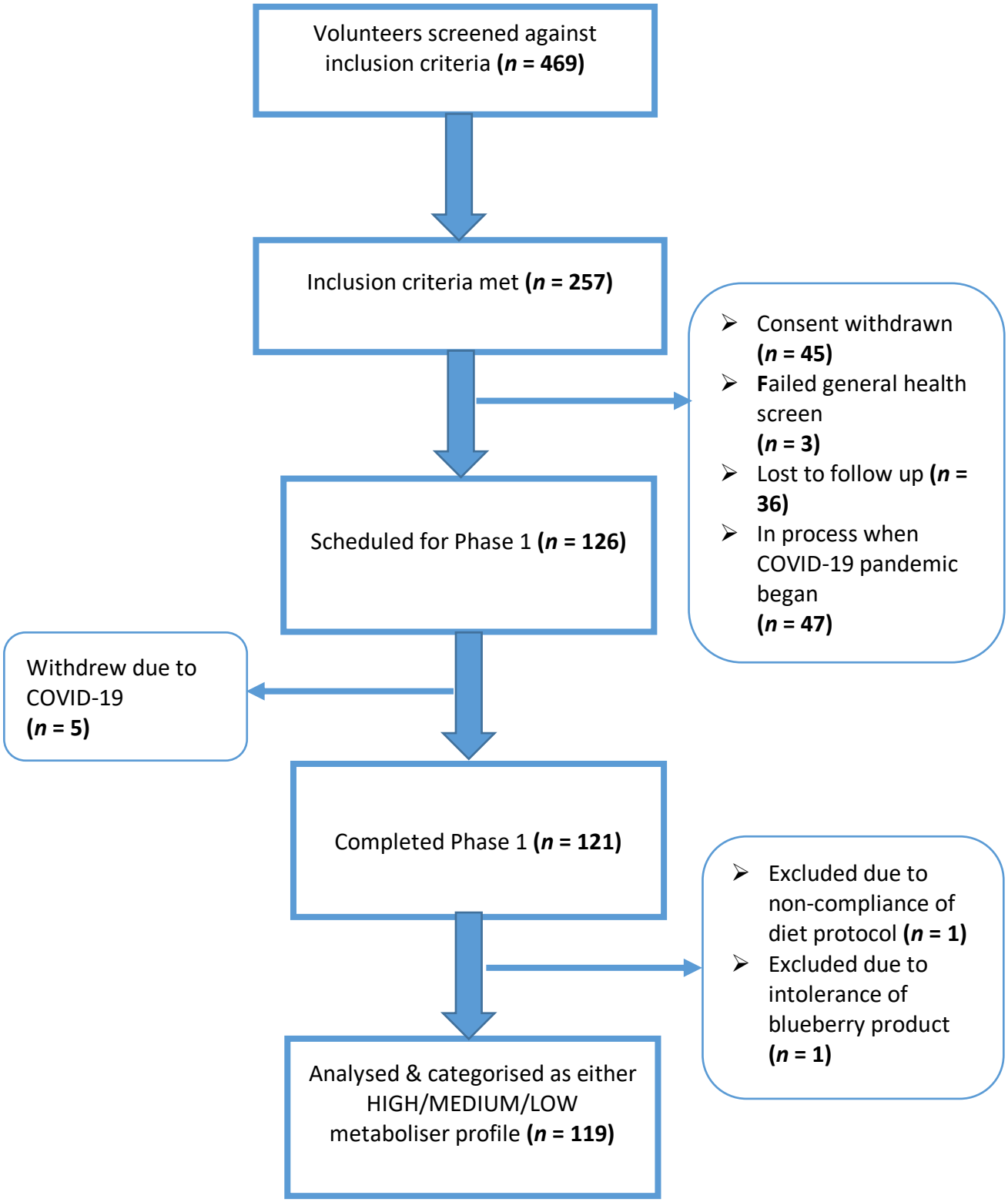


Figure 3.1: Consort diagram displaying recruitment and Phase 1 of study.

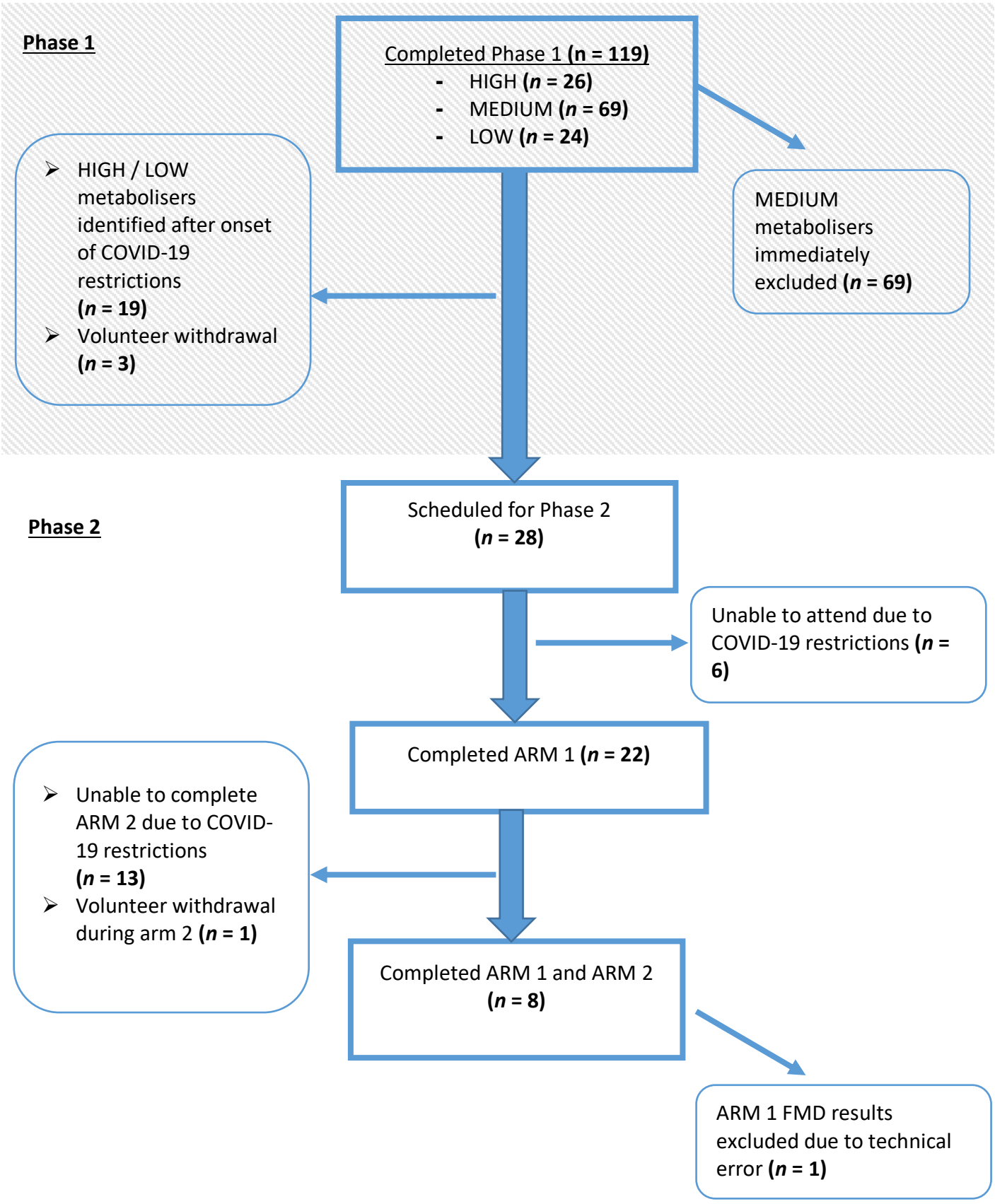


Figure 3.2: Consort diagram showing the progression of participants from Phase 1 to Phase 2 of the study.

3.3 Participants and methods

3.3.1 Study population:

Our planned target was to recruit 82 adults to Phase 2 of the study (see power calculation; section 3.6.1); with *n* = 41 LOW and *n* = 41 HIGH anthocyanin metabolisers required to complete the cross-over study. Participants were aged between 50-80 years, with a BMI ≥25 kg/m^2, and were non-smokers (either 'never smokers' or those ceasing ≥ 6 months prior to general health screening), without a GP diagnosis of diabetes, cardiovascular disease (CVD), or any other conditions that may influence flavonoid metabolism, or vascular or metabolic function. The use of a range of prescribed medications with known effects on vascular function or glucose control (i.e., vasodilators, anti-hypertensive and diabetes medications) was not permissible. Other medications were assessed on a case-by-case basis, in consultation with the study's clinical advisor. Flavonoid supplements were not allowed; other supplements (e.g., fish oil capsules) were reviewed on a case by case basis to determine whether they had concomitant modes of action which may affect the outcome markers in the study. The study inclusion / exclusion criteria are show in tables 3.1 and 3.2.

To ensure the suitability of potential study volunteers, their medical histories were compared against inclusion/exclusion criteria through two processes:

1) GPs hosting the research study, targeted patients meeting the study inclusion criteria through searches of the practice database – with only those patients deemed eligible receiving information about the study, and an invitation to take part.

2) An additional assessment (a health and lifestyle questionnaire) was then conducted on those people who expressed an interest to participate. In this questionnaire, information to confirm all exclusion criteria was requested – including items that would not be routinely recorded on a GP practice database i.e., SystmOne.

Inclusion Criteria
Assessed via health and lifestyle questionnaire
Adults aged 50 to 80 years
Assessed from the clinical screening at CRTU
BMI ≥25 kg/m^2
Successful blood pressure assessment, biochemical, haematological and urinalysis assessed by research nurse and clinical research facility clinical advisor.

Table 3.1: Study inclusion criteria

Exclusion Criteria
Assessed via health and lifestyle questionnaire
Current smokers, or ex-smokers ceasing < 6 months ago.
Subjects with existing or significant past medical history of vascular disease or medical conditions likely to affect the study measures i.e. vascular disease, circulatory (i.e. Reynaud's), diabetes, hepatic, renal, digestive (including coeliac disease), haematological, neurological, cancer (excluding Basal-cell carcinoma) or thyroidal disease – to be judged by the study clinical advisor.
Fructose or lactose intolerant subjects, those with known allergy to blueberries.
Those unprepared to adhere to dietary restrictions for 5d preceding the 'blueberry challenge' and each of the two, test meal phases or unwilling to comply with the assessments *per protocol*.
Those unprepared to consume provided food (meals and drinks) for 4d during each test-meal assessment phase
Parallel participation in another research project involving dietary intervention and/or sampling of biological fluids/material.
Those on therapeutic diets or having experienced substantial weight loss (i.e. ≥5% body weight; to be judged by research nurses and the clinical advisor) within 3 months of screening.
Those taking flavonoid, nitrate / nitrite containing or fish oil containing supplements (and unwilling to cease intake during, and 1 month preceding the trial) or unwilling to maintain existing intake of other supplements. Examples of flavonoid supplements we will be asking participants to refrain from taking are: blueberry extract, beetroot extract, spinach capsules and acai capsules.
Prescribed hypoglycaemic, anti-hypertensive, vasodilator or HRT medication. Other medications to be assessed for suitability by the clinical advisor.
Assessed from the clinical screening at clinical research facility
Clinical advisor judged: abnormal biochemical, haematological or urinary results or measurements considered to be counter indicative for the study: including kidney and liver function, fasting glucose (especially if indicative of diabetes), lipid abnormalities, full blood count.
Undiagnosed hypertension (elicited at screening; i.e. *≥ 180 / 110mmHg*) at a level requiring immediate referral back to general practitioner for follow-up.

Table 3.2: Study exclusion criteria

3.3.2 Recruitment strategy:

All participant facing recruitment materials provided the study team contact details; with the instruction that potential volunteers could express an interest to participate by returning a slip with their contact details to the research team. Participants were approached for involvement in the study in two main ways:

3.3.2.1 Media calls:

Poster advertisements were placed in the clinical research facility and surrounding University buildings and across the Norwich Research Park. A call for volunteers for the study was featured in local media articles (Eastern Daily Press, ITV News) and were shared by respective outlets on social media. The research team also participated in community engagement, by presenting information about the benefits of blueberry intake within the science zone at the Royal Norfolk Show; here, interacting with potential study participants and answering questions about nutrition and research to engage prospective study participants.

3.3.2.2 GP involvement:

n = 15 GP surgeries within a 25-mile radius of the clinical facility agreed to identify potential participants for the study. Each participating GP practice was given a source document folder which contained the study protocol, ethical approval documentation, and a copy of all information to be provided to their patients. GP practices then searched their practice database to identify potential eligible patients with medical histories that fit key inclusion criteria. Those identified patients were sent, by post, a study pack which included; 1) letter of introduction from the GP, 2) an invitation to participate from the study team, 3) a participant information leaflet, 4) a health and lifestyle questionnaire, and 5) a prepaid envelope addressed to the study team. To ensure patient anonymity of those not wishing to take part, no patient details or details of the GP search were shared with the research team. The research team were only aware of potential study participants when they contacted the research team themselves. Participating GP practices were also asked to display study posters in their practice with contact details of the study team.

3.3.3 Eligibility assessment

3.3.3.1 Summary

The study had three stages; a health and eligibility screen, followed by a metaboliser profile assessment (Phase 1 - which was a gate-way assessment to participate further), and a cross-over intervention study (Phase 2). In the first stage, written informed consent was obtained before all study related procedures. This was followed by a general health screen which required no excluded medical conditions (table 3.2) for subsequent involvement. The second stage was confirming the metaboliser profile following a 'blueberry challenge' (Phase 1). Finally in the third stage, participants with a HIGH or a LOW metaboliser profile were then eligible to enrol in the intervention study (Phase 2).

3.3.3.2 Health and lifestyle questionnaire

The health and lifestyle questionnaire collected general health information, and also information about diet and lifestyle that was not captured in medical records. On the basis of reviewing the questionnaire, many volunteers were ruled out prior to a consent visit or a general health screening visit. This was an effective use of both researcher time and of participant's time. All self-reported information provided by the study volunteer was voluntarily given and was checked and confirmed by the research / clinical team if there were any ambiguous responses.

The health and lifestyle questionnaire captured the following information:

1. Age.
2. Estimated weight and height (used to calculate BMI, which was confirmed later in person).
3. Recent change in bodyweight (a ≥ 5% change in the previous 3 months was an exclusion), attempts to lose weight, therapeutic or restrictive diets (with weight loss as an aim).
4. Past and present smoking status.
5. Medical history of any past vascular disease, diabetes, hepatic, renal, digestive, haematological, neurological, cancer (excluding basal- cell carcinoma) or thyroidal disease (immediate exclusion). Other medical conditions were assessed on a case-

by-case basis by the clinical team to determine whether they might affect the study outcome measures.

6. The prescription of anti-hypertension, hypoglycaemic, vasodilators or hormone replacement therapy medications.
7. The frequency of over-the-counter medication use; including long-term pain relief, anti-inflammatories and antihistamines.
8. Food allergy and intolerances; including fructose, lactose, or known allergy to blueberries, and those with coeliac disease.
9. Concurrent participation in any other scientific studies that include consuming food or the donation of biological samples.
10. The intake of dietary and non-dietary supplements.
11. Typical intakes of anthocyanin rich fruits, tea and coffee, dark chocolate, oily fish and alcohol.

Once returned to the study team (by email or by post), the questionnaire was reviewed and those meeting the inclusion criteria were invited to attend an in-person consent visit at the University of East Anglia.

3.3.3.3 Consent

Eligible participants were invited to a group consent visit (*n* = 8 max. volunteers per session). One-to-one consent sessions were also offered; however, nobody requested this option. The group consent visit was led by a member of the research team (up to 4 members, including CF, rotated at leading consent sessions) and was an opportunity to fully inform potential participants about the study and answer any volunteer questions. The format of the meeting was a 30-minute PowerPoint presentation followed by a question and answers period. The PowerPoint presentation gave information on the study design, study hypothesis, what would be expected of the volunteer (with specific details about any fasting, food restrictions, food provision, type and frequency of biological collections and time at the clinical research facility required). Participants were aware no fee was given for participating in the study.

If volunteers were happy with the information provided, a number of forms were signed (and a copy retained by the volunteer) to provide the approval to proceed with the study and ensure that volunteers were fully informed. These forms were as follows:

- **Consent form:** This provided written confirmation that volunteers agreed to participate in the study.
- **Medical declaration form:** This was collected from each volunteer which confirmed that they would inform the research team if they experienced changes in medication or health.
- **Key aspects of the research form:** This explained each of the key aspects of the research design which may impact the volunteer (i.e. what would be expected of them at each visit) and volunteers signed each statement to confirm they understood.

Volunteers were provided with a copy of these forms, and, with the consent of the study volunteer, a copy of the consent forms was also sent to their GP to confirm their participation in the study. After volunteers had consented, a 3 day 'cooling off' period was observed prior to making arrangements for the general health screening visit. This period allowed participants to consider their study involvement and determine whether they wished to withdraw their consent.

3.3.3.4 General health screening

The purpose of the in-person, general health screening, was to ensure that participants had the following:

1. BMI ≥25.0kg/m^2
2. Successful biochemical, haematological and urinalysis, which was assessed by the clinical team and signed off by the study clinical advisor

Study volunteers attended the clinical research facility having observed an overnight fast (≥10 hours, with only water permitted). A qualified NHS research nurse or health care assistant completed the screening visit which lasted around 45 minutes. The order of assessment was as follows: 1) a questionnaire to determine fitness to proceed (based on health on the day and preparation), 2) medical history and a medication assessment (including any dose changes), 3) height and weight were measured to determine BMI, 4)

blood pressure (BP) and pulse were measured at rest (using a calibrated Omron monitor, from Omron Healthcare Co., Kyoto Japan) in a seated position in quiet conditions. BP measurements were taken in triplicate (separated by 3 minutes) on each arm and an average of the six measurements were taken; those with a measurement of over 180/110 mmHg (systolic / diastolic respectively) were immediately excluded.

A urine sample was collected at the study visit and assessed with a dipstick test. Abnormal urinalysis results were sent to the GP and a clinical decision was made by the clinical research facility nursing team (with further input from the study clinical advisor when needed), to determine eligibility according to the inclusion/exclusion criteria.

Finally, for those volunteers progressing successfully through the screening, a fasting blood sample was taken (10ml total). From this, a full blood count analysis and assessments of glucose, liver function, kidney function, lipids, urea and electrolytes were performed at the phlebotomy department at the Norfolk and Norwich University Hospital (NNUH). After the screening appointment a breakfast voucher was provided to be redeemed at the Quadram Institute café.

Results from the blood analyses were received ~4-7 days after submission for analysis and were reviewed and signed off by the clinical team (research nurses and study clinical advisor). Results were shared with the volunteers GP and clinical notes were added by the clinical research facility clinical advisor (Dr Jane Ewing, GP) if a clinical need was determined. Those deemed eligible were invited to partake in phase 1, the ‘blueberry challenge’.

3.4 Study design:

3.4.1 Summary

The research study comprised of two phases;

- **PHASE 1 -** a metabolism screening phase (to identify suitable volunteers), and
- **PHASE 2 -** a placebo controlled double-blinded cross-over intervention period, designed to determine the acute effects of providing blueberry anthocyanins (with an energy-dense meal) on cardiometabolic responses. Due to the prospective recruitment of volunteers by anthocyanin metabolism type (LOW *versus* HIGH), the intention was to additionally determine the effect of metabolism type on vascular and metabolic responses.

3.4.2 Phase 1 study design

Urine containers and a food frequency questionnaire (FFQ) [169] were provided at the general health screening along with instructions to follow prior to the phase 1 visit. Participants were required to observe 5 days of dietary restrictions (including restricting flavonoid foods, fully described in section 3.4.6) prior to the blueberry challenge, and for a further 2 days after consuming the blueberry milkshake (a total of 7 days of dietary restrictions).

Prior to coming into the clinical research facility, volunteers were asked to collect all the urine they passed in the 24 hours preceding the visit (a 24-hour baseline urine sample). Participants then attended the clinical research facility, in a non-fasted state (but still following dietary restrictions), to consume the blueberry challenge milkshake. For this, a single dose of the intervention material (freeze dried blueberries; providing 364mg anthocyanins (see section 3.4.6, table 3.6 for a breakdown of nutrition) was mixed into 500ml semi-skimmed cow's milk. Participants were observed whilst they consumed the drink. Thereafter, urine was collected for a further 48 hours to assess the excretion of blueberry metabolites. As before, all urine passed was collected in 24-hour aliquots (thus, 2 x 24-hour collections were made) stored accordingly (i.e., in a cool place) by the volunteer

and returned to the research facility at 48 hours. Boric acid (15g) and ascorbic acid (100mg) was added to the urine containers to preserve the sample and deter microbial growth.

3.4.3 Phase 1 results

LH analysed a total of *n* = 119 volunteer urine samples for phase 1. Each urine sample was aliquoted (10ml) and centrifuged for 15 minutes before being acidified and stored in -80 degrees Celsius. Samples were then analysed using liquid chromatography tandem mass spectrometry and tandem mass spectrometry using electrospray ionisation. The metabolites included in the metabotype profile were hippuric acid, 3-hydroxyhippuric acid, 4-hydroxy-3-methoxyphenylacetic acid, and 3,5-dihydroxyphenylpropionic acid (see table 3.3). These metabolites were chosen due to their abundance and their association with improvements in FMD after 6 month of daily blueberry intake [65]. Phase 1 categorised these metabotypes as HIGH, MEDIUM and LOW metabolisers. Only those volunteers characterised with metabolism profiles of HIGH or LOW were invited to partake in phase 2.

Table 6-2. Urinary excretion of panel metabolites up to 48h after blueberry drink by group

Group	Time	3,5-DiOH-PPA	3-OH-HA	4-OH-3-OCH-PAA	HA
Low (n = 19)	-24 to 0 h	11.0 ± 13.6	31.3 ± 54.1	17.5 ± 11.6	1708.7 ± 1461.0
	0 to 24h	5.4 ± 10.7	18.4 ± 37.0	12.4 ± 9.3	2139.3 ± 1529.8
	24 to 48h	8.5 ± 11.8	15.3 ± 35.5	13.2 ± 10.9	759.3 ± 871.0
Medium (n = 78)	-24 to 0 h	6.1 ± 10.6	12.3 ± 44.4	16.7 ± 10.0	841.8 ± 1122.6
	0 to 24h	6.4 ± 8.6	17.0 ± 27.1	17.3 ± 12.8	2249.8 ± 1406.2
	24 to 48h	6.3 ± 7.2	14.0 ± 35.3	16.4 ± 10.1	1060.4 ± 1320.1
High (n = 22)	-24 to 0 h	2.7 ± 4.5	7.5 ± 9.7	12.3 ± 9.0	448.2 ± 896.8
	0 to 24h	6.4 ± 6.3	25.0 ± 24.8	17.4 ± 12.2	2196.5 ± 1568.3
	24 to 48h	11.6 ± 10.8	27.7 ± 35.3	17.6 ± 11.6	1148.0 ± 1573.9
All (n = 119)	-24 to 0 h	6.3 ± 10.5	14.4 ± 42.5	16.0 ± 10.2	907.5 ± 1198.6
	0 to 24h	6.2 ± 8.5	18.7 ± 28.4	16.6 ± 12.3	2222.3 ± 1444.6
	24 to 48h	7.6 ± 9	16.8 ± 35.4	16.1 ± 10.5	1028.5 ± 1307.2

Values are mean ± SD in µmol/24h. 3,5-DiOH-PPA: 3,5-dihydroxyphenylpropionic acid; 3-OH-HA: 3-hydroxyhippuric acid; 4-OH-3-OCH-PAA: 4-hydroxy-3-methoxyphenylacetic acid; HA: Hippuric acid

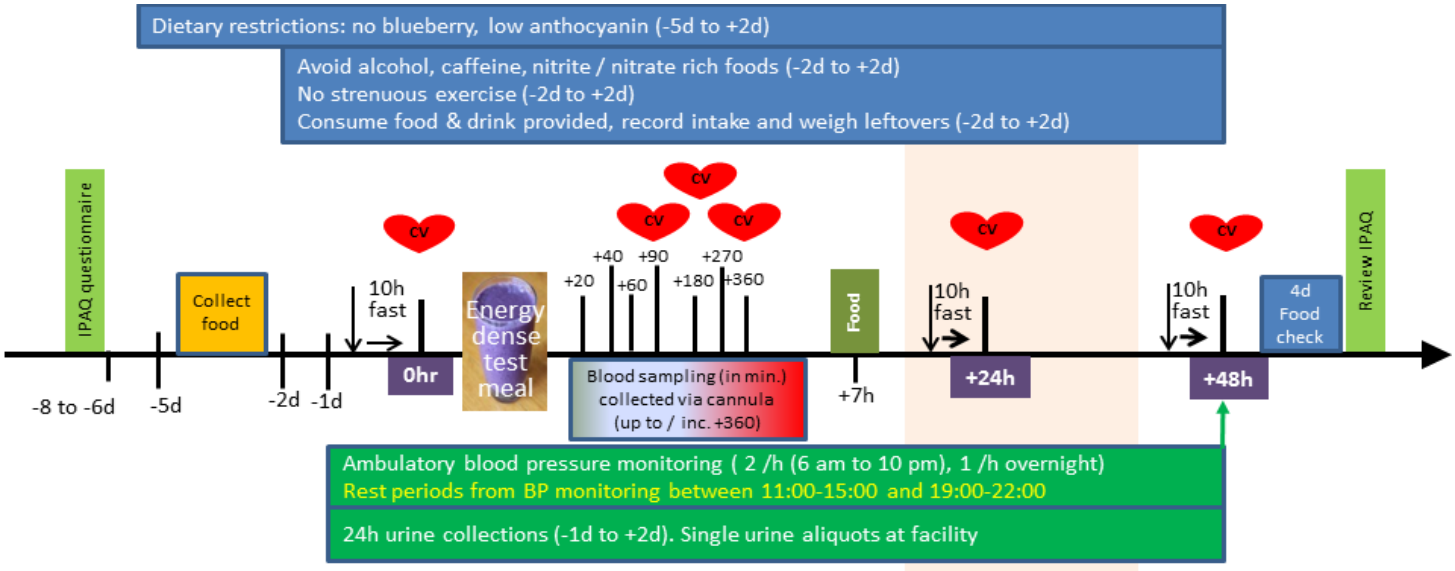
Table 3.3: Extracted table from Laura Haag's (LH) thesis showing the urinary metabolites for the LOW, MEDIUM and HIGH metabolisers

3.4.4 Phase 2 study design

The single-dose effects of blueberry intake, provided within a standardised energy-dense meal challenge, was tested on vascular function and a series of metabolically relevant biomarkers, at specific time-points following intake (over a 48-hour period). Fasted baseline

measurements were also taken, so that the change in response could be determined. Phase 2 was designed as a cross-over study however, the data collection phase was interrupted, and subsequently abandoned, due to the COVID-19 pandemic. As a result, 22 participants completed arm 1 of the cross-over (12 blueberry and 10 placebo), 8 completed both arms 1 and 2 and therefore completed the study.

Participants were asked to restrict flavonoid containing foods (see section 3.4.6.1) from their diet and avoid vigorous exercise 5 days before attending the facility for their test meal. For 48 hours before the test day, and the 48 hours after, participants were given all of their food and drink by the study team to control background diet. Participants were provided with a selection of foods to choose from (Appendix B2) and then were given a tailored menu which specified the weight of each food item which was reflective of their estimated energy requirements (based on standardised formulae; see section 3.4.6.2). Participants were provided with a food checklist and were asked to note down whether they ate the food items provided in the quantities advised, or whether they modified the intake amounts (weighing scales were provided and their use encouraged). These data were used for the second treatment week of the cross-over design to ask volunteers to ensure that dietary intake was comparable across both treatment arms. Urine was collected 1 day prior to the test day and for the 48-hour after test-meal intake. These samples were to be used to assess absorption, distribution, metabolism and elimination of anthocyanin parent compounds and secondary metabolites. As detailed in figure 3.3, a number of vascular function measures were repeatedly performed during the assessment visits at the clinical research facility; including at baseline and during the 48 hours which followed the test-meal intake. As noted in figure 3.3, ambulatory blood pressure monitoring (described in 3.4.5.3) was also performed in the participants domestic environment.



	Hours	Minutes								Hours	
	-24	0	20	40	60	90	180	270	360	+24	+48
Biological samples:											
- **Blood**		X	X	X	X	X	X	X	X	X	X
- **Urine**	X	Single aliquots in CRF								X	X
Cardiovascular measures											
- **Endothelial function (FMD)**		X				X	X		X	X	X
- **Resting blood pressure**		X				X	X		X	X	X
- **Ambulatory BP**	X	X	When possible throughout day							X	X
- **Artery stiffness (PWV & AIx)**		X					X		X	X	X

AIx, augmentation index; **BP**, blood pressure; **CRF**, clinical research facility; **CV**, cardiovascular; **FMD**, flow mediated dilatation; **IPAQ**, International Physical Activity Questionnaire; **PWV**, pulse wave velocity.

Figure 3.3: Phase 2 study design. Figures details the assessment timeline and endpoint overview for each of the intervention periods in phase 2. The timing of when food and questionnaires were provided is identified.

3.4.5 Phase 2 - Measures of vascular function

3.4.5.1 Rationale for vascular assessments chosen

Three key parameters of vascular function were assessed in this acute study: endothelial function, blood pressure and arterial stiffness. The gold-standard non-invasive method of assessing endothelial function is ultrasound determined flow-mediated dilation (FMD) and this was the primary endpoint of the study. Arterial stiffness was a secondary vascular endpoint; determined using two non-invasive techniques: pulse wave velocity (PWV) and augmentation index (AIx). PWV is recognised as a gold standard for measuring arterial stiffness [170]. Blood pressure was measured in the clinical setting at rest, and outside of the clinical setting using an ambulatory blood pressure monitor (over a period of 3 days).

Ambulatory blood pressure is described as the gold standard method of assessment because it takes into account fluctuations of blood pressure throughout the day and avoids 'white coat syndrome' [111].

3.4.5.2 Endothelial function (flow-mediated dilation)

Equipment: Researchers used either a Philips iE33 or a Philips EPIQ 5 ultrasound machine with a superficial linear transducer (L18-5 probe – flat head) ultrasound for image acquisition. The same equipment was used for each assessment made on an individual. Image sequence acquisition was initiated via a 3 lead ECG, which captured sequential image frames in sequence with each heartbeat. Data was captured on Vascular Imager computer software. Reactive hyperemia of the brachial artery was induced by cuff inflation to 220mmHg (using an Aneroid Sphygmomanometer; Hokanson, Bellevue, USA). Data analysis was conducted using Brachial Analyzer software (Medical Imaging Applications).

Data acquisition: One experienced research scientist (Dr Peter Curtis (PC)) - attended training course at St Thomas's Hospital London and has completed >100 hours of FMD scanning; the second researcher, Caoimhe Flynn (CF), was taught by PC and attained sufficient FMD experience prior to study commencement. PC and CF conducted all FMD measurements throughout the study. In preparation for the assessment day, participants fasted for ≥ 10 hours prior to arrival at the research facility and limited exercise and caffeine intake. FMD was performed in a quiet, dimly lit, temperature-controlled room (between 21-24°C) with the blinds shut to prevent any outside distraction and direct sunlight.

The researchers explained the procedure to study participants, ensuring they were aware that 5 minutes of cuff-occlusion at 220mmHg can cause some tingly sensation to the fingertips (and in some cases a small amount of pain and discomfort). Participants were made aware that they must remain still during the procedure. This was fully explained at the consent visit with the aid of a video of the FMD procedure. For the assessment, participants were placed in a supine position on a hospital bed for at least 15 minutes prior to the procedure. Researchers limited communication with participants in an effort to minimise distractions that may affect measurements being taken. A blood pressure cuff was placed on the participant's right forearm with the leading edge aligned with edge of the elbow.

Researchers used ultrasonography (Philips iE33 or Philips EPIQ 5) to attain a longitudinal image of the brachial artery and confirmed this with doppler flow to confirm the vessel chosen was arterial (as shown in figure 3.4 B). Once a clear image of the brachial artery was located, which was characterized by ensuring the vessel was level and the edges of the vessel were clear, the ultrasound probe was secured in a stable position within a clamp (as shown in figure 3.4 A) which was mounted on a magnetic plate which could be moved incrementally using a manometer screw. Notes were made if any difficulty was incurred during setup, including if for any reason the right arm was unable to be used and where on the arm the probe was placed so that the procedure could be replicated during the participant's next visit. Image capture was trigged by the participant's heartbeat via a 3-lead ECG and Vascular imager software (Vascular Imager software; Medical Imaging Applications LLC, Coralville, USA). The FMD assessment procedure lasted 11 minutes and was coordinated as follows; 1-minute capture at baseline (rest), 5 minutes occlusion with the blood pressure cuff inflated at 220 mmHg and 5 minutes post occlusion (cuff deflation). A clear image was maintained by the researcher throughout the 11-minute procedure. FMD measurements were taken at 0 mins (baseline) ,90 mins, 180 mins, 360 mins, 24 hours and 48 hours (shown in figure 3.3).

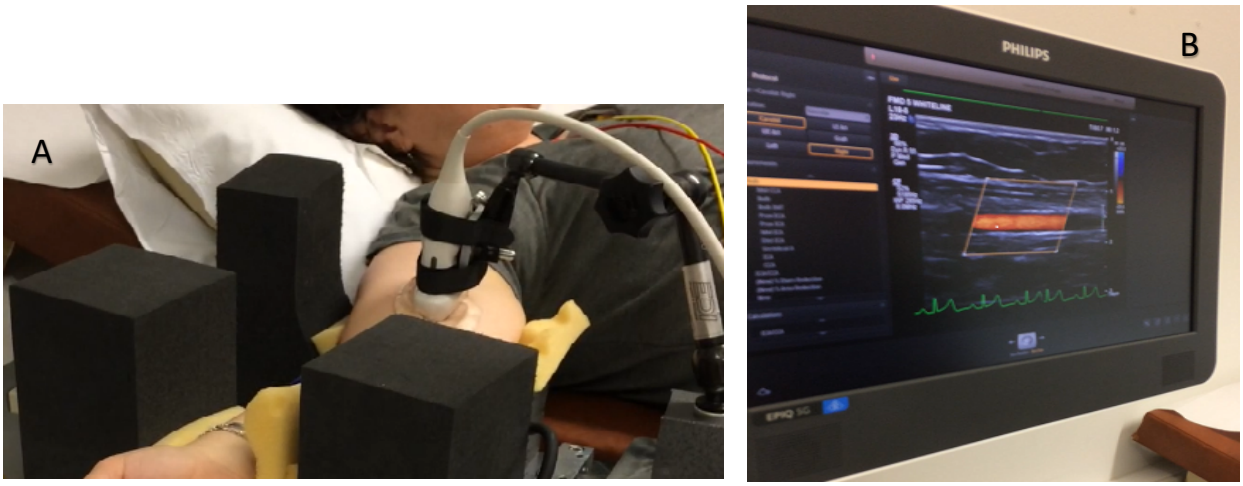


Figure 3.4: Flow-mediated dilation in practice. Image A shows the ultrasound probe secured by the clamp on an arm which is propped up by foam arm holds. Image B shows the longitudinal image acquisition of the brachial artery on the Philips ultrasound machine.

Data analysis: Analysis of the FMD image sequences was completed independently by the two researchers carrying out the FMD procedures, who were blinded to intervention allocation. A region of interest was identified, and automated edge-detection software was used (Brachial Analyzer v5; Medical Imaging Applications LLC, Coralville, Iowa) to minimise

human error. The entire 11-minute sequence was reviewed to establish that the region of interest was viable throughout the assessment and to identify any software issues with edge-detection. Any automated edge detection errors on a frame had to be either excluded or fixed semi-automatically (using the automated 'detect' function on an edge manually selected by the researcher). A mean baseline diameter was calculated as an average of all viable baseline frames. Peak FMD was calculated as percentage; $(diameter_{max} - diameter_{baseline}) / diameter_{baseline} \times 100$. LFMC was calculated as an average all viable frames 30 seconds prior to cuff release; $(diameter_{LFMC} - diameter_{baseline}) / diameter_{baseline} \times 100$ and given as a percentage. HFMC was calculated as an average of all viable frames 10 seconds after cuff release; $(diameter_{HFMC} - diameter_{baseline}) / diameter_{baseline} \times 100$ and given as a percentage. TTP was calculated as time of the peak frame – time of cuff release (in hh:mm:ss).

Data validation: To ensure data quality, two researchers (PC and CF) independently analyzed all FMD assessments (*n* = 174, 11-minute procedures in total) using Brachial Analyzer Software. All sequences were independently rated by the researchers and notes made if quality was insufficient. In Microsoft Excel CF took the average of both researchers results for each FMD endpoint and timepoint which was used for statistical analysis. In cases where both researchers agreed that data quality was poor for a particular endpoint and analysis not possible, these FMD assessments were excluded.

To reduce bias, the intention was that the two researchers would review all independent analyses where there was >1% or 10 seconds difference between the values of each researcher. This was to reduce bias in the results however, time constraints due to the interruption caused by COVID-19, meant this was not possible. Instead, both researchers reviewed a small selection of FMD analyses from the data set that were identified as having a substantial difference between the two researchers' values. Table 3.4 shows the results of this validation analysis and percentage change from original mean value can be seen to vary. For peak FMD for example, the joint review percentage change ranges from -2.44 to 1.02%.

Table 3.4: Flow-mediated dilation validation – joint review of a selection of flow-mediated dilation analyses

	Peak FMD			TTP			LFMC			HFMC		
FMD assessment	Original mean	Joint review – mean value	% Δ	Original mean	Joint review – mean value	% Δ	Original mean	Joint review – mean value	% Δ	Original mean	Joint review – mean value	% Δ
1 (a)	5.24	3.84	-1.40	00:00:33	00:00:36	0.08	2.56	1.40	-1.16	1.10	0.03	-1.07
2 (b)	6.78	4.34	-2.44	00:00:35	00:00:35	0.00	0.95	0.41	-0.54	1.24	-0.17	-1.41
3 (b)	4.12	3.26	-0.86	00:01:17	00:00:55	-0.40	0.20	-0.03	-0.23	0.20	-0.03	-0.23
4 (a)	11.73	12.76	1.02	00:00:50	00:00:50	0.00	-1.05	-0.37	0.68	-1.54	-0.77	0.77
5 (a)	1.69	1.09	-0.60	00:01:00	00:01:03	0.05	1.75	NP	NP	0.04	-0.56	-0.60
6 (c)	4.12	4.12	0.00	00:00:33	00:00:33	0.00	-1.38	-1.38	0.00	-2.39	-2.39	0.00
7 (a)	10.81	9.66	-1.15	00:00:34	00:00:33	-0.02	1.42	0.10	-1.32	1.02	0.03	-0.99
8 (d)	6.04	6.04	0.00	00:00:37	00:00:37	0.00	0.98	0.98	0.00	1.33	1.33	0.00

This table displays 8 FMD analyses, all with at least one mean value of an FMD endpoint >1% different between researchers and therefore chosen for this validation table. The joint review was carried out by CF and PC. Values for peak FMD, HFMC and LFMC are shown as percentage, TTP is shown in hours:minutes:seconds. Abbreviations; NP (not possible) , TTP (time to peak), FMD (flow-mediated dilation), LFMC (low flow-mediated constriction), HFMC (high flow-mediated constriction), Δ (change) (a) = instances where both reviewers agree discount one researchers readings and use other researchers readings instead; (b) = instances where a new analysis is conducted by researchers together and a new value is decided on; (c) instances where one researchers repeated their results and a new mean was created from other researchers original results; (d) = both researchers agreed a mean both original analyses was still to be used.

3.4.5.3 Blood Pressure (resting and ambulatory) and heart rate

Equipment: Resting blood pressure was taken with an automated blood pressure monitor (using a calibrated Omron monitor, from Omron Healthcare Co., Kyoto Japan). ABPM was taken using ambulatory blood pressure monitors (ABPM) from Spacelabs (Spacelabs Healthcare, Hereford UK) attached to a blood pressure cuff. The ABPM monitors connected via USB to a laptop with Spacelabs software which allowed the data to be downloaded.

Data acquisition: During Phase 2, following 15 minutes of supine rest in a quiet, dark, temperature-controlled room, resting BP measurements were taken in triplicate (separated by 3 minutes). Researchers and clinical nursing staff took BP readings and ensured there was no communication with the participant so as not to affect the results. A sustained BP reading of >180/100mmHg resulted in termination of the participant in the study (at any stage) and a visit to their GP recommended. Resting BP readings were taken at 0 mins (baseline), 90 mins, 180 mins, 360 mins, 24 hours and 48 hours (shown in figure 3.3).

ABPM was taken to measure habitual BP over a 24-hour period and assess the effect of placebo or blueberry treatment on BP. Participants had a 30-minute session with a researcher who explained how to wear and use the ABPM equipment as well as the safety precautions to be followed. In total, participants wore the ABPM monitor for three sequential days in a row at each assessment period (i.e. one day before the test meal, to assess habitual BP, then during day of the test meal, and also on the day after (72 hours in total). Participants were asked to wear the ABPM monitor during the day (from 06:00 to 22:00) 1 assessment per 30mins was made, whilst overnight (22:00 to 06:00) this was reduced to once per hour. To reduce participant burden, two periods (per day) were defined as times to remove the ABPM device (i.e. 11:00 to 14:59 and 19:00 to 21:59 – 7 hours in total). During the 3 days, participants noted their sleep and waking times, eating times and any unusual events which may have affected BP (e.g. a football match on TV or an argument). A researcher went through the ABPM readings from the previous day with the participants to identify any abnormalities, issues with the machines and to check that volunteer noted any abnormal events during the 24-hour period.

Data analysis: Resting BP measurements were taken in triplicate and an average reading was recorded. Systolic, diastolic and heart rate average for each time point was inputted into Microsoft Excel and used for statistical analysis separately. Spacelabs computer software (Spacelabs Healthcare, Hereford UK) recorded all ABPM readings from the ABPM monitors. A report was produced with an average systolic, diastolic, heart rate, mean arterial pressure and pulse pressure reading for each of the 3 days.

3.4.5.4 Arterial stiffness (pulse wave velocity and augmentation index)

Equipment: Vicorder equipment (Skidmore Medical, Bristol, UK) was used to acquire data for PWV and AIx. Two blood pressure cuffs were used (one for the neck, one for the upper thigh) which were connected to two tubes. The tubes were connected to the Vicorder equipment which was connected by USB to a laptop on which Vicorder software was installed.

Data acquisition: For PWV, participants were placed in supine rest prior to the procedure being carried out. Resting blood pressure was taken and inputted in the Vicorder software to enable PWV to be calculated. The participant had a pillow placed under their lower back so that their head and neck was sloping downwards enabling a more prominent carotid arterial pulse and clear wave traces (see figure 3.5). Both blood pressure cuffs (thigh and neck) were placed tightly on the participants body to ensure an accurate reading. The neck cuff was placed just over the carotid artery, which was found using human touch or if necessary, a handheld ultrasound device. The length between the carotid artery and femoral artery (midpoint of the thigh cuff) was obtained by taking an average of 2 measurements and inputted in the Vicorder software. Both cuffs were connected to the research laptop which had Vicorder software. AIx was taken alongside PWV and is an expression of the aortic pressure wave. BP was measured to establish pulse pressure (the difference between systolic BP and diastolic BP). The BP cuff was placed tightly on the participants upper arm, in the supine position, and was connected to the research laptop which recorded and calculated AIx measurements. During both PWV and AIx measurements participants were asked to be quiet and still. If the volunteer coughed, swallowed, talked during the measurement this was visible on the pulse wave recording shown on the computer. This disturbance would be noted by the researcher and the measurement would

be performed again for accuracy. PWV and AIx readings were taken at 0 mins (baseline), 180 mins, 360 mins, 24 hours and 48 hours (shown in figure 3.3).

Figure 3.5: Demonstrating the correct positioning for pulse wave velocity

Data analysis: The Vicorder software not only shows the pulse wave recording but also provides an instant automatic calculation of PWV and AIx. Each PWV and AIx measurement was repeated at least 3 times (recordings < 10% variation, if >10% recordings were repeated to a maximum of 6 times), an average taken and inputted into Microsoft Excel for further statistical analysis.

3.4.5.5 Measures of metabolic control

Rationale

Within this study there are two markers of metabolic control: blood and urine. Some of the blood samples were sent for analysis of a range of cardiometabolic health markers. Anthocyanins have found previously to reduce oxidative LDL and increase HDLC [72], [171]. Conversely, energy-dense meals high in fat have been shown to increase cholesterol and triglyceride levels acutely [37], [40]. Both blood and urine have metabolites present after the consumption of anthocyanins [81]. The rest of the blood samples were stored and banked in the UEA for future analysis on anthocyanin metabolites and any or any cardiometabolic markers that become of interest. Blood samples were taken regularly throughout the day to assess for changes given the evidence of high concentrations of anthocyanin metabolites in

the first 6 hours after anthocyanin consumption [81]. Blood samples were taken initially, following a 10 hour fast, at 0 mins (baseline) and then at 20 mins, 40 mins, 60 mins, 90 mins, 120 mins, 180 mins, 360 mins, 24 hours and 48 hours (shown in figure 3.3) after consumption of the intervention material (blueberry powder or placebo) with the energy-dense meal.

3.4.5.5.1 ***Bloods***

Blood sampling: Throughout the study a qualified NHS nurse or health care assistant, working at the clinical research facility, took all blood samples. At the general health screening 10ml of blood was taken. During phase 2, on the first day of each assessment period a canula was inserted by a nurse and all blood samples were taken by vacutainer tubes to reduce participant burden. Participants unable to have a cannula inserted were unable to partake in Phase 2. The blood draws at the 24-hour and 48-hour assessment visits were taken from a single venepuncture.

Data acquisition: During 1 arm of a phase 2 assessment visit 229.5mls of blood was taken. During phase 2 bloods were taken at 0 min, 20 mins, 40 mins, 60 mins, 90 mins, 180 mins, 270 mins, 360 mins, 24 hours and 48 hours. For the bloods taken at the general health screen visit, baseline of phase 2 (0 mins) and at 24 hours and 48 hours of the phase 2 visits participants were required to fast for at least 10 hours the night before (but were allowed water). Due to the volume of blood taken and the insertion of a cannula participants had a minimum of four weeks between assessment visits (arm 1 and arm 2) during phase 2. All test tubes were anonymised and only identifiable by participant study identification.

Sample processing: Blood samples were analysed at the haematology and biochemistry laboratories at the NHS Norfolk and Norwich Hospital. Analysis included full blood count, glucose, liver and kidney function, lipids and lipoproteins (i.e. cholesterol, HDLC, LDLC, triglycerides, cholesterol:HDLC), urea, electrolytes and HbA1c.

3.4.5.5.2 Urine

Urine was collected during phase 2 in same way as in phase 1; one day before consumption of intervention material (baseline) and two days after. The purpose of collecting urine was

for future analysis of excreted metabolites and their potential correlation with vascular function. Due to the abandonment of the study, no urine samples collected during phase 2 have currently been analysed.

3.4.6 Dietary restrictions and dietary intake monitoring

3.4.6.1 Dietary restrictions

Dietary restrictions were imposed in phase 1 and phase 2 to make the study design more robust and minimise the circulatory levels of anthocyanins. Other foods that could potentially influence the cardiometabolic outcomes being assessed were also restricted. For five days before Phase 1 and during Phase 2 participants were required to restrict intake of blueberries (and products made from blueberries), anthocyanin rich foods, flavonoid containing dietary supplements and other potentially 'bioactive' foods. Table 3.5 outlines an exhaustive list of foods participants were asked to avoid during each phase and alternatives to choose from.

During Phase 2, participants were required to follow the dietary restrictions for five days before the test day. 48 hours before the test day participants were required to follow additional restrictions including avoiding caffeinated drinks and nitrate and nitrite rich foods. To facilitate this extra burden, participants were provided with a menu (Appendix B2) of foods to select from and eat two days before the phase 2 test meal and two days after (four days in total).

	Foods to avoid	Alternatives examples
Any drinks, salads, sandwiches and products (e.g., ready meals, pizza, casserole) containing:		
Fruit	**<u>Berry fruits:</u>** Blueberries, blackberries, raspberries, cranberries, strawberries, black currants, gooseberries, elderberries, aronia (chokeberries), acai, lingonberries **<u>Other:</u>** Cherries, plums, red/purple grapes, citrus fruits (oranges, grapefruit, lemon, lime)	Bananas, apples, green grapes, figs, kiwi, melon, pineapple, peaches, avocado, pears
Vegetables	**<u>Red / purple vegetables:</u>** Beetroot, aubergine, red onion, radishes, red cabbage, black/red beans **<u>Other</u>**: Potatoes	White onion, courgette, peppers, tomatoes, peas, sweetcorn, mushrooms, green beans, white beans, asparagus Sweet potato
Other	**Dark chocolate and milk chocolate** **Foods containing dark or milk chocolate**	White chocolate Custard cream, Rich tea biscuits
Drinks	**Red wine** **Juice/smoothies of fruits and vegetables named above** **Drinks flavoured with fruits or vegetables (e.g., fruit squash/cordial, fizzy drinks such as lemonade, Fanta, Sprite)** **Coffee and Tea (black, green, oolong, fruit and herbal teas)** **Drinks containing chocolate (e.g., chocolate milk, Ovaltine)**	Water White wine other alcoholic drinks (e.g., lager/cider/spirits) Coke, Soda water, Ginger ale Milk

Table 3.5: List of dietary restriction for phase 1 and phase 2. Table was provided to participants in the participant information leaflet.

3.4.6.2 Estimation of energy requirements, food provision and exercise

The food menu provided during phase 2 consisted of 3-4 choices of foods to eat per meal (Appendix B2), participants were aware of the limited food choices prior to consenting to the study. The limited menu allowed stricter controls on background diet 2 days before the intervention (and during) adding to the robustness of the study. This also reduced the participant burden if volunteers had been asked to interpret the restrictions and then shop themselves accordingly. Practically, volunteers chose their main meals for 4 days and duplicate choices were allowed (for example the same breakfast for 4 consecutive days). Additional snack items were available, to provide further calories or choice if required. Food selected by the participants for these 4 days was ordered to be delivered directly to the

participants house by Tesco. If any food items were not delivered, researchers provided an alternative from a small stock supply at the research facility. Participants were each provided with a detailed menu plan, with suggested amounts of food (in grams or common household measurements such as teaspoons), for the 4 days based on their food choices and their estimated energy requirements. The menu plan acted as a guide on how to create meals with the food provided over 4 days and to encourage participants to be aware of their portions. If they made any changes to their menu plan, for example if they ate more or less than suggested, they were asked to note down these changes. Participants were asked where possible to be specific and use food weighing scales that were provided. Researchers explained this was so that any changes made by participants during the 4 days in in the first intervention could be added to their menu plan and replicated for the second intervention period.

Each individual had their energy requirements estimated by a registered dietitian (CF) using a standard metabolic rate equation. The Henry equation was used as at the time of writing the study protocol the current version of the Parenteral and Enteral Nutrition Group (PENG) pocketbook of the British Dietetic Association [172] supported this method. A Physical Activity Level (PAL) was added to the equation as participants were healthy and free-living (Appendix B1). PALs were estimated by asking participants to answer a number of questions about their physical activity which was based on tables within the Parenteral and Enteral Nutrition Group (PENG) pocketbook [172] with reference to Department of Health 2004 'At Least Five a Week' [173]. Intakes specified on the menus were broadly matched to their individual energy requirements. During the time of writing this thesis, the British Dietetic Association PENG released updated guidance (updated 2018, released and published for public December 2019) on calculating energy requirements and so it should be noted the protocol and methods (approved by the ethical committee in February 2019) used the 2011 guidance available at the time.

3.4.6.3 Treatment, placebo and energy-dense meal provision

The test meal, consisting of croissants, full fat cheddar cheese, and low-fat Greek yoghurt, was provided along with a study test drink – consisting of 500mls of semi-skimmed cow's

milk and 36g of either freeze dried blueberries (364mg anthocyanin, 879mg total phenolics – see table 3.6), or 36g of an isocaloric colour and taste matched control powder (no anthocyanin or phenolic acids). The meal was adjusted for each participant, on the basis of their BMR, which accounts for age, sex and bodyweight, and an estimate of their Physical Activity Level (PAL).

Table 3.6: Nutritional content of 36g of freeze-dried blueberries

Anthocyanins (mg)	364
Phenolics (mg)	879
Calories (kcal)	132.84
Protein (g)	1.06
Carbohydrates (g)	32.94
Fat (g)	0.66
Dietary Fiber (g)	8.14
Vitamin C (mg)	6.12

Nutritional analysis of treatment completed by U.S Highbush Blueberry council (provider of treatment)

Acknowledging each individual's different energy requirements fits with the rejection of 'one size fits all' approach and understanding that individuals differ in their energy and nutrient needs. The adjustment of the test meal for each individual was done by increasing the amount of cheese, yogurt or croissant in the meal. Every effort was made to aim for no more than 5% difference between the ratio for the different food constituents of the meal for all participants. However, the hierarchical emphasis was to prioritise having the correct amount of calories and fat for each individual participant. The combined meal provided 50% of the daily energy requirements, and 40% of calories which came from fat. Table 3.7 displays the mean, minimum and maximum energy-dense meal composition and nutrition of those participants taking part in phase 2 (excluding the test drink). A researcher, blinded to the intervention, made up the study meal and drink (treatment or placebo) for the participant using weighing scales in the research facility kitchen. To ensure blinding of the placebo and treatment, they were put in (pre-placed by the manufacturer) in packaging labelled A or B. Study drinks were prepared in an opaque shaker bottle and a researcher not involved in phase 2 data collection observed that participants finished the meal and drink within 20 minutes. The observing researcher also washed the participants drink out with low nitrate water and requested the participant drink it to ensure all the treatment was

consumed. Participants did not consume any food or drink (apart from low nitrate bottled water) until the end of the research day (typically 8 hours later). All participants were offered a snack from the allowed foods prior to leaving the research facility at the end of the day.

Table 3.7: Nutritional composition of energy-dense teat meal (excluding treatment milkshake)

Meal Composition	**Minimum**	**Maximum**
Croissant (g)	83.29	206.70
Yoghurt (g)	72.22	397.10
Cheese (g)	37.50	62.01
Nutrition		
Calories (Kcal)	585.00	1429.50
Fat (g)	33.11	68.73
Saturated fat (g)	20.56	40.78
Carbohydrates (g)	46.50	145.95
Protein (g)	21.90	56.74

Table displays minimum and maximum composition and nutrition of energy-dense meal from *n* = 22 participants who took part in phase 2. This table excludes nutrition from blueberry or placebo treatment and the 500ml of semi-skimmed cow's milk the treatment was mixed with.

3.4.6.4 Monitoring dietary intake

During phase 2 of the study a 4-day food checklist was used to analyse the quantity of foods provided that the participants had eaten. Participants were asked to weigh their foods where possible; scales were provided. The food checklist was reviewed by the researcher in the presence of the volunteer so any clarifications could be asked. This enabled researchers to provide participants with an exact replica of the foods they had eaten in arm 1 of the study for repetition in arm 2.

3.4.6.5 Assessment of habitual dietary intake

Habitual dietary intake was assessed one week before phase 1 with a validated food frequency questionnaire (FFQ) [169]. The FFQ was a 131-item questionnaire which included the six main subclasses of flavonoids (anthocyanins, flavanones, flavan-3-ols, flavones and oligomer and polymer flavonoids). Data collection was completed by all members of the study team however data collation was done by Dr Amy Jennings and Veronica Bion. For data quality, data was excluded if deemed contentious (2 SDs above the mean) however none of the *n* = 8 participants for the analysis of this thesis had data excluded.

3.4.7 Lifestyle maintenance and monitoring

3.4.7.1 Anthropometrics

Body size assessment: Participant's height (in metres) and weight (in kilograms) was taken using a calibrated scale and stadiometer at the general health screening to confirm a BMI ≥25kg/m^2. BMI was calculated using the equation weight/(height^2). Weight was taken again during arm 1 and arm 2 of the experiment (taken twice and a mean weight calculated). At the start of the study, participants were told they needed to maintain their weight throughout the study (+/- 5%) at the three weigh-ins, those who did not would be excluded (*n* = 0).

Body composition assessment: The proportion of fat mass was assessed in all participants during arm 1 and arm 2 of the study using a bioimpedance machine. Participants were asked to remove their shoes and socks prior two stepping onto the bioimpedance machine to allow detection of electrical currents. This helped to ensure accurate results which were also calculated using the participants age, sex, height and weight. Hip and waist measurements were also taken using a measuring tape and performed by one of the researchers. Measurements were taken twice and an average of the two measurements was used.

3.4.7.2 Exercise and body weight monitoring

Participants were asked to maintain their habitual exercise patterns during the study with the exception of restricting strenuous exercise two days before and during the phase 2 test days (as this can affect vascular measurements). The aim was that participants maintained their body size and fitness levels, changes in either could affect metabolic and cardiovascular measurements. To measure physical activity levels participants had to fill in a validated International Physical Activity Questionnaire (IPAQ) after the study days (this was usually done on test day 2). The IPAQ (Appendix B3) quantifies physical activity (work, commuting, leisure and household) for the previous seven-day period (therefore including two days before the study test day where strenuous exercise was asked to be avoided).

Body weight was confirmed at the general health screening and during each test meal period. Any substantial shifts (>5% change in bodyweight) were monitored for and participants were aware they would be withdrawn from the study (*n* = 0).

3.4.7.3 Medication and medical condition monitoring

At each assessment visit, participants were asked by a clinical research nurse if they had any medication changes, health changes or if they felt unwell or had felt unwell since the researchers had last been in contact with them. Any adverse event (AE) or serious adverse event (SAE) data was collected on forms. All trends in AEs and SAEs reporting were monitored and forms were signed by the clinical advisor to the study. Additionally, participants were asked to report if they have started, or changed dose of, any medications during the study. Any volunteers who started any excluded medications or had a new health condition (which was on the exclusion criteria) during the trial would be excluded (*n* = 0).

3.5 Retrospective analysis of energy-dense meal methodology and the application of QRISK 3 score (presented in chapter 6)

3.5.1 Examining the impact of absolute fat content in the energy-dense meal provision

Within the phase 2 study design, as described, each individual had their energy-dense meal specifically tailored to their estimated energy requirements, with the meal containing 50% of their daily energy requirements, and 40% of the calories being from fat. Though other nutrition interventions often use this method within flavonoid research many studies provide a standardised meal [36], [174], [175]. We examined the cardiometabolic responses of those volunteers completing the placebo intervention week (*n* = 14) by comparing those participants with a lower absolute fat test meal with those participants with a higher absolute test meal. Groups were determined by splitting *n* = 14 volunteers at the median for absolute fat (7 below the median *versus* 7 above the median).

3.5.2 Application of QRISK3 score

The QRISK score is a model using an electronic database (QRESEARCH) where an individual's information on sex, age, systolic BP, ethnicity etc can be inputted to give a score indicating 10-year risk of a cardiovascular event within the next 10 years. It is widely used in GP practices and is recommended for use by National Institute of Clinical Excellence (NICE) in the primary prevention of CVD in patients [176].At the time of this analysis QRISK3 was the most up to date model [177], [178]. For the purpose of the analysis and to understand the potential impact of QRISK3 score on cardiometabolic outcomes we assessed, data from participants who completed the placebo arm only were used (*n* = 14). Within the model we inputted information for each participant including age, sex, post code, smoking status, diabetes status, Cholesterol / HDLC ratio, systolic blood pressure (and standard deviation of 2 readings), height and weight. This analysis was retrospective and therefore we did not have access to all information QRISK3 required, in these instances the response was left blank for all participants. This included information such as 1st degree relative history of angina or heart attack, migraines, and ethnicity. We examined the cardiometabolic responses of those volunteers completing the placebo intervention week (*n* = 14) by comparing those participants with a lower QRISK3 score with those participants with a higher QRISK3 score. Groups were determined by splitting *n* = 14 volunteers at the median for QRISK3 score (7 below the median *versus* 7 above the median).

3.6 Statistics

3.6.1 Power calculation

The primary endpoint in the planned cross-over study, was the change in percentage peak FMD from baseline within each metaboliser group (LOW and HIGH). A cross-over design was chosen to reduce interindividual variation by participants acting as their own control. A formal power calculation was performed, using FMD data from the research group's most recent blueberry anthocyanin study [65]; whereby a change of 1.06% in FMD (between placebo and blueberry intake) was observed, with a population SD of 1.87. At the p = 0.05 level with 90% power, *n* =37 volunteers per metabotype group were required to complete.

A drop-out rate of 10% was predicted; therefore, 41 = LOW and 41 = HIGH participants were targeted (total *n* = 82) to allow for withdrawals. Based on best estimates at the start of the study, it was anticipated that a total of *n* =274 participants was needed to complete phase 1 to identify these participants (and a total of 315 participants recruited initially accounting for a 15% drop-out before or during phase 1).

Due to minor delays in starting recruitment, and slower throughput of volunteers through the metaboliser profile phase (phase 1), it was agreed that this thesis should collect as much phase 2 data as possible within the allocated period for data collection. As detailed previously, 6 months before the end of year 3 (and part-way through the phase 2 visits that had been arranged), the trial was halted due to the first national COVID-19 lockdown. The trial was subsequently abandoned due to the ongoing impacts of the pandemic and thus, the data presented within this thesis includes all available data from this study.

3.6.2 Blinding and randomisation

Both treatment and placebo were coded and only known to one researcher not involved in the conducting phase 2 or doing its statistical analysis. Treatment was randomised using block-randomisation. To ensure blinding during meal administration either a clinical research facility NHS nurse or a researcher not involved in the analysis of phase 2 prepared and observed the test meal. The researcher (CF) involved in the statistical analysis of phase 2 remained blinded until statistical analysis was complete.

3.6.3 Data organisation

Data processing was handled in Microsoft Excel. For the *post hoc* parallel analysis arm 1 data was used except for 2 volunteers: *n* = 1 who did not have ABPM for arm 1 and *n* = 1 who did not have arm 1 FMD data, their arm 2 data was used. Vascular measurements were excluded for *n* = 2 volunteers who did not fast at 24 hours or 48 hours (ABPM data was still included). Some serum cardiometabolic markers are missing for volunteers at different timepoints due to failed cannulation or sample labelling error (*n* = 3).

3.6.4 Planned statistical analysis – chapters 4 and 5

As previously outlined, the primary endpoint of the cross-over study was change in percentage peak FMD from baseline between the two metaboliser groups (HIGH *versus* LOW). The power calculation outlined needing a total of 37 HIGH and 37 LOW participants completing the crossover study (PHASE 2) in order to reach significance at the p value of 0.05. Due to the impact of COVID-19 lockdowns these numbers were never reached, and the original statistical plans had to be abandoned.

If adequate participant numbers had been achieved, the results from vascular function measures and biomarker assessments would have been analysed using SPSS statistical software. A process of blind review would have been undertaken, to identify outliers (i.e. ≥ +/- 3 standard deviations of the mean) and also to test the data for the normality of distribution. For non-normally distributed data, a series of data transformations (e.g. log transformation) would have been applied to attempt to normalise the distribution (so that parametric testing could be used). Any participants identified as not completing the study per protocol (e.g. not adhering to dietary restrictions) would have been immediately excluded from the analysis. A statistical model such as ANCOVA (analysis of covariance) would have been chosen, with covariate contributions attributable to participant characteristics, e.g. sex, controlled for in the model. The selected ANCOVA would have been designed to account for the repeated data collection within the trial (i.e. designed to account for treatment and time effects). A statistical assessment was planned which looked at responsiveness to treatment (blueberry *versus* placebo) regardless of metaboliser groups (i.e. HIGH and LOW). Further analysis would then compare the difference in response of HIGH *versus* LOW metabolisers and test for significance. Results would be considered statistically significant at the $p < 0.05$ level. In Chapter 4 the statistical focus would be on change in FMD response by treatment group, and by metaboliser group. In Chapter 5 the statistical focus would be on changes in blood pressure, arterial stiffness, serum lipids and lipoproteins, and glucose, by treatment group, and by metaboliser group. Adjustments for multiple testing would have been made

(such as the Bonferroni test) to generate adjusted pairwise comparisons when testing for significance.

3.6.5 Completed statistical analysis

3.6.5.1 Summary

In total there were *n* = 8 who completed the cross-over design and *n* = 22 who completed at least 1 arm of the cross-over design. Due to reduced participant numbers expected for the cross-over, and a larger number who had completed at least 1 arm, statistical analysis was performed on the data as a *post hoc* parallel study as well as a crossover design. SPSS statistics software package was used throughout. A p value of <0.05 was considered significant.

3.6.5.2 Statistical analysis - chapters 4 and 5

Prior to COVID-19 and the national lockdown, *n* = 22 of these participants finished arm 1 (randomised to treatment: blueberry *n* = 10 and placebo *n* = 12). Of those who completed the entire cross-over study (*n* = 8), *n* = 1 FMD results were excluded as the baseline FMD image acquisitioned did not meet quality standards (set out in Chapter 3, the methods chapter). Therefore *n* = 7 are presented in the cross-over FMD data set (*n* = 3 LOW and *n* = 4 HIGH). In the parallel analysis,*n* = 1 TTP baseline score was removed due to the score being vastly different between the two independent researcher's analyses. In the cross-over analysis *n* = 1 HFMC baseline assessment did not meet either of the independent researcher's quality standards and was therefore excluded.

3.6.5.2.1 Participants that completed at least one arm

A sample size of 22 participants completed at least one arm of the study. Of the 22, *n* = 10 consumed the placebo powder and *n* = 12 consumed the blueberry powder. Effects of the dietary intervention (regardless of metaboliser type) on all the study end points were analysed using non-parametric tests due to lack of normality (Levene test to assess variance of the data) in the data and small sample size. Baseline change for each end point was

calculated and then analysed using a Mann-Whitney U test or the Kruskal-Wallis test. Due to the exploratory nature of this study and small sample size, tests were not further adjusted for covariates nor was multivariate analysis performed. A p value of <0.05 was considered significant. Further exploration of the correlation for baseline LFMC and HFMC were plotted. The closer the R^2 value was to 1, to stronger the correlation.

3.6.5.2.2 Cross-over

A sample size of *n* = 8 participants completed both arms of the study and therefore had both the placebo and blueberry powder with an energy-dense meal. Of those 8 participants, *n* = 3 were LOW and *n* = 5 were HIGH metabolisers. First, effects of the dietary intervention on all endpoints were analysed in blueberry (*n* = 7) *versus* placebo powder (*n* =7). Baseline change was calculated and used to test for significance between the 2 groups. Due to small sample size a non-parametric test (Mann-Whitney U) was used and not a specific linear mixed model for crossover studies. A p value of <0.05 was considered significant.

Further observational analysis into the difference between LOW and HIGH metabolisers was conducted. Change for all individuals at each endpoint, for every outcome, from the placebo result to the blueberry anthocyanin result was calculated. The data was not analysed as it was considered inappropriate due to small participant numbers.

3.6.5.3 Statistical analysis - chapters 6

3.6.5.3.1 Energy-dense meal

A sample size of *n* = 14 participants who completed the placebo intervention only were included in this *post hoc* analysis. As described in section 3.5.1, participants were grouped according to whether they fell below ('lower' fat group) or above ('higher' fat group) the median. Due to small sample size and lack of normality in the data, a non-parametric test (Mann-Whitney U) was used on change from baseline at each timepoint. A p value of <0.05 was considered significant. Further exploration of the differences between absolute fat

content and change in percentage peak FMD for both sexes was plotted. The closer the R^2 value was to 1, to stronger the correlation.

3.6.5.3.2 QRISK3

A sample size of *n* = 14 participants who completed the placebo intervention only were included in this *post hoc* analysis. As described in section 3.5.2, participants were grouped according to whether they fell below ('lower' QRISK3 group) or above ('higher' QRISK3 group) the median. Due to the small sample size and lack of normality in the data, a non-parametric test (Mann-Whitney U) was used on change from baseline at each timepoint. A p value of <0.05 was considered significant.

CHAPTER 4. Acute changes in flow-mediated dilation after a single dose of blueberry anthocyanins in older adults

4.1 Aims and objectives of the chapter

This chapter addresses three key aims:

1) To assess correlations between participant characteristics at baseline and parameters of endothelial function.

And in those completing the cross-over intervention:

2) To determine endothelial function by metaboliser type (HIGH / LOW).

3) To assess parameters of endothelial function by intervention week (blueberry *versus* placebo).

To achieve these aims, data is used from the study outlined in Chapter 3 – which tested blueberry efficacy, when presented within an energy dense meal.

Herein, it is investigated whether a single dose of blueberry anthocyanins affects endothelial function over 48 hours, and whether anthocyanin metaboliser type mediates this response.

4.2 Hypotheses tested in the chapter

This chapter addresses two hypotheses:

1) A single dose of blueberry anthocyanins will improve endothelial function.

2) HIGH metabolisers of anthocyanins will have significantly improved endothelial function compared with LOW metabolisers of anthocyanins.

4.3 Introduction

Epidemiological evidence has previously suggested that higher intakes of dietary anthocyanins are associated with reduced risk for cardiovascular disease (CVD) development [53], [163]. A potential mechanism for this risk reduction, which has been

explored in multiple randomised controlled trials (RCTs), is the favourable effect that anthocyanin-rich foods appear to have on endothelial function [65], [161]. In a preceding chapter of this thesis (Chapter 2), the importance of a novel series of endothelial function parameters was explored, through retrospective analysis of a previously completed blueberry study. The current chapter extends this analysis, by assessing the acute effect of blueberry intake on these parameters, when prospectively incorporated into the data collection and analysis plan at the inception of the 'AMP study'.

The endothelium is of paramount importance to the vascular system, fulfilling several functions including; anticoagulation, smooth muscle cell proliferation and thrombosis [7], [179]. In addition to these functions, the endothelium also regulates vascular tone; controlling and balancing vasoconstriction with vasodilation of blood vessels, to ensure adequate flow and pressure [179]. It is this core function which is the focus of this chapter. The association between endothelial dysfunction and CVD outcomes is well established (illustrated in figure 4.1) [180]. In an analysis of over 2,500 participants (from 10 studies with follow-up between 1 and 92 months) coronary, brachial and overall endothelial dysfunction (tested by shear stress dependent flow-mediated dilation (FMD) or infusion of acetylcholine vasomotor test) were independently associated with CVD events - including mortality [180]. CVD encompasses many different diseases including coronary heart disease, heart failure and stroke and is one the leading causes of death in the UK [106]. As outlined in figure 4.1, there is a similarity in the effect size on cardiovascular events irrespective of whether endothelial dysfunction is established as coronary or brachial (peripheral) endothelial dysfunction. These data suggest that dysfunction of the endothelium (measured at a specific site) is reflective of systemic dysfunction, rather than restricted to the target vessel. Endothelial dysfunction has been identified as the primary step leading to the development of atherosclerosis and progression of CVD [7] and can present in many different ways; such as reduced vasodilation [76], reduced number of circulating endothelial progenitor cells [181] and vasoconstriction [136]. Though processes such as atherosclerosis cannot be reversed, endothelial function can be improved through intervention. For example, pharmaceutical medications (e.g. statins), diet (e.g. flavonoids) and exercise, have all been shown to ameliorate endothelial dysfunction with data suggesting both acute and

long-term benefits [36], [155], [156], [161], [182]. However the benefits of intervention, such as exercise, are lost upon cessation [182].

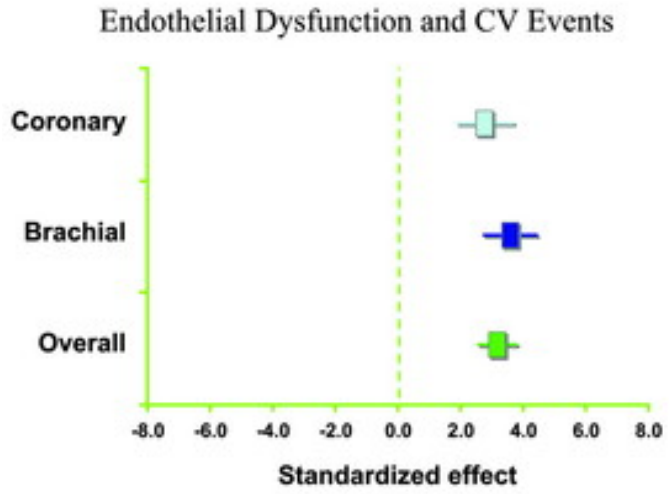


Figure 4.1: The effect of endothelial function (coronary, brachial and overall) on incidence of cardiovascular events. Figure from Lerman and Zeiher, 2005.

Endothelial function can be tested non-invasively via ultrasound methods; with the FMD technique recognised as the gold-standard. This 11-minute procedure involves measuring brachial artery vasodilation in response to the 5 minute brachial artery occlusion instigated via an inflated blood pressure cuff [99]. The artery's maximum ability to vasodilate after occlusion is called peak FMD, and is shown as a percentage change compared with the diameter of the artery at rest [100]. The vasodilation after occlusion in FMD is thought to be, at least in part, mediated by nitric oxide (NO); a vasodilatory mediator [114]. FMD is frequently used in research to test the effectiveness of an intervention, such as diet, by assessing change in percentage peak FMD. It is used in the research setting as it is non-invasive (compared to other measures of endothelial function), prognostic of CVD and robust and reproducible [76], [180], [183], [184]. Additional parameters of vascular function which are attainable from FMD analyses include low flow-mediated constriction (LFMC), high flow-mediated constriction (HFMC) and time to peak (TTP) which were discussed in depth in Chapter 2 of this thesis. These measures, which consider vasoconstriction during occlusion of the artery (LFMC and HFMC) and the time taken to reach peak FMD (TTP) in FMD, may compliment the well-studied, prognostic, percentage peak FMD. Currently,

research is continuing (in observational studies, in different populations) to establish the significance of LFMC, HFMC and TTP on vascular health and endothelial function.

Anthocyanins, a subclass of flavonoids, have been shown to improve endothelial function in both shorter and long-term research studies. Treatment with a blueberry drink (310mg of anthocyanins) in 10 young (age 27$\pm$1.3, BMI 25$\pm$0.8kg/m^2), healthy men, resulted in improvements in FMD 2 and 6 hours after consumption, when compared with matched controls [161]. This study found the maximum improvement in FMD at a dose of 310mg anthocyanins – assessed against varying doses from 129-724mg anthocyanins. As larger doses of anthocyanin (517mg and 724mg) had no further benefit on FMD, it has been suggested that the effects of anthocyanins on endothelial function eventually plateau [161]. In a different assessment of 12 hypercholesterolemic volunteers given purified, isolated anthocyanins (320mg), improvements in FMD at 1 and 2 hours (from baseline) were observed when compared with the control group [62]. The effect of processing blueberry anthocyanins, by adding them to baked products, also improved FMD after a single dose in healthy volunteers [63]. Sustained changes in FMD have also been observed after daily consumption of anthocyanins. Curtis *et al.*, 2019 found that 6 months of daily blueberry supplementation (containing 374mg anthocyanins), was associated with improved FMD in volunteers with metabolic syndrome, when compared to the placebo control group. Likewise, Stull et al., 2015 found that 6 weeks of daily blueberry smoothies (containing 290.3mg anthocyanin) improved reactive hyperaemia index (RHI), a measure of endothelial function, in volunteers with metabolic syndrome [66]. However, not all studies have observed similar improvements. For example, a single dose of blueberries (containing 348mg anthocyanins) did not affect acute RHI in 10 healthy male volunteers [185]. Similarly, 6 weeks of a daily blueberry drink (containing 375mg anthocyanins) had no effect on RHI in 18 healthy males [67]. The variability in response to anthocyanins is not limited to endothelial function, it has also been shown with other intermediate markers of cardiometabolic health such as blood pressure, arterial stiffness and lipid profiles (discussed further in Chapter 5).

In 2013, a paper by Czank *et al.*, 2013 found that anthocyanins were more bioavailable and remained in circulation for longer than previously thought [186]. In the study, a bolus dose

of 500mg 13C isotopically labelled cyanidin-3-glucoside (a type of anthocyanin) was given to 8 male participants; with samples of blood, urine, expired breath and faeces collected at various points over 48 hours. Metabolites of the 13-C labelled cyanidin-3-glucoside were found in greater abundance than had previously been reported and were still circulating at the final kinetic assessment at 48 hours (figure 4.2). Within the study there was considerable variation between volunteers (recovery from samples ranged from 15.1-99.3%) demonstrating interindividual variation in the absorption, distribution, metabolism and elimination of anthocyanins [81]. Notably, peak concentrations of the 13C labelled metabolites were found at 24 hours – which were at much higher concentrations than the parent cyanidin-3-glucoside compound.

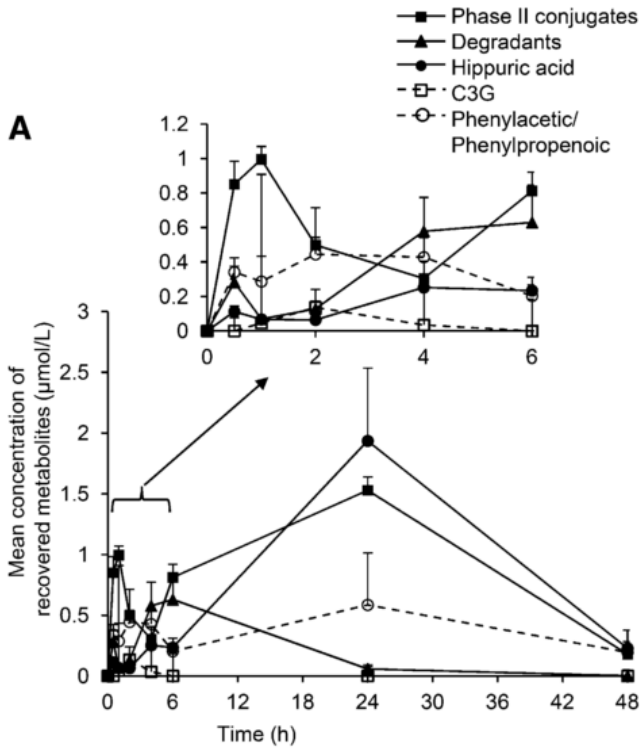


Figure 4.2: Anthocyanin metabolite activity in serum over 48 hours. Figure from Czank *et al.*, 2013 [81].

The concept that interindividual variation in metabolism may be responsible for variations in functional responses to intervention is not limited to anthocyanin research. It has also been established that reproducible metabotypes exist (for example, for isoflavones [94] and ellagitannins [93]) which group individuals, based on metabolism-type, and that this may influence health. In a RCT by Hazim *et al.*, 2016, only equol producers had vascular

improvements following isoflavone intake (especially when equol levels were elevated) [94]. Similarly, differences in serum cardiometabolic markers were observed in intervention participants when retrospectively categorised by two different urolithin production metabotypes [93], [164]. Urolithin, is a metabolite of ellagitannins which are polyphenols found in walnuts and pomegranates, amongst other foods. These data support the potential for metabotype-driven responses for anthocyanins and emerging evidence has suggested that anthocyanin metabolites may similarly mediate cardiometabolic effects - specifically vascular function [167]. Thus, establishing if an anthocyanin metabolism signature may predict the vascular response to anthocyanin rich food intake is considered a necessary next step in the field to improve understanding. Having established a favourable metabolic signature, research is then required to prospectively recruit participants with differing anthocyanin metabotypes, to establish if there is a difference in vascular response to intervention. This chapter reports on these aims and objectives.

This chapter will examine the existing gap in our understanding by exploring the vascular response over a 48-hour period following a single dose of blueberries incorporated into an energy-dense test meal. Data from an acute RCT (described in chapter 3) will be examined to assess the following: 1) observational findings of emerging analysis techniques (LFMC, HFMC and TTP) in a population of healthy older adults (aged 50-80 years) 2) the effect of a single dose of blueberry anthocyanins, compared with a placebo matched control, on endothelial function 3) observational findings of the novel HIGH / LOW blueberry anthocyanin metabotype on acute endothelial function measured by FMD.

4.4 Results

4.4.1 Metabotype profile (HIGH / LOW) analysis

Table 4.1: Characteristics of study participants who completed cross-over flow-mediated dilation analysis by metaboliser type (HIGH/LOW)

	LOW					HIGH				
	N	Mean	SD	Min	Max	N	Mean	SD	Min	Max
Age (y)	3	64.33	4.62	59.00	67.00	4	60.20	5.67	52.00	66.00
Sex (male)	3	2.00	/	/	/	4	1.00	/	/	/
BMI (kg/m^2)	3	26.77	0.91	26.10	27.80	4	29.63	3.61	27.30	34.90
Test meal fat content (g)	3	59.39	17.14	46.09	78.73	4	52.24	8.60	46.09	64.98
Statin (NO)	3	3.00	/	/	/	4	4.00	/	/	/
Body fat %	3	25.47	8.02	20.30	34.70	4	36.15	9.62	21.90	42.90
Trunk fat %	3	24.87	5.81	20.30	31.40	4	34.90	7.93	23.40	41.00
Hip (cm)	3	105.20	5.77	100.85	111.75	4	107.84	4.61	103.00	113.00
Waist (cm)	3	88.08	12.80	74.00	99.00	4	94.41	4.33	89.00	99.45
Peak FMD %	3	2.53	1.71	1.14	4.44	4	0.82	1.48	-1.31	2.09
HFMC %	3	1.07	1.40	0.10	2.68	4	0.45	1.54	-1.34	2.32
LFMC %	3	1.40	2.56	-0.37	4.33	3	-0.02	0.89	-1.02	0.67
TTP (seconds)	3	4.33	17.01	-13.00	21.00	4	-6.00	24.36	-35.00	17.00
PWV (m/s)	3	0.60	1.44	-0.60	2.20	4	0.08	0.83	-0.93	1.10
AIx (%)	3	-0.33	8.01	-9.33	6.00	4	-0.65	1.77	-2.67	1.00
SBP (mmHg)	3	4.67	1.15	4.00	6.00	4	-6.75	9.54	-18.00	3.00
DBP (mmHg)	3	5.00	3.46	1.00	7.00	4	-6.75	9.54	-18.00	3.00
HR (BPM)	3	-3.67	5.03	-9.00	1.00	4	-1.63	2.69	-5.00	1.00
ABPM SBP (mmHg)	2	3.00	8.49	-3.00	9.00	4	-6.25	2.87	-8.00	-2.00
ABPM DBP (mmHg)	2	3.00	1.41	2.00	4.00	4	-3.75	3.59	-7.00	1.00
ABPM MAP (mmHg)	2	3.50	6.36	-1.00	8.00	4	-4.25	2.87	-6.00	0.00
ABPM PP (mmHg)	2	0.50	6.36	-4.00	5.00	4	-2.25	2.06	-4.00	0.00
ABPM HR (BPM)	2	-3.00	2.83	-5.00	-1.00	4	-4.00	6.68	-14.00	0.00
Glucose (mmol/L)	3	-0.23	0.12	-0.30	-0.10	4	-0.05	0.30	-0.40	0.20
Cholesterol (mmol/L)	3	0.23	0.31	-0.10	0.50	3	-0.03	0.25	-0.30	0.20
LDLC (mmol/L)	3	0.00	0.26	-0.20	0.30	3	-0.07	0.06	-0.10	0.00
HDLC (mmol/L)	3	0.10	0.09	0.03	0.20	4	-0.01	0.13	-0.17	0.16

Mean calculated from placebo to blueberry intervention week for those participants who completed the cross-over intervention week. Measure of BMI refers to arm 1 baseline measurements taken (n = 7). Abbreviations: SD (standard deviation), FMD (flow-mediated dilation), HFMC (high flow-mediated dilation), LFMC (low flow-mediated dilation), TTP (time to peak), PWV (pulse wave velocity), AIx (augmentation index), SBP (systolic blood pressure), DBP (diastolic blood pressure), HR (heart rate), BPM (beats per minute), ABPM SBP (ambulatory blood pressure monitor systolic blood pressure), ABPM DBP (ambulatory blood pressure monitor diastolic blood), ABPM MAP (ambulatory blood pressure monitor mean arterial pressure), ABPM PP (ambulatory blood pressure monitor pulse pressure), ABPM HR (ambulatory blood pressure monitor heart rate), LDLC (low density lipoprotein cholesterol) and HDLC (high density lipoprotein cholesterol).

Table 4.2: Measures of endothelial function assessed by metaboliser type (HIGH/LOW) in those participants who completed the cross-over intervention (*n* = 7).

		BASELINE					1.5HR				3HR		
		N	Mean	SD	Min	Max	N	Mean	SD	BL Δ	N	Mean	SD	BL Δ
Peak FMD %	LOW	3	2.53	1.71	1.14	4.44	3	-0.44	1.25	-2.98	3	0.74	4.10	-1.80
	HIGH	4	0.82	1.48	-1.31	2.09	4	1.60	2.73	0.78	4	0.43	2.94	-0.40
LFMC %	LOW	3	1.40	2.56	-0.37	4.33	3	-1.78	2.21	-3.18	3	0.94	4.34	-0.45
	HIGH	4	0.45	1.54	-1.34	2.32	4	1.17	3.58	0.72	3	0.73	0.72	-0.70
HFMC %	LOW	3	1.07	1.40	0.10	2.68	3	-1.44	1.70	-2.52	2	-0.95	1.08	-2.34
	HIGH	3	-0.02	0.89	-1.02	0.67	3	1.50	1.73	1.52	2	0.05	0.62	-1.00
TTP (seconds)	LOW	3	4.33	17.01	-13.00	21.00	3	32.67	64.53	28.33	3	13.67	5.03	9.33
	HIGH	4	-6.00	24.36	-35.00	17.00	4	3.50	14.71	9.50	4	4.75	10.81	10.75

		6HR					24HR				48HR			
		N	Mean	SD	BL Δ	P	N	Mean	SD	BL Δ	N	Mean	SD	BL Δ
Peak FMD %	LOW	3	1.93	3.61	-0.60	0.72	3	0.06	3.02	-2.47	2	-1.91	1.39	-4.71
	HIGH	4	2.13	6.62	1.30		4	-0.31	2.4	-1.13	4	-0.69	3.75	-1.51
LFMC %	LOW	3	1.20	1.54	-0.20	0.72	3	0.63	0.67	-0.77	2	0.28	1.26	0.36
	HIGH	4	-0.37	2.05	-0.82		4	-1.35	1.79	-1.81	4	-0.36	0.85	-0.82
HFMC %	LOW	3	1.24	1.48	0.17	0.51	3	-0.96	2.42	-2.03	2	0.43	0.94	0.16
	HIGH	3	-0.31	1.91	-0.29		3	-1.00	0.82	-0.98	3	-0.58	1.99	-0.56
TTP (seconds)	LOW	3	2.00	7.81	-2.33	0.72	3	-2.00	1.00	-6.33	2	-3.50	4.95	-7.50
	HIGH	4	2.75	11.90	8.75		4	-5.75	24.23	0.25	4	9.00	28.81	15.00

Mean change calculated from placebo to blueberry intervention week for those participants who completed the cross-over intervention. Mean and BL Δ for FMD, LFMC and HFMC are shown in percentage. Mean BL Δ for TTP is shown in seconds. Abbreviations: FMD (flow-mediated dilation), HFMC (high flow-mediated dilation), LFMC (low flow-mediated dilation), TTP (time to peak), BL (baseline) SD (standard deviation), Δ (change), min (minimum) max (maximum).

The analysis method for tables 4.1 and 4.2 was based on mean change, for each individual volunteer from the placebo week to blueberry group for each time point – analysis was then conducted on mean change from baseline between HIGH and LOW metabolisers. As described in the methodology, no statistical testing was applied to the HIGH / LOW data due to small study numbers. Table 4.1 displays the mean baseline characteristics of, grouped by HIGH / LOW metaboliser type. The LOW group tended to have more men and a higher BP at baseline (for both systolic BP and diastolic BP). At 1.5 hours the LOW group had a lower, more constrictive HFMC (mean = -1.44%, mean change = -2.52%) compared with the HIGH group (mean = 1.50%, mean change = 1.52%) (table 4.2). Noticeably at 6hr, 24hr and 48hr TTP had reduced from baseline for the LOW group, however the SD for both groups were large and suggest high variation between participants. Figure 4.3 shows each participants peak FMD by metaboliser type, where no clear differences were observed.

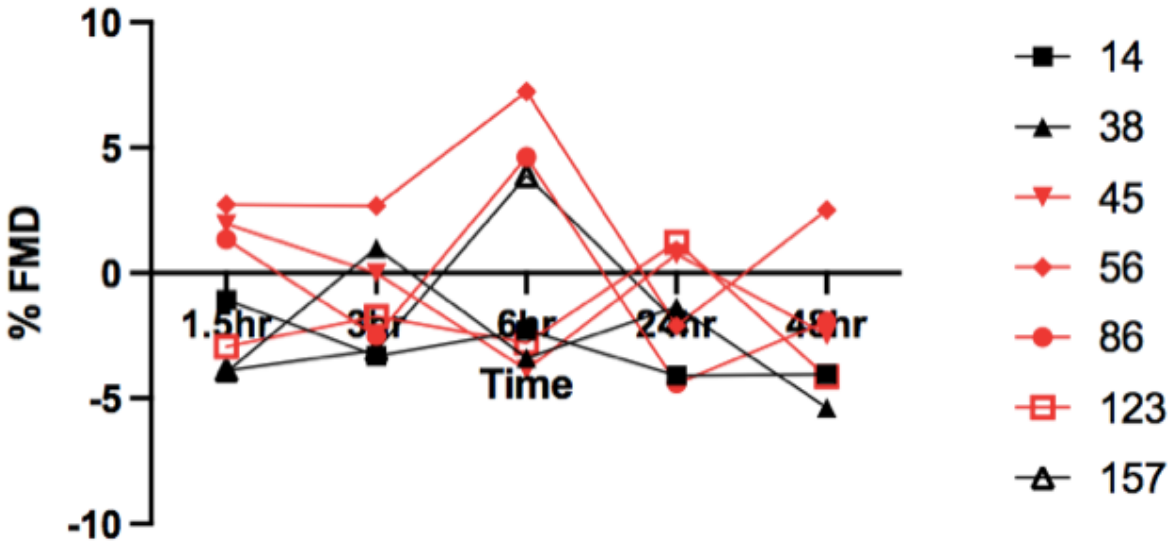


Figure 4.3: Difference in each individual volunteers mean peak percentage FMD change from baseline, between placebo and blueberry, at each time point and identified as HIGH or LOW metaboliser. HIGH metabolisers are plotted on the graph in red, those who are LOW metabolisers are plotted in black. Each symbol represents a different volunteer who had FMD measured for both treatment weeks. X axis indicates each of the timepoints FMD was measured at. Y axis represents percentage peak FMD. Abbreviations: FMD (flow-mediated dilation), % (percentage).

4.4.2 Crossover analysis

Table 4.3: Characteristics of study participants who completed cross-over flow-mediated dilation analysis

	N	Mean	SD	Minimum	Maximum
Age (y)	7	61.71	5.82	52.00	67.00
Sex (male)	7	3	/	/	/
BMI (kg/m^2)	7	28.40	3.02	26.10	34.90
Statin (NO)	7	7	/	/	/
Body fat %	7	31.57	10.02	20.30	42.90
Trunk fat %	7	30.60	8.45	20.30	41.00
Hip (cm)	7	106.71	4.87	100.85	113.00
Waist (cm)	7	91.70	8.68	74.00	99.45
Test meal fat content (g)	7	55.30	12.23	46.09	78.73

Abbreviations: BMI (body mass index), SD (standard deviation).

Table 4.3 displays the baseline characteristics for participants completing the cross-over study (*n* = 7); with the repeat data from this group of 7 participants shown for their mean baseline levels on the blueberry treatment and the placebo days.

As show in table 4.4, no baseline differences were observed between the baseline measures on each assessment day (blueberry *versus* placebo). There were also no differences detected for any of the postprandial FMD measurements in the cross-over analysis (table 4.5).

Table 4.4: Characteristics of study participants, for each intervention week, who completed cross-over flow-mediated dilation analysis

	Blueberry Week					Placebo Week					
	N	Mean	SD	Min	Max	N	Mean	SD	Min	Max	P
FMD %	7	5.06	4.47	1.03	13.83	7	3.50	4.02	-0.33	11.73	0.46
HFMC %	6	-0.54	1.01	-2.30	0.42	6	-1.07	1.24	-2.60	0.43	0.38
LFMC %	7	-0.06	1.41	-1.29	2.71	7	-0.92	1.71	-3.50	1.68	0.70
TTP (seconds)	7	46.29	9.32	33.00	61.00	7	49.29	17.82	23.00	78.00	0.81
PWV (m/s)	7	9.89	1.80	8.23	13.30	7	9.58	1.01	8.20	11.10	0.90
AIx %	7	27.33	7.45	17.67	36.00	7	27.85	10.09	16.67	44.33	1.00
SBP (mmHg)	7	138.21	8.45	128.00	151.00	7	140.07	12.44	124.00	162.00	0.81
DBP (mmHg)	7	85.57	8.77	72.00	98.00	7	86.57	13.33	65.00	104.00	0.71
HR (BPM)	7	62.86	3.93	58.00	70.00	7	65.36	5.66	59.00	72.50	0.62
ABPM SBP (mmHg)	6	126.33	5.99	116.00	132.00	6	129.50	8.69	117.00	140.00	0.49
ABPM DBP (mmHg)	6	78.67	5.75	68.00	83.00	6	80.17	8.84	64.00	89.00	0.49
ABPM MAP (mmHg)	6	94.67	3.93	89.00	99.00	6	96.33	7.09	85.00	105.00	0.59
ABPM PP (mmHg)	6	47.50	6.35	39.00	58.00	6	48.83	5.64	39.00	54.00	0.49
ABPM HR (BPM)	6	72.83	6.31	67.00	81.00	6	76.50	6.75	68.00	82.00	0.24
Glucose (mmol/L)	7	4.51	0.16	4.30	4.70	7	4.64	0.35	4.10	5.00	0.26
Cholesterol (mmol/L)	6	5.52	0.92	4.40	6.80	6	5.42	1.16	3.90	7.10	0.94
HDLC (mmol/L)	7	1.45	0.23	1.01	1.73	7	1.42	0.25	1.03	1.72	0.71
LDLC (mmol/L)	6	3.50	1.03	2.40	5.20	6	3.53	1.15	2.10	5.30	0.82
Triglycerides (mmol/L)	7	1.19	0.18	0.86	1.36	7	1.11	0.28	0.66	1.60	0.38

Statistical differences between the blueberry and placebo group was established using a Mann Whitney U test. A p value of < 0.05 was considered statistically significant however no results indicated differences between the intervention weeks. Abbreviations: SD (standard deviation), FMD (flow-mediated dilation), HFMC (high flow-mediated dilation), LFMC (low flow-mediated dilation), TTP (time to peak), PWV (pulse wave velocity), AIx (augmentation index), SBP (systolic blood pressure), DBP (diastolic blood pressure), HR (heart rate), BPM (bests per minute), ABPM SBP (ambulatory blood pressure monitor systolic blood pressure), ABPM DBP (ambulatory blood pressure monitor diastolic blood pressure), ABPM MAP (ambulatory blood pressure monitor mean arterial pressure), ABPM PP (ambulatory blood pressure monitor pulse pressure), ABPM HR (ambulatory blood pressure monitor heart rate), HDLC (high density lipoprotein cholesterol), and LDLC (low density lipoprotein cholesterol).

Table 4.5: Crossover analysis mean change from baseline in markers of endothelial function for participants completing both intervention crossover arms (blueberry *versus* placebo)

		BASELINE					1.5HR					3HR				
		N	Mean	SD	Min	Max	N	Mean	SD	BL Δ	P	N	Mean	SD	BL Δ	P
Peak FMD %	Blueberry	7	5.06	4.47	1.03	13.83	7	4.54	4.04	-0.52	0.46	7	6.31	4.53	1.25	0.26
	Placebo	7	3.50	4.02	-0.33	11.73	7	3.82	1.82	0.31		7	5.75	2.40	2.25	
LFMC %	Blueberry	7	-0.06	1.41	-1.29	2.71	7	0.82	2.61	0.88	0.90	7	0.83	2.84	0.89	0.71
	Placebo	7	-0.92	1.71	-3.50	1.68	7	0.92	0.90	1.83		7	0.56	1.50	1.48	
HFMC %	Blueberry	6	-0.54	1.01	-2.30	0.42	6	0.11	1.86	0.66	0.70	5	-0.52	2.53	0.22	0.31
	Placebo	6	-1.07	1.24	-2.60	0.43	6	0.09	0.53	1.16		5	0.47	2.29	1.75	
TTP (seconds)	Blueberry	7	46.29	9.32	33.00	61.00	7	59.29	11.88	13.00	0.90	7	53.43	10.94	7.14	0.54
	Placebo	7	49.29	17.82	23.00	78.00	7	73.86	46.25	24.57		7	48.57	12.37	-0.71	

		6HR					24HR					48HR				
		N	Mean	SD	BL Δ	P	N	Mean	SD	BL Δ	P	N	Mean	SD	BL Δ	P
Peak FMD %	Blueberry	7	5.75	5.15	0.69	0.81	7	4.09	3.75	-0.97	0.13	6	5.10	7.12	-0.27	0.13
	Placebo	7	3.71	1.75	0.20		7	4.24	3.69	0.84		6	6.19	4.19	2.31	
LFMC %	Blueberry	7	-0.02	1.30	0.05	0.71	7	-0.67	1.59	-0.61	0.21	6	-0.01	0.82	0.51	1.00
	Placebo	7	-0.32	1.38	0.60		7	-0.16	0.51	0.76		6	0.13	0.33	0.94	
HFMC %	Blueberry	6	-0.69	1.36	-0.15	1.00	6	-1.74	1.79	-1.19	0.24	5	-0.65	1.41	0.08	1.00
	Placebo	6	-1.16	1.16	-0.09		6	-0.76	0.63	0.31		5	-0.48	0.39	0.36	
TTP (seconds)	Blueberry	7	47.86	8.97	1.57	0.38	7	54.86	9.48	8.57	1.00	6	54.83	16.62	9.33	0.59
	Placebo	7	43.43	7.35	-5.86		7	58.71	11.77	9.43		6	50.00	14.14	1.83	

Mean and BL Δ for FMD, LFMC and HFMC are shown in percentage. Mean BL Δ for TTP is shown in seconds. Statistical differences between the blueberry and placebo group was established using a Mann Whitney U test. A p value of < 0.05 was considered statistically significant however no results indicated differences between the groups at any timepoint. Abbreviations: FMD (flow-mediated dilation), HFMC (high flow-mediated dilation), LFMC (low flow-mediated dilation), TTP (time to peak), BL (baseline) SD (standard deviation), Δ (change), min (minimum) max (maximum).

4.4.3 Correlations – low flow-mediated constriction, high flow-mediated constriction and time to peak

Table 4.6: Baseline characteristics of all participants *(n* = 22) taking part in at least one arm of the intervention study

	N	Mean	Minimum	Maximum	SD
Age (y)	22	59.55	51.00	71.00	5.80
Sex (male)	22	55% (12)	/	/	/
BMI (kg/m^2)	22	28.60	25.20	34.90	2.58
Body Fat %	22	30.70	18.70	46.60	8.31
Trunk Fat %	22	30.06	18.70	46.60	7.32
Hip (cm)	22	107.28	100.85	122.25	5.22
Waist (cm)	22	94.13	74.00	112.75	9.59
Peak FMD (%)	22	4.88	0.22	11.73	3.25
LFMC (%)	21	-0.12	-2.21	2.71	1.45
HFMC (%)	21	-0.72	-2.60	1.29	1.34
TTP (seconds)	21	43.38	23.00	69.00	11.27
SBP (mmHg)	22	141.64	119.50	165.00	11.25
DBP (mmHg)	22	85.39	72.00	104.00	7.98
HR (BPM)	21	60.17	43.00	75.50	8.31
ABPM SBP (mmHg)	22	131.05	117.00	149.00	9.62
ABPM DBP (mmHg)	22	80.32	68.00	96.00	7.62
ABPM MAP (mmHg)	22	96.91	88.00	110.00	6.28
ABPM PP (mmHg)	22	50.50	38.00	67.00	7.50
ABPM HR (BPM)	22	70.45	53.00	88.00	8.61
Statin Use (Yes)	22	4.55% (1)	/	/	/
Aix (%)	22	25.95	17.67	38.67	5.33
PWV (m/s)	21	10.43	8.20	13.30	1.40
Glucose (mmol/l)	22	4.74	4.10	5.50	0.36
Cholesterol (mmol/L)	21	5.50	2.70	8.50	1.25
HDLC (mmol/L)	22	1.38	0.83	2.03	0.31
LDLC (mmol/L)	21	3.55	1.50	5.60	1.10
Triglyceride (mmol/L)	22	1.25	0.70	2.02	0.34

Abbreviations: SD (standard deviation), BMI (body mass index), FMD (flow-mediated dilation), HFMC (high flow-mediated dilation), LFMC (low flow-mediated dilation), TTP (time to peak), PWV (pulse wave velocity), AIx (augmentation index), SBP (systolic blood pressure), DBP (diastolic blood pressure), HR (heart rate), ABPM SBP (ambulatory blood pressure monitor systolic blood pressure – showing baseline measurement), ABPM DBP (ambulatory blood pressure monitor diastolic blood pressure – showing baseline measurement), ABPM MAP (ambulatory blood pressure monitor mean arterial pressure – showing baseline measurement), ABPM PP (ambulatory blood pressure monitor pulse pressure – showing baseline measurement), ABPM HR (ambulatory blood pressure monitor heart rate – showing baseline measurement), LDLC (low density lipoprotein cholesterol) and HDLC (high density lipoprotein cholesterol).

Table 4.7: Pearson's Correlations for baseline markers of endothelial function with select baseline cardiometabolic characteristics in those completing at least one intervention arm (*n* =22)

	Mean	SD	Min	Max	**1**	**2**	**3**	**4**	**5**	**6**	**7**
1. LFMC	-0.69	1.74	-4.42	2.47							
Pearson Correlation						**0.87**	0.09	-0.18	**-0.53**	-0.43	**-0.45**
Sig. (2-tailed)						**0.00**	0.69	0.44	**0.02**	0.05	**0.04**
N						**14.00**	21.00	20.00	**21.00**	21.00	**21.00**
2. HFMC	-1.01	1.59	-4.99	2.43							
Pearson Correlation					**0.87**		0.08	-0.28	**-0.56**	-0.40	**-0.59**
Sig. (2-tailed)					**0.00**		0.74	0.23	**0.01**	0.08	**0.01**
N					**14.00**		21.00	20.00	**21.00**	21.00	**21.00**
3. FMD	4.88	3.25	0.22	11.73							
Pearson Correlation					0.09	0.08		-0.05	-0.10	0.10	-0.23
Sig. (2-tailed)					0.69	0.74		0.83	0.67	0.65	0.30
N					21.00	21.00		21.00	22.00	22.00	22.00
4. TTP	43.38	11.27	23.00	69.00							
Pearson Correlation					-0.18	-0.28	-0.05		-0.12	-0.12	0.19
Sig. (2-tailed)					0.44	0.23	0.83		0.61	0.60	0.40
N					20.00	20.00	21.00		21.00	21.00	21.00
5. DBP	85.39	7.98	72.00	104.00							
Pearson Correlation					**-0.53**	**-0.56**	-0.10	-0.12			
Sig. (2-tailed)					**0.02**	**0.01**	0.67	0.61			
N					**21.00**	**21.00**	22.00	21.00			
6. ABPM DBP	80.32	7.62	68.00	96.00							
Pearson Correlation					-0.43	-0.40	0.10	-0.12			
Sig. (2-tailed)					0.05	0.08	0.65	0.60			
N					21.00	21.00	22.00	21.00			
7. Glucose	4.74	0.36	4.10	5.50							
Pearson Correlation					**-0.45**	**-0.59**	-0.23	0.19			
Sig. (2-tailed)					**0.04**	**0.01**	0.30	0.40			
N					**21.00**	**21.00**	22.00	21.00			

Statistically assessed using Pearson's Correlation. Mean, max and min for LFMC, HFMC and FMD shown as percentage. TTP is shown in seconds, DBP and ABPM DBP shown in mmHg and glucose shown in mmol/L. Abbreviations: SD (standard deviation), FMD (flow-mediated dilation), HFMC (high flow-mediated dilation), LFMC (low flow-mediated dilation), TTP (time to peak), DBP (diastolic blood pressure) and ABPM DBP (ambulatory blood pressure monitor diastolic blood pressure – showing baseline measurement).

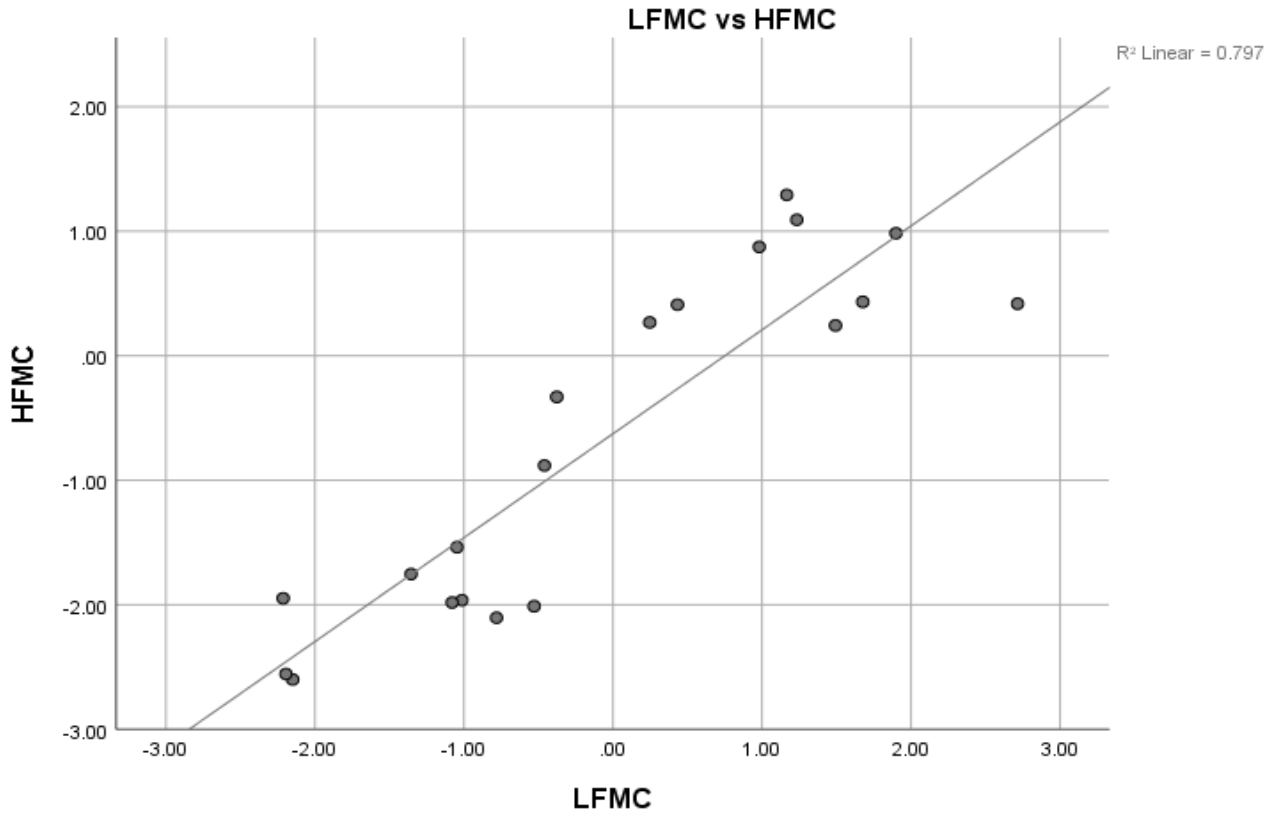


Figure 4.4: Scatter plot correlation for low flow-mediated constriction and high flow-mediated constriction, $R^2 = 0.797$. A strong positive linear relationship for LFMC and HFMC (values shown as percent). Abbreviations: HFMC (high flow-mediated dilation), LFMC (low flow-mediated dilation).

Similar to Chapter 2 results, significant Pearson's correlation was observed between LFMC and HFMC ($p < 0.00$) (shown in table 4.7 and figure 4.4 – $R^2 = 0.797$). Correlations between participant characteristics and baseline FMD and emerging analysis techniques discussed in Chapter 2 (LFMC, HFMC and TTP) were analysed. The characteristics of the *n* = 22 participants baseline assessments used are described in table 4.6. LFMC was inversely correlated with diastolic BP as measured in clinic ($p = 0.02$) but not when measured by ABPM ($p = 0.05$). Similarly, diastolic BP measured in clinic at rest (but not ABPM) was inversely correlated with HFMC ($p = 0.01$) (shown in table 4.7). As show in table 4.7, baseline glucose levels were inversely correlated with LFMC ($p = 0.04$) and HFMC ($p = 0.01$). TTP did not correlate with any of the select cardiometabolic characteristics at baseline.

Supplementary data (Appendix C1) table 4.8 shows the comparative baseline characteristics between those participants that, on their first assessment, completed either the blueberry, or the placebo intervention group. As shown in supplementary data (Appendix C1) table 4.8,

there were key differences between the blueberry and placebo groups with those taking the blueberry intervention being mostly female (*n* = 7 vs *n* = 3; p = 0.04), having a higher body fat (35% vs 27%, p = 0.02) and a non-significant trend towards more trunk fat (33% vs 27%, p = 0.06). As shown in supplementary data (Appendix C1) table 4.9, no differences in postprandial FMD measurements were found when grouping by blueberry or placebo treatment allocation (supplementary data (Appendix C1) table 4.9).

4.5 Discussion

We hypothesised that a single dose of blueberry anthocyanins would have a sustained effect on flow-mediated dilation over 48 hours and that this effect would be mediated by an individual's anthocyanin metabotype (HIGH / LOW). It would also be the first time the effect of blueberry anthocyanins on vascular function would be assessed over 48 hours. Additionally, following from Chapter 2, HFMC, LFMC and TTP would be explored in a different population and in another blueberry intervention to assess their importance. The impact of COVID-19 meant there were a reduced number of volunteers who completed the cross-over study (*n* =7 for FMD analysis) who were analysed firstly by metabotype (HIGH / LOW) and then by intervention type (blueberry *versus* placebo). The limited study data meant we were considerably underpowered and therefore unable to test our hypothesis. Our data shows no improvement from blueberry anthocyanin intervention in a parallel or cross-over analysis when compared with the placebo control. In the cross-over study looking at the effect of blueberry anthocyanins on the novel HIGH / LOW metabotype no consistent changes in FMD parameters were observed.

Due to only a small number of participants completing FMD measures in the crossover study (*n* = 7) our hypothesis on the HIGH / LOW metabotype could not be tested. No statistical analysis was performed on this data (tables 4.1-4.2) as given the reduced numbers it was deemed inappropriate. The primary endpoint of the study and which the HIGH / LOW metabotype was constructed on was change in percentage peak FMD. It was hypothesised that the HIGH group would experience improved endothelial function after the blueberry intervention compared to the LOW metabotype group. However, whilst we did not formally test the comparisons with statistical methods, a visual assessment of the data did not reveal any notable consistent changes between the two groups. It was noted at 1.5 hours HFMC appeared to be more constrictive in the LOW group and dilate more in the HIGH group (table 4.2). However, given this is an isolated finding, and the standard deviations of both HIGH and LOW for HFMC is as large as the mean suggesting substantial variation between participants. These results should therefore be interpreted with caution.

Following on from Chapter 2 and to further our understanding of other FMD parameters, an analysis of LFMC, HFMC and TTP was performed to assess for associations with the participants characteristic data (table 4.6 and 4.7). Several correlations were observed, the first being an inverse correlation between diastolic BP and LFMC and HFMC (table 4.7). Although not statistically significant, the assessment of ABPM diastolic BP (measured over a 24-hour period), a more robust method of capturing blood pressure than at rest, went some way to support the inverse correlation with resting BP (table 4.7). To our knowledge a lower LFMC and HFMC have not previously been associated with a higher diastolic BP, however in a large cross-sectional study (*n* = 5314) a similar observation for peak FMD was noted. diastolic BP was inversely correlated with peak FMD, meaning that a lower diastolic BP was associated with a higher percentage peak FMD level [187]. This interesting finding relates back to the myogenic response of artery's (the artery's ability to respond to pressure and shear stress by either vasodilation or vasoconstriction) described in chapter 2 [136]. It is possible that a high diastolic BP, which is associated with a degree of endothelial dysfunction (as measured by peak FMD), is also associated with a greater magnitude of constriction (seen in the inverse associations with LFMC and HFMC). Blood pressure somewhat mediates the myogenic response as the artery responds to changes in shear stress and flow. Consistent high levels of arterial pressure, for example from higher blood pressure, could impact the arterial wall and disrupt this response, and further decrease peak FMD [188]. It is important to note the complexities of the endothelium, with CVD progression generally being multi-factorial. For example decreased peak FMD does not independently predict hypertension [189]. Although these observational data suggest some interesting correlations, they are not conclusive, are limited in numbers and only add to the descriptive data about endothelial vasoconstriction in a healthy, overweight population.

We observed no changes in markers of endothelial function in those who completed the crossover study when categorised by HIGH / LOW data (table 4.2). The are several possibilities why this may be the case. One potential reason is that the study does not meet the required number of participants completing the crossover study (*n* = 37 HIGH, *n* = 37 LOW metabolisers) set out in the power calculation (Chapter 3) and therefore having small study numbers in the HIGH / LOW analysis (*n* = 7) (table 4.2). Due to limited RCTs on blueberry anthocyanins in older adults in the UK, our power calculation was based on the

changes in percentage peak FMD in Curtis *et al.*, 2019 population which was a 6 month, parallel intervention study. Although in an underpowered study it is unlikely to have made a difference, it was also a potential limitation to have used chronic data to predict change in acute, postprandial FMD responses after a single dose of blueberries. Whilst there are currently limited acute anthocyanin studies that measure FMD in healthy older adults upon which we could have based our power calculation on, a cross-over study in young men found blueberry anthocyanins acutely increased peak percentage FMD [161]. However, this was when consumed without an energy-dense or high-fat meal [161]. As discussed in Chapter 1, high-fat and energy-dense meals (as provided in this study) challenge the vascular system and affect FMD response [36]. Another cross-over study found improvements acutely in percentage peak FMD in 12 hypercholesterolemic men and women (ranging from 45-65 years of age) [62]. There were increases in FMD 1 hour postprandially after 320mg purified anthocyanins, when compared with the control [62]. Similarly, this study did not include an energy-dense or high-fat meal, which may mean differences in FMD would be detected in a smaller sample size.

Another potential reason for the lack of effect seen in endothelial function for the HIGH / LOW metabotype is that the metabolites in the HIGH / LOW profile (described in detail in chapter 3) were created from a chronic intervention. The novel metabolic profile was based on chronic changes in FMD over 6 months following daily consumption and not, acutely over a 48-hour period. The metabolic profile was also based on a population with metabolic syndrome compared to our healthy older adults. It is also possible that the cluster of metabolites that make-up the profile was not correct. An analysis by Rodriguez-Mateos *et al.*, 2019 found 14 blueberry anthocyanin metabolites have been previously identified as mediating FMD improvements acutely. A further 7 metabolites (n = 21 total) were involved in chronic (>28 days) FMD improvements suggesting there are differences in the metabolites produced from a single dose of anthocyanins compared with repeated doses. Of these 14 metabolites associated with acute change FMD none matched the novel metabotype described in Chapter 3. However 2 metabolites from the HIGH / LOW metabotype were included in the 21 metabolites associated with chronic improvements in FMD (Hippuric acid and 3-Hydroxyhippuric acid) [167]. Although speculative, with adequate power, it remains possible that the HIGH / LOW metabotype classification, based on chronic

metabolite changes, may have not been appropriate for an acute study. However, it should be noted that Rodriguez-Mateos *et al.*, 2019 only looked at acute metabolites for up to 2 hours and thus does not account for the extensive anthocyanin metbaolite activity known to be present for up to 48 hours [81].

The added challenge of this study was the addition of the energy-dense meal with the intervention material. Research shows that energy-dense meals or 'high-fat' meals can have deleterious effects on the cardiovascular system [35]–[38]. In an intervention such as this, they have the potential to blunt the effect of anthocyanins on the vascular system but also provide a more 'real world' condition to test the intervention material in. It can make the results of a trial feel more applicable to everyday UK life rather than reserving testing anthocyanins in a clinical and fasted state with a test meal that may not be reflective of the current UK diet. Despite this barrier, Alqurashi *et al.*, 2016 tested an acai berry smoothie (with 493mg anthocyanins and 50g of fat) *versus* a control smoothie on acute vascular function in 23 healthy men aged between 30-65 years with a BMI between 25-30kg/m^2. Alqurashi *et al.*, 2016 found the acai berry group had significant postprandial increases in percentage peak FMD when compared with the control however struggled to reach their baseline FMD levels until 6 hours after the energy-dense meal.

Some limitations of these data presented in this chapter are important to note. Firstly, the study was intended to be a cross-over intervention assessment (blueberry *versus* matched placebo arms), with sufficient participants recruited to analyse the effect of HIGH and LOW metaboliser profile status on the effectiveness of the intervention. However, because of the effects of the COVID-19 pandemic and a national lockdown in March 2020 the study was placed on hold before being stopped completely in early 2021 – with only n = 7 participants completing the study *per* protocol. This has resulted in an inability to fully test the primary hypothesis in the study.

Despite this, n = 22 participants had completed at least one arm of the crossover intervention. These data were therefore analysed as a *post hoc* parallel arm analyses of the blueberry and placebo treatment arms for the various FMD endpoints however no improvement was seen following blueberry intake (table 4.9, Appendix C1). These analyses

had limitations due to baseline between-group differences. Differences in sex and body fat has the potential to influence the response to the blueberry anthocyanins intervention and the cardiovascular responses. The blueberry group were predominantly female ($p = 0.04$) and unsurprisingly, based on our pre-existing knowledge of different fat distribution by sex, the predominantly female group had a higher body fat percentage ($p = 0.02$) (table 4.8, Appendix C1). Whilst it was unfortunate that there was an imbalance in the groups on the basis of sex, this experience clearly shows the importance of accounting for sex in the random allocation to treatment order for future studies. Younger women have a significantly better FMD, and endothelial function, than their age matched male counterparts [190]. This effect appears to stop when females reach 60-69 years of age, likely when the protective effects of oestrogen have decreased [154], [190]. The mean age of the blueberry group was 58 (table 4.8, Appendix C1) and so it is possible there was a beneficial advantage for FMD. However notably no differences in FMD were detected at any time point in this analysis (table 4.9, Appendix C1).

In conclusion, no effect was detected in the parallel or crossover analyses. Due to small study numbers, the study hypothesis on the HIGH / LOW metabotype could not be tested. Several limitations were identified in the data sets, namely the effect of COVID-19 limited data collection and thus study volunteer numbers. Future, well powered studies, assessing the effect of the novel blueberry anthocyanin study are required to test the hypothesis.

CHAPTER 5. Acute cardiometabolic responses after a single dose of blueberry anthocyanins in older adults

5.1 Aims and objectives of the chapter

This chapter addresses three key aims:

1) To compare food frequency questionnaire data at baseline by metaboliser type (HIGH/LOW).

And in those completing the cross-over intervention; to assess measures of cardiometabolic function (i.e blood pressure, arterial stiffness, augmentation index and blood markers) by:

2) metaboliser type (HIGH/LOW).

3) intervention week (blueberry *versus* placebo).

This chapter determines whether the novel HIGH/LOW anthocyanin metabotype influences postprandial vascular function, assessed over 48 hours. Measures assessed included ambulatory and within clinic blood pressure measurements, arterial stiffness, serum lipid and lipoproteins, and glucose concentrations. The HIGH/LOW metabotype was generated by Laura Haag (PhD student) using mathematical modelling techniques, to explore the interaction between changes (i.e. from baseline to 6 months) in anthocyanin metabolism and flow-mediated dilation. Previously, it has been shown that anthocyanin metabolites remain in circulation for at least 48 hours [81]. This novel assessment of 48-hour cardiometabolic responses following a single dose of blueberries aimed to confirm whether this metabolite exposure impacted clinically relevant outcomes.

5.2 Hypotheses tested in the chapter

This chapter addresses two hypotheses:

1) A single dose of blueberry anthocyanins, presented within an energy dense meal, will significantly improve cardiometabolic function* over a 48-hour period.

2) HIGH metabolisers of anthocyanins will have greater cardiometabolic responses than LOW anthocyanin metabolisers.

* a portfolio of clinically relevant cardiometabolic function assessments will be made and tested e.g. blood pressure, arterial stiffness, serum lipids, serum lipoproteins and serum glucose.

5.3 Introduction

The structure of the arterial wall involves three main layers (figure 5.1). The outer most layer is the tunica externa, which is a layer of connective tissue carrying elastic and collagenous fibres. The middle layer is referred to as the tunica media and is where smooth muscle cells are located [15]. As discussed in Chapters 2 and 3, the smooth muscle cells are key in regulating blood pressure as they are involved in both vasoconstriction and vasodilation (especially, nitric oxide mediated) in response to changes in blood flow [5]. The innermost layer, called the tunica intima, is where endothelial cells are located in the endothelium and its elastin and collagen fibres allow the vessel to accommodate changes in blood flow and are crucial to a healthy vascular system [15]. With age, there are structural changes which occur i.e. elastin fibres lose functionality and collagen concentration increases. This shift in balance leads to the stiffening and thickening of the artery [191].

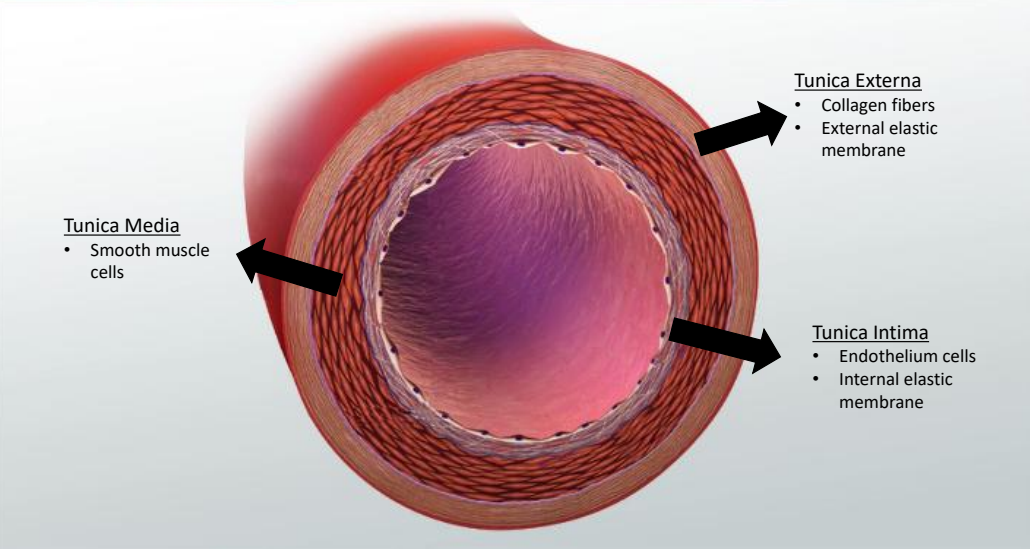


Figure 5.1: Adapted from [192], shows the structure of the arterial wall composing of 3 layers; tunica externa, tunica media and tunica intima.

From a clinical perspective, arterial stiffness has been shown to be an independent indicator of both hypertension and cardiovascular disease (CVD) [193]. In the UK, CVD alone accounts for a quarter of all annual deaths and an estimated 15 million people have hypertension (half of whom are suspected to be undiagnosed) [106]. The NHS defines hypertension as a blood pressure (BP) reading of over 140/90mmHg [107] and NICE guidance suggests this should be confirmed with ambulatory blood pressure monitoring (ABPM) [110]. Along with age, another contributing factor to the progression of arterial stiffness is hypertension [193]. There are many modifiable contributing factors to hypertension including diet, alcohol, obesity and smoking however there are also factors which can predispose individuals to a greater risk of hypertension such as genetics, ethnicity and older age [194].

The consequence of sustained hypertension and it's resultant high pressure on the arterial wall, is endothelial dysfunction [187] – which is viewed as the initial step in the development of atherosclerosis [3]. Similarly, increasing age and lipid levels, having obesity or hypertension, and smoking are all associated with damage to the endothelium [195]. What follows this damage is circulating macrophages ingesting low-density lipoprotein cholesterol (LDLC) and begin forming foam cells. Next, the development of a fatty streak lesion occurs when the macrophages die and accumulate on the endothelium. Then, with the deposition of cholesterol crystals, proliferation of smooth muscle cells and calcification of this lipid core, an atherosclerotic plaque with a fibrous cap is formed. The development of an atherosclerotic plaque can partially occlude an artery making blood flow more difficult as well as promoting thrombosis [116].

Measuring the serum lipid and lipoprotein profile has become an important part of assessing CVD risk. Elevated LDLC levels [196], and more recently triglyceride concentrations [197], have been shown to be independent indicators of CVD risk. High density lipoprotein cholesterol (HDLC) is involved in the reverse cholesterol transport of LDLC to the liver [198]. In epidemiology studies, lower HDLC levels are associated with increased risk of coronary heart disease [199], [200].

Diet undoubtedly plays a key role in mediating factors which influence the risk of CVD. For example, a high sodium diet has been associated with rises in blood volume, and thus blood

pressure, and consequently an increased risk of developing hypertension [198]. Likewise, high saturated fat intakes are linked to increased cholesterol and triglyceride levels which, as discussed earlier, contribute to atherosclerotic plaque development [198].

Although the negative impacts of various dietary components on vascular health have been relatively well established, the last two decades has seen a marked shift towards identifying potentially 'bioactive' dietary constituents with cardio-protective properties, such as anthocyanins. To this extent, higher intakes of anthocyanins (compared to lower intakes) have been associated with a reduced risk of CVD (RR 0.68-0.88) [27]–[29], [53], lower arterial stiffness (i.e. decreased pulse wave velocity (PWV), lower augmentation index (AIx) [54] and a reduced incidence of hypertension in women (RR 0.87, 95% CI) [45].

To explore the potential underlying mechanisms of these epidemiology observations, randomised controlled trials (RCTs) have tested the effectiveness of single or repeated doses of anthocyanins (and foods containing anthocyanins) on intermediate markers of CVD health; including arterial stiffness, BP and serum lipid and lipoprotein markers (the key studies in this area are tabulated in Chapter 1, tables 1.4 and 1.5). However, the results have been somewhat equivocal.

A cross-over study examining the effects of 300ml of cherry juice (207mg anthocyanins) in 13 younger and older adults found significant reductions in systolic BP ($p < 0.01$), diastolic BP ($p < 0.01$) and heart rate (HR) ($p = 0.03$) 2 hours after consumption (but not when tested at 6 hours) [68]. An 8 week study giving post-menopausal women, with pre and stage-1 hypertension, 22g of freeze dried blueberries (470mg anthocyanins) daily saw reductions in diastolic BP and systolic BP [71]. At the end of the 8 weeks, there were significant improvements in arterial stiffness when measured by brachial to ankle PWV ($p <0.01$) [71]. A study in men and women with metabolic syndrome examined 50mg freeze dried blueberry powder (742mg of anthocyanins - split into 2 doses, given 8 hours apart) daily for 8 weeks. They found significant improvements in systolic BP ($p <0.05$) and diastolic BP ($p <0.05$) [72]. In contrast many other studies found that anthocyanins have no effect on BP, either with a single dose [61], [161] or repeated doses [65]–[67], [69]. Additionally, a meta-analysis of six studies (472 participants) investigating the effect of longer-term anthocyanin

supplementation on systolic BP and diastolic BP found no effect [201]. The inconsistent effect of anthocyanins on BP is also found for arterial stiffness. Similar to the findings of Johnson *et al.*, 2015 [71], a study in 14 healthy men and women who consumed 288mg anthocyanins for 14 days (from the purple majesty potato) had significantly improved arterial stifness as measured by carotid to femoral PWV compared with the control group [74]. However several anthocyanin RCTs found no improvement in arterial stiffness (measured by PWV or AIx) following single [60], [61], [64] or repeated doses of anthocyanins [75].

Lipid and lipoprotein levels, including LDLC, HDLC and triglycerides have also been assessed in several anthocyanin RCTs. Whilst HDLC has been shown to increase in two studies (durations of 12 weeks and 6 months; anthocyanin doses of 320mg and 374mg/day respectively [62], [65] several other studies found no effect. Those examining anthocyanin doses of between 288-742mg of anthocyanins for between 2 and 8 weeks found no difference in HDLC [66], [67], [72], [74], [75]. Decreased oxidative LDL levels were observed after 8 weeks of freeze dried blueberries (742mg) in men and women with metabolic syndrome however there were no differences in HDLC, triglycerides, or glucose [72]. Many RCTs that measured LDLC, triglycerides and glucose have found no effect from anthocyanins [62], [65], [66], [74], [75].

The discrepancy in the effectiveness of anthocyanins on serum lipids, BP and arterial stiffness is also seen for endothelial function as explored in Chapter 4. The variation in response across vascular and lipid outcomes may be due to interindividual variation in the metabolism of anthocyanins. The role of interindividual variation is demonstrated by Czank *et al.*, 2013 who described wide variation in metabolite concentrations after ingestion of 500mg 13-C isotopically labelled cyanidin-3-glucoside (anthocyanin) in eight male participants though notably no health endpoints were assessed [81]. As previously described (Chapter 1 and Chapter 4), there are data suggesting that grouping individuals by metabolism type may influence the response to anthocyanins and subsequent health outcomes, as has been reported for ellagitannins and isoflavones [93], [94].

This chapter aims to explore the relevance of the novel HIGH / LOW metabotype on postprandial measures of vascular function (assessed over 48 hours) including BP (both ambulatory and clinic measurements), arterial stiffness, serum lipids and lipoprotein and glucose. The HIGH / LOW metabotype was generated to explore the relationship between changes in anthocyanin metabolism and flow-mediated dilation over a 6-month period. The assessment of acute cardiometabolic responses to a single dose of blueberries, tested over 48 hours, is novel and aims to examine whether the prolonged circulation of anthocyanin metabolites that has previously been reported has an impact on clinically relevant cardiometabolic outcomes [81].

5.4 Results

5.4.1 Analysis by metaboliser type

The analysis method for tables 5.1-5.4 was based on mean change, for each individual volunteer from the placebo week to blueberry group for each time point – analysis was then conducted on mean change from baseline between LOW and HIGH metabolisers.

Table 5.1 shows the characteristics of *n* = 8 study participants, there were more females in the LOW group, and at baseline systolic BP was higher in the LOW group. At 3 hours, after consuming blueberries, the decrease in AIx was greater in the LOW group than observed for the HIGH group (table 5.2). At 24 and 48 hours, in clinic diastolic BP was lower in the LOW group than the HIGH group after blueberry treatment. Similarly, in clinic systolic BP was lower in the LOW group at 24 hours (table 5.2). When BP was assessed with ABPM, in table 5.3, ABPM diastolic BP was lower in the LOW group on day 2 (48 hours). At 24 hours glucose was lower in the HIGH group while total cholesterol was lower in the LOW group (table 5.4).

When looking habitual dietary intake (table 5.5) and metabolite levels (table 5.6) several observations were apparent. For example, tea and wholegrain consumption was higher in the LOW group while beer and fat consumption were lower (table 5.5). The metabolite data shows that the LOW group had higher amounts of the four key metabolites at baseline (table 5.6). The four key metabolites in the HIGH group all increased postprandially however it was notable that 3,5-DiOH-PPA and 3-OH-HA did not reach the baseline levels of the LOW group. All four key metabolites in the LOW group dropped from baseline at 24 hours but levels increased again at 48 hours (except for hippuric acid (HA)).

Table 5.1: Characteristics of study participants who completed cross-over analysis by HIGH or LOW metaboliser type

	LOW					HIGH				
	N	Mean	SD	Min	Max	N	Mean	SD	Min	Max
Age (y)	3	64.33	4.62	59.00	67.00	5	60.20	5.67	52.00	66.00
Sex (male)	3	2.00	/	/	/	5	1.00	/	/	/
BMI (kg/m^2)	3	26.77	0.91	26.10	27.80	5	29.54	3.13	27.30	34.90
Test meal fat content (g)	3	59.39	17.14	46.09	78.73	5	50.41	8.49	43.11	64.98
Statin (NO)	3	3.00	/	/	/	5	5.00	/	/	/
Body fat %	3	25.47	8.02	20.30	34.70	5	35.34	8.53	21.90	42.90
Trunk fat %	3	24.87	5.81	20.30	31.40	5	32.98	8.10	23.40	41.00
Hip (cm)	3	105.20	5.77	100.85	111.75	5	106.67	4.77	102.00	113.00
Waist (cm)	3	88.08	12.80	74.00	99.00	5	92.03	6.52	82.50	99.45
Peak FMD %	3	-1.73	8.00	-10.76	4.44	4	1.44	5.23	-3.51	8.48
HFMC %	3	1.07	1.40	0.10	2.68	3	-0.02	0.89	-1.02	0.67
LFMC %	3	1.40	2.56	-0.37	4.33	4	0.45	1.54	-1.34	2.32
TTP (seconds)	3	4.33	17.01	-13.00	21.00	4	-6.00	24.36	-35.00	17.00
PWV (m/s)	3	0.60	1.44	-0.60	2.20	5	0.07	0.72	-0.93	1.10
AIx (%)	3	-0.33	8.01	-9.33	6.00	5	-0.25	1.77	-2.67	1.33
SBP (mmHg)	3	4.67	1.15	4.00	6.00	5	-6.00	8.43	-18.00	3.00
DBP (mmHg)	3	5.00	3.46	1.00	7.00	5	-3.80	3.83	-6.00	3.00
HR (BPM)	3	-3.67	5.03	-9.00	1.00	5	-0.50	3.43	-5.00	4.00
ABPM SBP (mmHg)	2	3.00	8.49	-3.00	9.00	5	-3.20	7.26	-8.00	9.00
ABPM DBP (mmHg)	2	3.00	1.41	2.00	4.00	5	-2.20	4.66	-7.00	4.00
ABPM MAP (mmHg)	2	3.50	6.36	-1.00	8.00	5	-2.40	4.83	-6.00	5.00
ABPM PP (mmHg)	2	0.50	6.36	-4.00	5.00	5	-0.80	3.70	-4.00	5.00
ABPM HR (BPM)	2	-3.00	2.83	-5.00	-1.00	5	-2.80	6.38	-14.00	2.00
Glucose (mmol/L)	3	-0.23	0.12	-0.30	-0.10	5	0.08	0.39	-0.40	0.60
Cholesterol (mmol/L)	3	0.23	0.31	-0.10	0.50	4	-0.18	0.35	-0.60	0.20
LDLC (mmol/L)	3	0.00	0.26	-0.20	0.30	4	-0.18	0.22	-0.50	0.00
HDLC (mmol/L)	3	0.10	0.09	0.03	0.20	5	-0.04	0.13	-0.17	0.16

Abbreviations: SD (standard deviation), FMD (flow-mediated dilation), HFMC (high flow-mediated dilation), LFMC (low flow-mediated dilation), TTP (time to peak), PWV (pulse wave velocity), AIx (augmentation index), SBP (systolic blood pressure), DBP (diastolic blood pressure), HR (heart rate), ABPM SBP (ambulatory blood pressure monitor systolic blood pressure – showing baseline measurement), ABPM DBP (ambulatory blood pressure monitor diastolic blood pressure – showing baseline measurement), ABPM MAP (ambulatory blood pressure monitor mean arterial pressure – showing baseline measurement), ABPM PP (ambulatory blood pressure monitor pulse pressure – showing baseline measurement), ABPM HR (ambulatory blood pressure monitor heart rate – showing baseline measurement), LDLC (low density lipoprotein cholesterol) and HDLC (high density lipoprotein cholesterol).

Table 5.2: Measures of arterial stiffness and blood pressure assessed by metaboliser type (HIGH/LOW) in those participants who completed the cross-over intervention (n = 8).

		BASELINE					1.5hr				3HR			
		N	Mean	SD	Min	Max	N	Mean	SD	BL Δ	N	Mean	SD	BL Δ
PWV (m/s)	LOW	3	0.60	1.44	-0.60	2.20					3	0.21	0.22	-0.39
	HIGH	5	0.07	0.72	-0.93	1.10					4	-0.41	0.66	-0.46
AIx (%)	LOW	3	-0.33	8.01	-9.33	6.00					3	-2.03	7.85	-1.69
	HIGH	5	-0.25	1.77	-2.67	1.33					4	1.73	1.11	1.37
SBP (mmHg)	LOW	3	4.67	1.15	4.00	6.00	3	6.67	8.39	2.00	3	4.33	12.86	-0.33
	HIGH	5	-6.00	8.43	-18.00	3.00	5	4.70	11.03	10.70	4	-0.63	3.94	7.10
DBP (mmHg)	LOW	3	5.00	3.46	1.00	7.00	3	7.67	7.51	2.67	3	5.33	5.51	0.33
	HIGH	5	-3.80	3.83	-6.00	3.00	5	2.10	3.36	5.90	4	2.88	3.07	7.10
HR (BPM)	LOW	3	-3.67	5.03	-9.00	1.00	3	-5.67	6.43	-2.00	3	-2.23	5.41	1.43
	HIGH	5	-0.50	3.43	-5.00	4.00	5	-2.20	2.71	-1.70	4	-0.50	1.73	-1.13

		6HR					24HR				48HR			
		N	Mean	SD	BL Δ	P	N	Mean	SD	BL Δ	N	Mean	SD	BL Δ
PWV (m/s)	LOW	3	0.38	0.49	-0.22	0.83	3	0.69	0.21	0.09	3	0.61	1.92	0.01
	HIGH	3	0.02	0.27	-0.05		5	0.05	0.28	-0.02	5	-0.01	0.76	-0.07
AIx (%)	LOW	3	0.69	4.30	1.03	0.66	3	-0.64	3.58	-0.31	3	-1.89	4.88	-1.56
	HIGH	5	0.87	2.35	1.12		5	-1.10	3.55	-0.85	5	1.60	2.95	1.85
SBP (mmHg)	LOW	3	-0.33	6.51	-5.00	0.30	3	-4.67	6.51	-9.33	3	-1.67	10.79	-6.33
	HIGH	5	2.80	8.04	8.80		5	3.20	9.31	9.20	5	-0.10	6.54	5.90
DBP (mmHg)	LOW	3	1.33	3.79	-3.67	0.10	3	-3.33	11.02	-8.33	3	-6.00	8.72	-11.00
	HIGH	5	-0.10	2.79	3.70		5	0.80	4.15	4.60	5	-2.40	3.51	1.40
HR (BPM)	LOW	3	-0.33	7.77	3.33	0.29	3	-4.33	3.06	-0.67	3	-3.00	3.00	0.67
	HIGH	5	-0.40	5.81	0.10		5	3.80	2.59	4.30	5	-0.10	4.98	0.40

Mean change calculated from placebo to blueberry week, for each volunteer who completed the crossover. Abbreviations: PWV (pulse wave velocity), AIx (augmentation index), SBP (systolic blood pressure), DBP (diastolic blood pressure), HR (heart rate), BL (baseline) SD (standard deviation), Δ (change), min (minimum) max (maximum). Mean and BL Δ for SBP and DBP are shown in millimetres of mercury (mmHg). Mean and BL Δ for PWV is shown in meter per second squared (m/s), AIx is shown in percentage (%) HR are shown in beats per minute (BPM).

Table 5.3: Ambulatory blood pressure monitor measurements assessed by metaboliser type (HIGH/LOW) in those participants who completed the cross-over intervention (n = 8).

		BASELINE (DAY 0)					DAY 1				DAY 2			
		N	Mean	SD	Min	Max	N	Mean	SD	BL Δ	N	Mean	SD	BL Δ
ABPM SBP (mmHg)	LOW	2	3.00	8.49	-3.00	9.00	2	5.50	12.02	2.50	2	-2.50	7.78	-5.50
	HIGH	5	-3.20	7.26	-8.00	9.00	5	1.40	1.67	4.60	4	1.00	6.48	3.00
ABPM DBP (mmHg)	LOW	2	3.00	1.41	2.00	4.00	2	5.00	7.07	2.00	2	-1.00	1.41	-4.00
	HIGH	5	-2.20	4.66	-7.00	4.00	5	0.00	1.87	2.20	4	1.25	4.11	2.50
ABPM HR (BPM)	LOW	2	-3.00	2.83	-5.00	-1.00	2	-3.50	7.78	-0.50	2	-1.00	7.07	2.00
	HIGH	5	-2.80	6.38	-14.00	2.00	5	0.60	4.39	3.40	4	2.25	3.40	2.25
ABPM PP (mmHg)	LOW	2	0.50	6.36	-4.00	5.00	2	0.50	4.95	0.00	2	-2.00	5.66	-2.50
	HIGH	5	-0.80	3.70	-4.00	5.00	5	1.40	1.52	2.20	4	-0.25	2.63	0.50
ABPM MAP (mmHg)	LOW	2	3.50	6.36	-1.00	8.00	2	6.50	9.19	3.00	2	-1.00	2.83	-4.50
	HIGH	5	-2.40	4.83	-6.00	5.00	5	0.80	2.95	3.20	4	1.00	4.32	2.50

Mean change calculated from placebo to blueberry week, for each volunteer who completed the crossover. Abbreviations: ABPM SBP (ambulatory blood pressure monitor systolic blood pressure), ABPM DBP (ambulatory blood pressure monitor diastolic blood pressure), ABPM MAP (ambulatory blood pressure monitor mean arterial pressure), ABPM PP (ambulatory blood pressure monitor pulse pressure), ABPM HR (ambulatory blood pressure monitor heart rate), BL (baseline) SD (standard deviation), Δ (change), min (minimum) max (maximum). Mean and BL Δ for ABPM SBP, ABPM DBP ABPM MAP and ABPM PP are shown in millimetres of mercury (mmHg). Mean BL Δ for ABPM HR is shown in beats per minute (BPM).

Table 5.4: Serum cardiometabolic markers assessed by metaboliser type (HIGH/LOW) in those participants who completed the cross-over intervention (n = 8).

		BASELINE					20MIN				40MIN			
		N	Mean	SD	Min	Max	N	Mean	SD	BL Δ	N	Mean	SD	BL Δ
Glucose (mmol/L)	LOW	3	-0.23	0.12	-0.30	-0.10					2	-1.65	0.07	-1.45
	HIGH	5	0.08	0.39	-0.40	0.60					4	-0.80	0.68	-0.85
Cholesterol (mmol/L)	LOW	3	0.23	0.31	-0.10	0.50	2	2.10	3.54	1.90	2	-0.15	0.49	-0.35
	HIGH	4	-0.18	0.35	-0.60	0.20	4	-0.40	0.12	-0.13	3	-0.43	0.21	0.00
HDLC (mmol/L)	LOW	3	0.10	0.09	0.03	0.20					2	-0.01	0.00	-0.07
	HIGH	5	-0.04	0.13	-0.17	0.16					4	-0.13	0.08	-0.04
		60MIN					**90MIN**				**180MIN**			
		N	Mean	SD	BL Δ		N	Mean	SD	BL Δ	N	Mean	SD	BL Δ
Glucose (mmol/L)	LOW	2	-0.75	1.34	-0.55		2	-0.30	1.41	-0.10	3	-0.90	0.44	-0.67
	HIGH	4	0.00	1.73	-0.05		4	0.23	1.26	0.18	5	-0.72	0.54	-0.80
Cholesterol (mmol/L)	LOW	2	-0.75	1.34	-0.95		2	-0.30	0.28	-0.50	3	0.57	0.12	0.33
	HIGH	4	0.00	1.73	1.10		4	-0.25	0.49	-0.03	4	0.03	0.78	0.25
HDLC (mmol/L)	LOW	2	0.04	0.04	-0.02		2	-0.07	0.07	-0.13	3	0.19	0.10	0.09
	HIGH	4	-0.05	0.12	0.05		4	-0.05	0.18	0.04	5	0.02	0.22	0.06
		360MIN					**24HR**				**48HR**			
		N	Mean	SD	BL Δ		N	Mean	SD	BL Δ	N	Mean	SD	BL Δ
Glucose (mmol/L)	LOW	3	-0.03	0.35	0.20		3	0.03	0.12	0.27	3	-0.13	0.59	0.10
	HIGH	4	-0.18	0.13	-0.23		5	-0.24	0.17	-0.32	5	0.00	0.31	-0.08
Cholesterol (mmol/L)	LOW	3	0.07	0.38	-0.17		3	-0.37	0.76	-0.60	3	-0.07	0.23	-0.30
	HIGH	4	-0.28	0.68	-0.23		5	0.02	0.55	0.30	5	-0.04	0.57	0.03
HDLC (mmol/L)	LOW	3	0.10	0.09	0.00		3	-0.05	0.17	-0.16	3	-0.01	0.11	-0.12
	HIGH	4	-0.06	0.07	0.03		5	0.04	0.14	0.11	5	-0.03	0.13	0.01

Mean change calculated from placebo to blueberry week, for each volunteer who completed the crossover. Mean and BL Δ for glucose, cholesterol and HDLC is shown in millimoles per litre (mmol/L). Triglycerides and low-density lipoprotein due to small numbers from missing participant data. No data for glucose and HDLC at 20 mins due to low participant numbers. Abbreviations HDLC (high density lipoprotein cholesterol), BL (baseline) SD (standard deviation), Δ (change), min (minimum) max (maximum).

Table 5.5: Food frequency questionnaire data for LOW (n = 3) and HIGH (n = 5) metabolisers

Nutrients / Food	**LOW** (*n* = 3) Mean	SD	Median	IQR	**HIGH** (*n* = 5) Mean	SD	Median	IQR
Calories (kcals)	2232.026	1042.66	1878.00	1645.22 - 2641.75	2312.71	850.84	1882.02	1776.11 - 2477.16
Protein (g/d)	92.95	27.26	78.09	77.22 - 101.25	97.52	37.49	89.93	78.28 - 109.71
Fat (g/d)	74.55	33.57	62.99	55.64 - 87.68	103.61	36.87	104.25	77.03 - 106.13
Carbohydrates (g/d)	298.37	179.64	225.49	196.06 - 364.24	232.53	111.20	213.55	161.43 - 232.33
Total Flavonoids (mg/d)	1001.08	469.42	745.32	730.20 - 1144.08	960.02	247.83	854.68	835.18 - 921.40
Anthocyanins (mg/d)	19.63	2.23	19.32	18.45 - 20.66	21.07	12.76	15.20	13.90 - 34.17
Fruit (g/d)	224.48	268.66	110.34	71.04 - 320.85	188.69	84.42	155.98	140.60 - 209.33
Vegetables (g/d)	182.00	22.42	184.86	171.57 - 193.86	207.00	59.31	196.07	182.04 - 220.29
Tea (g/d)	674.05	203.72	665.00	570.00 - 773.57	561.86	171.83	488.57	475.00 - 502.14
Coffee (g/d)	343.81	227.23	475.00	278.21 - 475.00	385.43	200.29	475.00	475.00 - 475.00
Beer (g/d)	96.67	114.09	41.43	31.07 - 134.64	203.00	296.73	124.29	41.43 - 124.29
Red Wine (g/d)	51.00	49.57	54.00	27.00 - 76.50	52.20	35.09	54.00	54.00 - 54.00
Wholegrains (g/d)	84.88	50.18	84.29	59.64 - 109.82	68.00	54.40	32.14	30.00 - 104.64

Data is presented as the mean (with SD = standard deviation), median and IQR (inter quartile range). Food frequency questionnaire data was obtained prior to completion of PHASE 1.

Table 5.6: Metabolite profile for LOW (*n* =3) and HIGH (*n* = 5) metabolisers from PHASE 1

Group	Time	3,5-DiOH-PPA (absolute)	Range	3-OH-HA (absolute)	Range	4-OH-3-OCH-PAA (absolute)	Range	HA (absolute)	Range
LOW	0	37.86	0.04-49.13	60.45	4.43-183.5	29.35	0.75-44.23	2528.82	794.94-3465.25
	24	2.03	0.04-46.5	4.43	3.30-134.24	4.51	0.75-37.39	2116.61	800.85-3574.05
	48	32.72	0.04-37.15	15.53	4.43-66.03	18.23	0.75-32.55	1965.07	327.92-2752.5
HIGH	0	3.08	0.05-15.62	4.43	0.20-21.17	12.79	5.55-27.98	7.81	2.28-1524.97
	24	10.45	0.05-22.43	25.43	18.15-83.25	31.26	9.57-54.56	2625.82	1711.07-4117.13
	48	19.97	0.05-34.85	46.56	4.43-108.76	11.04	6.33-63.13	1160.99	7.81-1796.18

All data shown has been analysed by Laura Haag (LH) and has approval for use in this thesis. Shown is the profile of the four metabolites which make up the HIGH / LOW metabotype. Metabolites include hippuric acid (HA), 3-hydroxyhippuric acid (3-OH-HA), 4-hydroxy-3-methoxyphenylacetic acid (4-OH-3-OCH-PAA), and 3,5-dihydroxyphenylpropionic acid (3,5-DiOH-PPA). Data is presented as the median value with the IQR (inter quartile range).

5.4.2 Analysis of participants who completed at least one arm of the intervention – blueberry *versus* placebo

Table 5.10: Serum cardiometabolic markers assessed by intervention treatment (blueberry *versus* placebo) in those participants who completed at least one arm of the study

		BASELINE					20MIN					40MIN				
		N	Mean	SD	Min	Max	N	Mean	SD	BL Δ	P Value	N	Mean	SD	BL Δ	P Value
Glucose (mmol/L)	BLUEBERRY	12	4.74	0.33	4.40	5.50	11	6.22	0.60	1.45	0.68	11	5.95	1.30	1.18	0.05
	PLACEBO	10	4.68	0.42	4.10	5.30	8	6.14	1.07	1.34		8	6.84	0.79	2.04	
Cholesterol (mmol/L)	BLUEBERRY	12	5.61	1.56	2.70	8.50	10	5.89	1.72	0.17	0.06	11	5.66	1.62	0.09	0.49
	PLACEBO	9	5.41	0.66	4.40	6.80	8	6.13	0.82	0.39		7	5.91	0.91	0.09	
HDLC (mmol/L)	BLUEBERRY	12	1.35	0.33	0.83	2.03	11	1.39	0.37	0.07	0.93	11	1.33	0.32	0.01	0.23
	PLACEBO	10	1.43	0.31	1.02	1.96	8	1.53	0.37	0.08		8	1.49	0.39	0.04	
LDLC (mmol/L)	BLUEBERRY	12	3.65	1.33	1.50	5.60	8	3.88	1.39	0.06	0.13	9	3.69	1.40	0.09	0.90
	PLACEBO	9	3.48	0.67	2.60	4.90	7	3.83	0.73	0.27		6	3.68	0.75	0.07	
Triglycerides (mmol/L)	BLUEBERRY	12	1.32	0.37	0.77	2.02	9	1.47	0.46	0.12	0.71	9	1.40	0.46	0.05	0.87
	PLACEBO	10	1.16	0.30	0.70	1.60	7	1.27	0.30	0.10		7	1.28	0.42	0.12	

		60MIN					90MIN					180MIN				
		N	Mean	SD	BL Δ	P Value	N	Mean	SD	BL Δ	P Value	N	Mean	SD	BL Δ	P Value
Glucose (mmol/L)	BLUEBERRY	11	5.35	1.29	0.59	0.23	11	5.06	1.08	0.30	0.70	11	4.73	0.76	0.05	0.16
	PLACEBO	8	6.01	0.56	1.33		10	5.08	0.96	0.40		8	5.29	0.87	0.64	
Cholesterol (mmol/L)	BLUEBERRY	11	5.35	1.29	-0.22	0.36	11	5.37	1.56	-0.20	0.34	10	5.47	1.49	-0.10	0.15
	PLACEBO	8	6.01	0.56	0.36		10	5.45	0.74	-0.11		8	5.36	0.83	-0.22	
HDLC (mmol/L)	BLUEBERRY	11	1.35	0.37	0.03	0.38	11	1.26	0.32	-0.06	0.44	11	1.29	0.30	-0.07	0.21
	PLACEBO	8	1.39	0.35	-0.01		10	1.35	0.33	-0.08		8	1.34	0.36	-0.11	
LDLC (mmol/L)	BLUEBERRY	9	3.66	1.43	0.06	0.91	10	3.29	1.28	-0.19	0.17	9	3.40	1.24	-0.30	0.79
	PLACEBO	7	3.47	0.74	0.04		9	3.39	0.61	-0.09		7	3.11	0.77	-0.31	
Triglycerides (mmol/L)	BLUEBERRY	9	1.45	0.44	0.09	0.71	10	1.55	0.45	0.20	0.35	9	1.94	0.41	0.65	0.46
	PLACEBO	7	1.37	0.36	0.14		9	1.27	0.40	0.11		7	1.64	0.50	0.51	

CONTINUED. Table 5.10: Serum cardiometabolic markers assessed by intervention treatment (blueberry *versus* placebo) in those participants who completed at least one arm of the study

		360MIN					24HR					48HR				
		N	Mean	SD	BL Δ	P Value	N	Mean	SD	BL Δ	P Value	N	Mean	SD	BL Δ	P Value
Glucose (mmol/L)	BLUEBERRY	11	4.17	0.61	-0.60	0.08	11	4.73	0.26	-0.04	0.62	11	4.57	0.26	-0.10	0.14
	PLACEBO	10	4.44	0.27	-0.24		10	4.59	0.31	-0.09		9	4.38	0.39	-0.27	
Cholesterol (mmol/L)	BLUEBERRY	11	5.29	1.30	-0.17	0.89	11	5.77	1.55	0.09	0.88	11	5.75	1.52	0.07	0.38
	PLACEBO	10	5.40	0.78	-0.16		10	5.62	0.74	0.06		9	5.53	0.82	-0.03	
HDLC (mmol/L)	BLUEBERRY	11	1.24	0.29	-0.12	0.62	11	1.42	0.29	0.04	**0.02***	11	1.36	0.31	-0.01	0.47
	PLACEBO	10	1.29	0.35	-0.14		10	1.38	0.30	-0.05		9	1.36	0.33	-0.04	
LDLC (mmol/L)	BLUEBERRY	10	3.16	1.15	-0.44	0.87	11	3.74	1.31	0.05	0.73	11	3.76	1.30	0.05	0.77
	PLACEBO	9	3.09	0.71	-0.39		10	3.68	0.71	0.07		9	3.64	0.79	0.03	
Triglycerides (mmol/L)	BLUEBERRY	10	2.16	0.72	0.86	0.62	11	1.54	0.49	0.17	0.16	11	1.37	0.43	0.13	0.15
	PLACEBO	9	2.00	0.87	0.83		10	1.19	0.30	0.03		9	1.18	0.28	-0.03	

Mean change from baseline for participants who completed at least one arm of the intervention (n = 22). Some missing data as unable to take participant blood sample at particular timepoints. Mean and BL Δ for glucose cholesterol, HDLC, LDLC and triglycerides is shown in millimoles per litre (mmol/L). Statistical differences between the blueberry and placebo group was established using a Kruskal Wallis test. A P value of < 0.05 was considered statistically significant and indicated with a * above. Abbreviations LDLC (low density lipoprotein cholesterol), HDLC (high density lipoprotein cholesterol), BL (baseline) SD (standard deviation), Δ (change), min (minimum) max (maximum).

Table 5.7 (Appendix C2– supplementary data) shows that those that were randomly allocated to have the blueberry test drink first, in their cross-over sequence, were disproportionately female (p = 0.04) and had higher bodyfat (mean = 35.09%, p = 0.02) and trunk fat (mean = 33.37%, p = 0.06 –not significant). As shown in table 5.8 and 5.9 (Appendix C2– supplementary data), there were no significant differences between the two groups (blueberry *versus* placebo) in postprandial PWV, AIx, resting BP or ABPM measurements. HDLC significantly increased (p = 0.02) when compared with the control group at 24 hours (table 5.10).

5.4.3 Analysis of those who completed the crossover intervention – analysis by intervention type

Tables 5.11 and 5.12 (Appendix C2– supplementary data) show the characteristics for the participants, *n* = 8, who completed the cross-over study analysis (blueberry intervention arm *versus* placebo intervention arm). There were no differences between the baseline data at the start of each intervention week. No differences between the treatments were observed for postprandial PWV, AIx, clinic BP nor ABPM measurements throughout the 48-hour observation period (tables 5.13 and 5.14). With the exception of change in glucose at 180 minutes (from baseline levels), which was significantly lower after blueberries (table 5.15, Appendix C2– supplementary data), there were no other differences detected in postprandial serum measurements (glucose, cholesterol, HDLC, LDLC or triglycerides) (table 5.15, Appendix C2– supplementary data).

Table 5.13: Crossover analysis mean change from baseline in measures of arterial stiffness and blood pressure for participants completing both intervention crossover arms (blueberry *versus* placebo)

		BASELINE					1.5hr					3HR				
		N	Mean	SD	Min	Max	N	Mean	SD	BL Δ	P	N	Mean	SD	BL Δ	P
PWV (m/s)	BLUEBERRY	8	9.92	1.67	8.23	13.30						7	9.21	1.12	-0.68	0.46
	PLACEBO	8	9.66	0.96	8.20	11.10						7	9.35	1.06	-0.25	
AIx (%)	BLUEBERRY	8	27.88	7.07	17.67	36.00						7	24.70	7.73	-2.01	1.00
	PLACEBO	8	28.16	9.39	16.67	44.33						7	24.58	10.48	-2.07	
SBP (mmHg)	BLUEBERRY	8	138.44	7.84	128.00	151.00	8	133.88	8.88	-4.56	0.16	8	133.25	8.29	-5.19	0.57
	PLACEBO	8	140.44	11.56	124.00	162.00	8	128.44	6.26	-12.00		8	130.94	11.01	-9.50	
DBP (mmHg)	BLUEBERRY	8	85.25	8.17	72.00	98.00	8	80.44	7.39	-4.81	0.07	8	82.06	7.97	-3.19	0.16
	PLACEBO	8	85.75	12.56	65.00	104.00	8	76.25	9.41	-9.50		8	78.00	9.70	-7.75	
HR (BPM)	BLUEBERRY	8	62.00	4.38	56.00	70.00	8	68.19	6.28	6.19	0.28	7	66.61	6.31	4.90	0.87
	PLACEBO	8	63.69	7.06	52.00	72.50	8	71.69	7.30	8.00		8	69.25	8.68	5.56	

		6HR					24HR					48HR				
		N	Mean	SD	BL Δ	P	N	Mean	SD	BL Δ	P	N	Mean	SD	BL Δ	P
PWV (m/s)	BLUEBERRY	6	9.55	1.32	-0.28	1.00	8	9.72	1.30	-0.20	0.88	8	9.50	1.23	-0.42	0.51
	PLACEBO	7	9.58	1.07	0.00		8	9.43	1.16	-0.23		8	9.28	0.66	-0.38	
AIx (%)	BLUEBERRY	8	28.43	7.47	0.55	0.33	8	28.33	7.61	0.46	0.38	8	29.90	7.81	2.02	0.57
	PLACEBO	8	27.63	9.61	-0.53		8	29.26	9.82	1.10		8	29.60	10.36	1.45	
SBP (mmHg)	BLUEBERRY	8	138.31	11.40	-0.13	0.38	8	132.13	11.27	-6.31	0.88	8	136.94	8.11	-1.50	0.44
	PLACEBO	8	136.69	7.71	-3.75		8	131.88	6.36	-8.56		8	137.63	5.53	-2.81	
DBP (mmHg)	BLUEBERRY	8	85.94	9.72	0.69	0.51	8	81.44	11.82	-3.81	0.88	8	81.38	10.74	-3.88	0.23
	PLACEBO	8	85.50	9.53	-0.25		8	82.19	9.31	-3.56		8	85.13	8.34	-0.63	
HR (BPM)	BLUEBERRY	8	64.88	6.29	2.88	0.80	8	64.69	6.51	2.69	0.28	8	62.44	7.68	0.44	0.72
	PLACEBO	8	65.25	9.16	1.56		8	63.94	6.10	0.25		8	63.63	8.45	-0.06	

Mean and BL Δ for SBP and DBP are shown in millimetres of mercury (mmHg). Mean and BL Δ for PWV is shown in meter per second squared (m/s^2), AIx is shown in percentage (%) HR are shown in beats per minute (BPM). Statistical differences between the blueberry and placebo group were established using a Mann Whitney U test. A P value of < 0.05 was considered statistically significant however no results indicated differences between the groups at any timepoint. Abbreviations: PWV (pulse wave velocity), AIx (augmentation index), SBP (systolic blood pressure), DBP (diastolic blood pressure), HR (heart rate), BL (baseline) SD (standard deviation), Δ (change), min (minimum) max (maximum).

Table 5.14: Crossover analysis mean change from baseline for ambulatory blood pressure measurements in participants completing both intervention crossover arms (blueberry *versus* placebo)

		BASELINE (DAY 0)					DAY 1					DAY 2				
		N	Mean	SD	Min	Max	N	Mean	SD	BL Δ	p	N	Mean	SD	BL Δ	p
ABPM SBP (mmHg)	BLUEBERRY	7	127.14	5.87	116.00	132.00	7	125.43	5.09	-1.71	0.10	6	121.50	7.01	-5.50	0.39
	PLACEBO	7	128.57	8.30	117.00	140.00	7	122.86	7.15	-5.71		6	121.67	4.55	-5.67	
ABPM DBP (mmHg)	BLUEBERRY	7	79.29	5.50	68.00	83.00	7	78.00	6.24	-1.29	0.32	6	76.50	8.55	-2.17	0.49
	PLACEBO	7	80.00	8.08	64.00	89.00	7	76.57	9.69	-3.43		6	76.00	8.00	-2.50	
ABPM HR (BPM)	BLUEBERRY	7	72.14	6.04	67.00	81.00	7	71.29	5.65	-0.86	0.32	6	74.17	5.12	1.33	0.39
	PLACEBO	7	75.00	7.33	66.00	82.00	7	71.86	6.72	-3.14		6	73.00	4.73	-0.83	
ABPM PP (mmHg)	BLUEBERRY	7	47.71	5.82	39.00	58.00	7	47.57	5.50	-0.14	0.46	6	45.00	7.29	-3.17	0.49
	PLACEBO	7	48.14	5.46	39.00	54.00	7	46.43	4.35	-1.71		6	45.83	5.27	-2.67	
ABPM MAP (mmHg)	BLUEBERRY	7	95.43	4.12	89.00	100.00	7	94.29	3.99	-1.14	0.07	6	92.17	5.64	-2.67	0.82
	PLACEBO	7	96.14	6.49	85.00	105.00	7	91.86	6.79	-4.29		6	91.83	4.54	-2.83	

Mean and BL Δ for ABPM SBP, ABPM DBP ABPM MAP and ABPM PP are shown in millimetres of mercury (mmHg). Mean BL Δ for ABPM HR is shown in beats per minute (BPM). Statistical differences between the blueberry and placebo group were established using a Mann Whitney U test. A p value of < 0.05 was considered statistically significant however no results indicated differences between the groups at any timepoint.

Abbreviations: ABPM SBP (ambulatory blood pressure monitor systolic blood pressure), ABPM DBP (ambulatory blood pressure monitor diastolic blood pressure), ABPM MAP (ambulatory blood pressure monitor mean arterial pressure), ABPM PP (ambulatory blood pressure monitor pulse pressure), ABPM HR (ambulatory blood pressure monitor heart rate), BL (baseline) SD (standard deviation), Δ (change), min (minimum) max (maximum).

5.5 Discussion

This study aimed to answer the hypotheses of whether a single dose of blueberry anthocyanins will have an effect on vascular measurements (BP, PWV, AIx, serum lipids, lipoproteins and glucose) and whether this effect is influenced by metabotype. The interruption to data collection due to COVID-19 March 2020 lockdown resulted in a small number of participants completing the study. Statistical testing on the influence of metabotype was therefore deemed inappropriate and data are presented without p values. Despite small numbers, these data suggest that the novel Phase 1 blueberry anthocyanin HIGH / LOW metabotype may influence vascular health, specifically arterial stiffness (measured by AIx) and blood pressure (both at rest and ambulatory) after blueberry anthocyanin consumption (table 5.2 and 5.3). This impact of a single dose was unexpected, since the metabotype was based on change in FMD after 6 months of chronic blueberry intake (where changes were observed in the equivalent HIGH metabotype group).

We also observed improvements in systolic BP, diastolic BP and arterial stiffness (measured by AIx) in the LOW metabotype group (table 5.2). The improvements in BP – seen in systolic BP at 24 hours and diastolic BP at 24 and 48 hours are consistent. This is accompanied by improvements in ABPM diastolic BP on day 2 (48 hours), which captures diastolic BP over a 24-hour period (table 5.3). The evidence for anthocyanins benefiting blood pressure is equivocal with a recent meta-analysis of seven randomised controlled trials suggesting no effect of long-term blueberry intake [201]. By contrast, Jennings *et al.*, 2012 found that those women with highest anthocyanin intake had lower central systolic BP ($p = 0.02$) and arterial stiffness (only for PWV, not for AIx) when compared with those women who had the lowest habitual anthocyanin intakes. Smaller marginal improvements were seen for central diastolic BP ($p = 0.05$) and peripheral diastolic BP ($p = 0.06$) for those women with the highest anthocyanin intakes [54]. There are other RCTs suggesting advantageous effects, notably Kent *et al.*, 2016 who found significant differences in systolic BP ($p < 0.05$), diastolic BP ($p < 0.05$) and HR ($p < 0.05$) 2 hours after a cherry drink (containing 207mg anthocyanins) [68]. They also measured phenolic metabolites and found associations between increases of eight metabolites and reductions in BP and phenolic metabolite concentrations two hours after the cherry drink. The authors noted that the high interindividual variability in the

concentrations of the metabolites and their small study population (*n* = 13) made it difficult to draw conclusions from the metabolite and BP changes [68]. None of the metabolites mentioned by Kent *et al.*, 2016 are included in our HIGH / LOW metabolite profile, potentially because of their short duration of postprandial measurement (2 hours compared to our 48 hour metabolite profile). Despite this, their study supports our findings that there is high interindividual variation in anthocyanin metabolism and that this variation may drive variable blood pressure responses. There was no effect on blood pressure or arterial stiffness, when the data were not analysed by metaboliser type (Supplementary data Appendice C2 tables 5.8, 5.9 and presented in this chapter 5.13 and 5.14).

The observed changes seen in BP in the HIGH / LOW analysis (tables 5.2 and 5.3) were seen at 48 hours. To our knowledge the effects of a single dose of anthocyanins on vascular function at 48 hours is novel and has not been previously analysed. We included this timepoint because Czank *et al.*, 2013 found that after a single dose of 13-C isotopically labelled cyanidin-3-glucoside (an anthocyanin) metabolites were still in circulation at 48 hours, and had not returned to baseline levels. We speculate that vascular effects of anthocyanins are still present at 48 hours and may be mediated by circulating metabolites. This is consistent with prior epidemiological evidence suggesting that just three portions of anthocyanin rich food a week may be needed to achieve cardiovascular benefits [53]. Additionally our data, showing sustained BP effects at 24 and 48 hours, perhaps explains why just one serving of blueberries a week appears to reduce the incidence in hypertension [45].

Our observations that the LOW metabotype may also influence vascular health were unexpected. Previously, it was hypothesised that anthocyanins go through phase 1 and phase 2 metabolism, and that enterohepatic recirculation follows which results in further colonic and microbial metabolism of anthocyanins [91]. On this basis it was expected that the HIGH metabotype would reflect prolonged exposure to these metabolites in the circulation. In our study baseline metabolite levels were not adjusted but we calculated 'change' as the absolute change from baseline to 24 and 48 hours. Thus, our focus was on the HIGH metabolisers, whose urinary metabolite levels increased after the consumption of the blueberry drink (phase 1) as it was speculated this would be associated with similar

improvements in vascular function. However, it was those with high habitual baseline levels (LOW group) of the metabolites that responded best, metabolites which are not unique to blueberries – potentially suggesting they are an indicator of effective metabolism. Notably, the LOW metabolisers also had a postprandial decline in all metabolite levels (table 5.6).

Our data suggests there may also be another potential mechanism. The trend of both the metabolite and food frequency questionnaire (FFQ) data shown in tables 5.5 and 5.6 between the LOW and HIGH groups is similar to the Phase 1 observations (from Laura Haag's PhD thesis - *n* = 104 for the FFQ data and *n* = 119 for the metabolite data). The LOW group had baseline dietary intakes (table 5.5) that more closely reflect the UK dietary guidelines than the HIGH group; i.e. intakes of wholegrains and tea, lower levels of fat and beer. Similarly, the LOW group exhibited much higher levels of the metabolites which comprise the metabolite panel at baseline – which suggests that their habitual diet may contain more foods which are metabolised by the colonic microbiota; i.e. wholegrain fibres, fruits and vegetables. This seeming difference, in habitual background diet, may also have a pre-biotic effect on the gut microbiota and explain why the LOW group had more favourable responses for arterial stiffness and BP (tables 5.2 and 5.3).

Habitual diet is very important for the gut microbiome and the promotion of bacterial growth. Nutrients from the food we eat can act directly with the gut and its bacteria can cause physiological changes to our body's health [202]. This well-established concept has been used in diet therapy for various different conditions. For example, for irritable bowel syndrome the low FODMAP (fermentable oligosaccharides, disaccharides, monosaccharides, and polyols) diet is used to help alleviate symptoms by lowering intake of indigestible carbohydrates. Studies examining the low FODMAP diet show changes in certain bacterial concentrations, greater microbial diversity a reduction in irritable bowel syndrome symptoms (pain) over a short time period [203], [204]. We speculate that the habitual dietary intakes, outlined by the food frequency questionnaire, of our LOW participants may have influenced their gut microbiome, their metabotype and ultimately allowed them to obtain the cardiovascular benefits from blueberry anthocyanins.

Regarding the apparent greater habitual intake of wholegrains by the LOW group; a diet rich in wholegrains has been associated with a reduced risk of mortality due to CVD and cancer [205], [206]. Likewise, in RCT studies, a cross-over study found 8 weeks of a diet rich in wholegrains (compared with the control – refined grains) saw significant decreases in diastolic BP ($p = 0.01$) [207]. Another study found that three daily portions of wholegrains, for 12 weeks, significantly reduced systolic BP and pulse pressure in older men and women [208]. It is noteworthy that two of the four metabolites which make up the HIGH / LOW metabotype used in this study (table 5.6); i.e. hippuric acid and 3-hydroxyhippuric acid, have been previously reported to be in abundance after wholegrain consumption [209], [210]. Similarly, a diet lower in fat has been associated with increased α-diversity in the gut microbiome and a reduction of bacteria associated with poorer cardiometabolic outcomes [211]. Beer consumption and the microbiome is less well understood – beer has polyphenolic compounds thought to be beneficial for the gut however the alcohol content of beer appears to interfere and alters this beneficial effect [212]. In our study, faecal samples were collected but not analysed due to the pandemic related abandonment of the RCT and therefore insufficient power to examine study effects. Consequently, further research is needed to understand the effect of the gut microbiome and whether bacterial species differed between the HIGH / LOW metabotype groups.

There were some changes seen in the serum results from the *post hoc* parallel analysis which suggest a favourable response in the blueberry group at 24 hours HDLC ($p = 0.02$) at 24 hours compared with the placebo (table 5.10). No other changes in HDLC were observed in the cross-over or HIGH / LOW analysis. This was a single statistical finding in a dataset not adjusted for multiple testing, therefore lone significant findings should be interpreted with some caution. There were comparable isolated findings for glucose (tables 5.15), however the lack of consistency in these findings (i.e. not observed at repeated time points in one analysis) and the small study numbers mean that these data should also be interpreted with caution.

Despite some findings of interest there were several key limitations. Firstly, because of the COVID-19 pandemic and national lockdowns our study was stopped prematurely. This meant that our sample size was severely limited, with only data for $n = 8$ available for the

HIGH / LOW cross-over analysis. This number was less than the power calculation which had predicted that *n* = 37 in each group would be needed. Although only a small number of participants (*n* =8) completed the cross-over study, we had a larger number who completed at least one arm of the study (*n* = 22). The data were therefore also analysed as a *post hoc* parallel analysis. We had not intended these *post hoc* parallel analyses and fully acknowledge that there are clear limitations. The blueberry and placebo groups also had baseline differences in sex and body fat (table 5.7- Appendix C2 supplementary data), which could have affected the results. Another limitation is that the metabolite data (table 5.6) represents the HIGH / LOW metabolite concentrations for the individuals from PHASE 1, and not for PHASE 2. It is therefore important to note the changes in metabolite concentration cannot be seen as a direct reflection of the effect seen on BP and arterial stiffness in PHASE 2.

In conclusion, these hypothesis generating data show some interesting observations that will require further evaluation in adequately powered studies to determine the effectiveness of blueberry anthocyanins. With that important caveat, it was interesting to note that the vascular benefits of blueberry anthocyanins, specifically for BP, may be influenced by an individual's metabolic profile. This potential benefit appears to last for up to 48 hours and future studies investigating the acute vascular responses to anthocyanins should include measurements at least up until this time point. Further research testing the change in metabolite concentrations in the HIGH / LOW metabotype in conjunction with BP measurements should also be considered.

CHAPTER 6. Exploration of personalised test meals and the application of a QRISK score

6.1 Aims and objectives of the chapter

This chapter addresses two key aims:

1) To determine how cardiometabolic outcomes are impacted by a personalised energy dense meal formulation.

2) To explore the effect of stratifying participants by baseline QRISK3 score, to determine the impact on global health on cardiometabolic response to treatment.

To achieve these aims, we compared those with a lower *versus* a higher fat test meal. Likewise, we re-assessed baseline screening data to determine the QRISK3 score for those taking part.

6.2 Hypotheses tested in the chapter

This chapter addresses two hypotheses:

1) Differences in fat content of an individualised energy dense meal does not influence cardiometabolic responses.

2) The application of the QRISK3 score is positively associated with a poorer cardiometabolic response.

6.3 Introduction

Randomised controlled trials (RCTs) are the gold standard in research design for testing the effectiveness of an intervention. They are highly controlled, in terms of limiting variation in potentially confounding variables, with the intention that attributable 'treatment' effect can be more clearly observed against a 'control' arm. Design characteristics which minimise bias and ensure similarity in the study population include: the stringent application of inclusion / exclusion criteria, and randomisation to intervention treatment. Nutritional RCTs offer

rigorous methodology for establishing the relationship between nutrients / foods and pre-specified primary and secondary health outcomes; these data help inform evidence-based dietary and public health guidelines. However, despite the control that an RCT provides, nutrition studies have acknowledged interindividual variation in response to interventions. Variation in response to flavonoids including anthocyanins [68], [81] often persist despite strict inclusion / exclusion criteria for body mass index, past medical history, age and biochemical markers of health (such as cholesterol levels).

Variation in response to a controlled nutrition intervention however, is not unique to plant bio-actives such as flavonoids. For example, researchers have examined the interindividual variation to a fixed macronutrient intervention, such as the oral glucose tolerance test (OGTT). In healthy volunteers they found there was a differential response and distinct phenotypes that indicated potential risk for future glucose intolerance [213]. Researchers examining interindividual variation in response to an oral lipid tolerance test (OLTT) found low variance occurred between participants (age 30.0$\pm$12.0, BMI 24.5$\pm$12.7kg/m^2) [214]. However, it should be noted the OLTT contained 533kcals, 54g of fat and 11g of carbohydrates using nutritional supplement powders [214]. In contrast, many of the studies we have reported on (table 1.1, Chapter 1) provided more fat, but particularly more carbohydrates and protein as the test meal was provided with food, in a mixed meal, to emulate 'real world' postprandial environments. Further research using macronutrient profiles most reflective of energy-dense, high-fat meal challenges using food rather than supplements would be of benefit.

Stratified nutrition is the intermediate position between a one size fits all traditional approach and personalised nutrition (for example the HIGH / LOW metabolisers). Individualised or personalised nutrition is generally accepted as delivering nutrition best suited to an individual [215]. Personalised nutrition has been used on some level for many years in the dietetic profession where tube feeding regimens and dietary plans are tailored to the energy and protein needs of the individual. There are many equations to calculate an individual's basal metabolic rate (BMR) such as the Harris and Benedict [216], Schofield [217] and Henry [218]. All recognise that body weight, age, sex and activity play a role in each person's differential metabolism.

In nutritional research studies which are designed to assess postprandial responses to intervention alongside a high-fat test meal challenge, there appears no consensus or 'gold-standard' approach regarding whether a test meal should be 'standardised' across participants or 'personalised' to account for interindividual variation in characteristics. In the field of postprandial flavonoid research, many studies have provided standardised meals – providing fixed amounts of macronutrients / foods to all participants [36], [174], [175]. Often, in many of these studies, participants have been given the same meal challenge regardless of different age, sex and body composition which may influence how they handle a test meal challenge. For these reasons, there has been a move towards personalised nutrition in postprandial studies, with individualised test meals provided which deliver a fixed proportion of energy from fat, based on the estimated energy requirements of individuals [219], [220].

Despite stringent inclusion / exclusion criteria we recognised the potential to further control our study by personalising the test meal to each individual (in the study reported in this thesis, methodology in Chapter 3). With our study design being a cross-over RCT, each individual would act as their own control. Due to the impact of COVID-19 there was a lack of participants who had finished the cross-over study, but many volunteers who had completed at least one arm of the cross-over (*n* =22), with a total of *n* =14 completing the placebo intervention arm. Within our study population the individual with the least amount of absolute fat in their test meal (43.11g) was female, 62 years, 70.7kgs, 1.55m in height and had an activity factor of 1.5 (suggesting light-moderate activity). Conversely the individual with greatest amount of absolute fat in their test meal (78.73g) was male, 59 years, 105kgs, 1.94m in height and had an activity factor of 1.7 (suggesting moderate activity). This indicates that, despite inclusion / exclusion criteria there was variation in phenotype. To address the potential limitations of previous studies, we used a basal metabolic rate (BMR) adjustment calculation to personalise the amount of energy provided (50% of an individual's energy requirements) and fat (40% of calories in the meal) for each individual having the postprandial assessment. As this was a less frequently utilised approach, the following research question was addressed in this chapter: *To what extent does an individualised meal, with 40% of energy derived from fat, affect acute cardiometabolic health outcomes?*

The second focus of this chapter is QRISK3 score. Whilst our study adhered to strict inclusion and exclusion criteria, a composite indicator of cardiovascular health was not part of the recruitment strategy. Framingham risk score is widely known for calculating CVD risk but is based on the U.S population, who have a differing CVD risk. The QRISK model was first established in 2007 with the aim of creating a 10-year risk of CVD score for the UK population [221]. The QRISK model was developed using an electronic database (QRESEARCH) from primary care which amalgamated the health data of over 10 million UK patients from 529 GP practices [221]. The QRISK model has been updated regularly, and is now in its 3rd iteration, to reflect the changing CVD risk in the UK, to include variables considered important in calculating risk [178]. QRISK is widely used in general practice to categorise a patient's CVD risk and is recommended for use by the National Institute of Clinical Excellence (NICE). In nutrition interventions the QRISK algorithm has been used as part of the screening phase, to recruit participants within certain cardiovascular thresholds – with the intention to examine the effectiveness of a dietary intervention on cardiometabolic health by risk stratification group [94], [222]. The causes of CVD are multi-factorial as have been outlined in Chapter 1, which include age, hypertension, endothelial dysfunction, arterial stiffness and serum lipid and lipoprotein levels. The QRISK3 score accounts for this and is a validated tool. As we did not include absolute risk of CVD in our recruitment process, in this chapter we examine if the placebo intervention participants (*n* =14) were recruited and categorised on the basis of a higher or lower QRISK3 score, would this have potentially influenced their cardiometabolic response.

6.4 Results

6.4.1 Exploring the impact of absolute fat content (in g) in the energy-dense meal on postprandial cardiometabolic responses: Placebo intervention participants only, retrospectively separated into 'lower' and 'higher' fat intake groups

Table 6.1: Characteristics of placebo volunteers who had a test meal containing <50.58g fat *versus* those consuming a test meal containing >51.80g fat

	Lower Fat					Higher Fat					P
	N	Mean	SD	Min	Max	N	Mean	SD	Min	Max	Value
Test meal fat content (g)	7	47.27	2.47	43.11	50.58	7	62.61	8.86	51.80	78.73	**0.01**
Age (y)	7	61.00	6.68	51.00	67.00	7	57.71	4.79	53.00	67.00	0.46
Sex (male)	7	0.00	/	/	/	7	6.00	/	/	/	**0.01**
BMI (kg/m$^{2)}$	7	28.64	3.09	25.60	34.90	7	28.46	2.56	25.90	33.50	1.00
Statin (NO)	7	6.00	/	/	/	7	7.00	/	/	/	0.30
Body fat %	7	36.46	4.41	31.80	42.90	7	27.71	9.04	20.30	46.60	**0.03**
Trunk fat %	7	32.70	6.25	25.30	41.00	7	28.97	8.80	20.30	46.60	0.26
Hip (cm)	7	106.63	4.26	102.00	113.00	7	107.01	5.13	100.85	114.65	0.81
Waist (cm)	7	85.86	7.68	74.00	95.50	7	98.14	7.35	90.00	112.15	**0.01**
Peak FMD %	7	4.91	3.97	0.22	11.73	7	4.21	4.33	-0.33	11.31	0.71
HFMC %	7	-0.68	1.53	-2.60	1.09	7	-1.97	1.63	-5.02	-0.03	0.26
LFMC %	7	-0.07	1.66	-2.15	1.90	7	-1.21	1.30	-3.50	0.20	0.26
TTP (seconds)	6	39.17	14.47	23.00	56.00	7	48.29	16.07	32.00	78.00	0.23
PWV (m/s)	7	10.26	1.09	8.20	11.73	6	9.58	0.63	8.60	10.30	0.10
AIx (%)	7	30.12	8.31	22.50	44.33	7	24.44	5.20	16.67	32.50	0.38
SBP (mmHg)	7	144.21	12.69	127.50	162.00	7	136.79	11.59	119.50	151.50	0.26
DBP (mmHg)	7	85.79	12.56	65.00	104.00	7	85.07	7.78	75.00	94.00	0.90
HR (BPM)	6	62.33	7.63	52.00	72.50	7	64.21	7.92	53.00	75.50	0.84
ABPM SBP (mmHg)	7	129.00	8.52	117.00	140.00	6	131.83	9.28	122.00	143.00	0.73
ABPM DBP (mmHg)	7	80.00	8.37	64.00	89.00	6	81.17	8.08	70.00	94.00	1.00
ABPM MAP (mmHg)	7	96.71	6.40	85.00	105.00	6	97.33	7.03	88.00	108.00	0.95
ABPM PP (mmHg)	7	48.57	7.30	39.00	60.00	6	50.50	4.72	44.00	58.00	0.73
ABPM HR (BPM)	7	74.57	7.87	65.00	82.00	6	73.17	9.11	63.00	88.00	0.73
Glucose (mmol/L)	7	4.50	0.36	4.10	5.00	7	4.94	0.21	4.70	5.30	0.50
Cholesterol (mmol/L)	6	5.42	0.57	4.40	6.10	7	5.57	1.11	3.90	7.10	1.00
LDLC (mmol/L)	6	3.38	0.53	2.60	4.10	7	3.77	1.10	2.10	5.30	1.00
HDLC (mmol/L)	7	1.60	0.24	1.23	1.96	7	1.21	0.17	1.02	1.53	1.00
Triglyceride (mmol/L)	7	1.01	0.19	0.70	1.20	7	1.28	0.36	0.66	1.60	1.00

Participants were divided into either lower or higher fat group dependent on where they fell below or above the median (methodology described in chapter 3). Statistical differences between the higher fat test meal group and the lower fat test meal group were established using a Mann Whitney U test, for all nominal data a Chi square test was performed. A P value of < 0.05 was considered statistically significant. Abbreviations: SD (standard deviation), min (minimum) max (maximum), BMI (body mass index), FMD (flow-mediated dilation), HFMC (high flow-mediated dilation), LFMC (low flow-mediated dilation), TTP (time to peak), PWV (pulse wave velocity), AIx (augmentation index), SBP (systolic blood pressure), DBP (diastolic blood pressure), HR (heart rate), ABPM SBP (ambulatory blood pressure monitor systolic blood pressure), ABPM DBP (ambulatory blood pressure monitor diastolic blood pressure), ABPM MAP (ambulatory blood pressure monitor mean arterial pressure), ABPM PP (ambulatory blood pressure monitor pulse pressure), ABPM HR (ambulatory blood pressure monitor heart rate), LDLC (low density lipoprotein cholesterol) and HDLC (high density lipoprotein cholesterol).

Table 6.2: Placebo volunteers mean change from baseline for markers of endothelial function in those in the 'lower fat' test meal *versus* those in the 'higher fat test meal '

		BASELINE					1.5HR					3HR				
		N	Mean	SD	Min	Max	N	Mean	SD	BL Δ	P	N	Mean	SD	BL Δ	P Value
Peak FMD %	LOW fat	7	4.91	3.97	0.22	11.73	7	5.25	2.83	0.33	0.90	7	6.75	3.93	1.84	1.00
	HIGH fat	7	4.21	4.33	-0.33	11.31	7	4.81	3.84	0.60		7	5.19	2.86	0.98	
LFMC %	LOW fat	7	-0.07	1.66	-2.15	1.90	7	0.92	1.16	0.98	0.71	7	0.88	1.67	0.95	0.62
	HIGH fat	7	-1.21	1.30	-3.50	0.20	7	0.55	1.34	1.75		7	-0.53	0.85	0.67	
HFMC %	LOW fat	7	-0.68	1.53	-2.60	1.09	7	0.37	0.87	1.05	0.62	7	0.41	1.94	1.09	0.62
	HIGH fat	7	-1.97	1.63	-5.02	-0.03	7	-0.13	1.02	1.84		7	-0.94	0.90	1.03	
TTP (seconds)	LOW fat	6	39.17	14.47	23.00	56.00	6	65.00	52.11	25.83	0.30	6	47.33	14.81	8.17	0.73
	HIGH fat	7	48.29	16.07	32.00	78.00	7	53.57	20.57	5.29		7	50.57	6.90	2.29	

		6HR					24HR					48HR				
		N	Mean	SD	BL Δ	P Value	N	Mean	SD	BL Δ	P Value	N	Mean	SD	BL Δ	P Value
Peak FMD %	LOW fat	7	5.69	3.57	0.78	0.81	7	5.23	3.72	0.32	1.00	7	5.85	4.57	0.94	0.54
	HIGH fat	7	5.04	2.39	0.83		7	4.64	2.80	0.43		7	6.50	2.60	2.29	
LFMC %	LOW fat	7	0.35	1.87	0.42	1.00	7	0.44	1.67	0.50	0.21	7	0.44	1.42	0.51	0.13
	HIGH fat	7	-0.36	1.18	0.84		7	0.18	0.74	1.39		7	0.59	1.01	1.80	
HFMC %	LOW fat	7	-0.56	1.59	0.13	1.00	7	-0.63	1.54	0.05	0.38	7	-0.63	0.69	0.05	0.07
	HIGH fat	7	-1.14	1.52	0.83		7	-0.69	0.74	1.28		7	-0.12	0.94	1.85	
TTP (seconds)	LOW fat	6	42.83	7.70	3.67	0.14	6	49.50	12.08	10.33	0.23	6	48.67	17.28	9.50	0.45
	HIGH fat	7	39.14	5.37	-9.14		7	50.43	14.93	2.14		7	43.43	5.50	-4.86	

Mean and BL Δ for FMD, LFMC and HFMC are shown in percentage. Mean BL Δ for TTP is shown in seconds. Statistical differences between the higher fat test meal group and the lower fat test meal group were established using a Mann Whitney U test. A P value of < 0.05 was considered statistically significant however no results indicated differences between the groups at any timepoint. Abbreviations: FMD (flow-mediated dilation), HFMC (high flow-mediated dilation), LFMC (low flow-mediated dilation), TTP (time to peak), BL (baseline) SD (standard deviation), Δ (change), min (minimum) and max (maximum).

Table 6.3: Placebo volunteers mean change from baseline for measures of arterial stiffness and blood pressure in those in the 'lower fat' test meal *versus* those in the 'higher fat test meal '

		BASELINE					**1.5hr**					**3HR**				
		N	Mean	SD	Min	Max	N	Mean	SD	BL Δ	P	N	Mean	SD	BL Δ	P
PWV (m/s)	LOW fat	7	10.26	1.09	8.20	11.73						6	10.13	1.33	-0.16	0.13
	HIGH fat	6	9.58	0.63	8.60	10.30						6	9.75	1.05	0.16	
AIx (%)	LOW fat	7	30.12	8.31	22.50	44.33						6	26.08	10.22	-2.62	0.53
	HIGH fat	7	24.44	5.20	16.67	32.50						7	20.79	4.57	-3.65	
SBP (mmHg)	LOW fat	7	144.21	12.69	127.50	162.00	7	130.00	8.50	-14.21	0.17	7	131.21	11.65	-13.00	0.54
	HIGH fat	7	136.79	11.59	119.50	151.50	7	131.21	8.29	-5.57		7	130.36	9.20	-6.43	
DBP (mmHg)	LOW fat	7	85.79	12.56	65.00	104.00	7	76.43	9.27	-9.36	0.46	7	78.86	9.88	-6.93	0.71
	HIGH fat	7	85.07	7.78	75.00	94.00	7	78.07	8.12	-7.00		7	79.86	8.48	-5.21	
HR (BPM)	LOW fat	6	62.33	7.63	52.00	72.50	6	72.00	7.07	9.67	0.07	6	71.42	6.99	9.08	**0.02***
	HIGH fat	7	64.21	7.92	53.00	75.50	7	69.57	7.04	5.36		7	65.07	9.21	0.86	
		6HR					**24HR**					**48HR**				
		N	Mean	SD	BL Δ	P	N	Mean	SD	BL Δ	P	N	Mean	SD	BL Δ	P
PWV (m/s)	LOW fat	5	10.23	1.27	-0.01	1.00	7	9.95	0.98	-0.30	0.63	7	9.57	0.81	-0.69	0.20
	HIGH fat	6	9.65	0.83	0.07		6	9.48	1.12	-0.10		5	9.41	0.48	-0.15	
AIx (%)	LOW fat	7	29.36	8.36	-0.76	0.32	7	31.37	8.95	1.25	0.71	7	31.02	9.02	0.90	0.26
	HIGH fat	7	22.42	5.53	-2.02		7	24.91	4.69	0.47		7	24.86	6.21	0.42	
SBP (mmHg)	LOW fat	7	142.93	8.99	-1.29	0.62	7	133.64	5.28	-10.57	1.00	7	138.43	5.06	-5.79	0.71
	HIGH fat	7	131.29	9.89	-5.50		7	128.29	6.73	-8.50		7	130.29	8.18	-6.50	
DBP (mmHg)	LOW fat	7	89.14	8.96	3.36	0.10	7	82.14	10.17	-3.64	0.81	7	85.07	9.09	-0.71	0.32
	HIGH fat	7	81.71	7.32	-3.36		7	81.50	6.08	-3.57		7	81.71	5.38	-3.36	
HR (BPM)	LOW fat	6	66.83	8.08	4.50	**0.04***	6	63.75	4.33	1.42	0.23	6	65.08	7.14	2.75	**0.02***
	HIGH fat	7	65.07	10.30	0.86		7	62.57	6.77	-1.64		7	62.43	9.07	-1.79	

PWV and AIx were not assessed at 1.5hrs. Mean and BL Δ for PWV is shown in meter per second squared (m/s^2), AIx is shown in percentage (%) HR are shown in beats per minute (BPM). Statistical differences between the higher fat test meal group and the lower fat test meal group were established using a Mann Whitney U test. A P value of < 0.05 was considered statistically significant and indicated with a * above. Abbreviations: PWV (pulse wave velocity), AIx (augmentation index), SBP (systolic blood pressure), DBP (diastolic blood pressure), HR (heart rate), BL (baseline) SD (standard deviation), Δ (change), min (minimum) max (maximum).

Table 6.4: Placebo volunteers mean change from baseline for ambulatory blood pressure of those in the 'lower fat' test meal *versus* those in the 'higher fat test meal '

		BASELINE (DAY 0)					DAY 1					DAY 2				
		N	Mean	SD	Min	Max	N	Mean	SD	BL Δ	P	N	Mean	SD	BL Δ	P
ABPM SBP (mmHg)	LOW fat	7	129.00	8.52	117.00	140.00	7	124.43	8.62	-4.57	0.73	7	124.43	6.80	-4.57	0.45
	HIGH fat	6	131.83	9.28	122.00	143.00	6	127.83	9.09	-4.00		6	125.00	6.42	-6.83	
ABPM DBP (mmHg)	LOW fat	7	80.00	8.37	64.00	89.00	7	76.29	10.13	-3.71	0.30	7	76.57	7.89	-3.43	0.95
	HIGH fat	6	81.17	8.08	70.00	94.00	6	80.17	6.37	-1.00		6	77.83	6.68	-3.33	
ABPM HR (BPM)	LOW fat	7	74.57	7.87	65.00	82.00	7	72.57	4.93	-2.00	0.84	7	73.00	4.93	-1.57	0.73
	HIGH fat	6	73.17	9.11	63.00	88.00	6	71.67	12.40	-1.50		6	71.83	10.30	-1.33	
ABPM PP (mmHg)	LOW fat	7	48.57	7.30	39.00	60.00	7	48.29	7.45	-0.29	0.10	7	48.00	6.51	-0.57	0.18
	HIGH fat	6	50.50	4.72	44.00	58.00	6	47.00	4.86	-3.50		6	47.17	3.54	-3.33	
ABPM MAP (mmHg)	LOW fat	7	96.71	6.40	85.00	105.00	7	92.86	7.40	-3.86	0.63	7	93.14	5.01	-3.57	0.37
	HIGH fat	6	97.33	7.03	88.00	108.00	6	94.50	6.22	-2.83		6	93.00	5.87	-4.33	

Mean and BL Δ for ABPM SBP, ABPM DBP ABPM MAP and ABPM PP are shown in millimetres of mercury (mmHg). Mean BL Δ for ABPM HR is shown in beats per minute (BPM). Statistical differences between the higher fat test meal group and the lower fat test meal group were established using a Mann Whitney U test. A P value of < 0.05 was considered statistically significant however no results indicated differences between the groups at any timepoint. Abbreviations: ABPM SBP (ambulatory blood pressure monitor systolic blood pressure), ABPM DBP (ambulatory blood pressure monitor diastolic blood pressure), ABPM MAP (ambulatory blood pressure monitor mean arterial pressure), ABPM PP (ambulatory blood pressure monitor pulse pressure), ABPM HR (ambulatory blood pressure monitor heart rate), BL (baseline) SD (standard deviation), Δ (change), min (minimum) max (maximum).

Table 6.5: Placebo volunteers mean change from baseline for serum cardiometabolic markers in those in the 'lower fat' test meal *versus* those in the 'higher fat test meal '

		BASELINE					20MIN					40MIN				
	N		Mean	SD	Min	Max	N	Mean	SD	BL Δ	P	N	Mean	SD	BL Δ	P
Glucose (mmol/L)	LOW fat	7	4.50	0.36	4.10	5.00	5	5.78	1.04	1.16	0.66	5	7.00	0.42	2.38	0.05
	HIGH fat	7	4.94	0.21	4.70	5.30	6	6.27	0.84	1.33		7	6.13	1.27	1.19	
Cholesterol (mmol/L)	LOW fat	6	5.42	0.57	4.40	6.10	5	6.42	0.78	0.48	0.54	4	6.30	0.92	0.15	0.65
	HIGH fat	7	5.57	1.11	3.90	7.10	6	6.22	0.89	0.37		7	5.81	1.06	0.24	
HDLC (mmol/L)	LOW fat	7	1.60	0.24	1.23	1.96	5	1.82	0.16	0.11	0.18	5	1.77	0.19	0.06	0.43
	HIGH fat	7	1.21	0.17	1.02	1.53	6	1.24	0.21	0.04		7	1.24	0.22	0.03	
LDLC (mmol/L)	LOW fat	6	3.38	0.53	2.60	4.10	3	3.50	0.30	0.23	0.38	2	3.35	0.49	0.05	1.00
	HIGH fat	7	3.77	1.10	2.10	5.30	6	4.37	0.96	0.32		6	4.20	0.92	0.15	
Triglycerides (mmol/L)	LOW fat	7	1.01	0.19	0.70	1.20	3	1.05	0.21	0.20	0.05	3	1.03	0.30	0.17	0.55
	HIGH fat	7	1.28	0.36	0.66	1.60	6	1.35	0.33	0.04		6	1.44	0.39	0.13	

		60MIN					90MIN					180MIN				
		N	Mean	SD	BL Δ	P	N	Mean	SD	BL Δ	P	N	Mean	SD	BL Δ	P
Glucose (mmol/L)	LOW fat	5	5.58	0.64	1.14	0.53	7	4.77	0.96	0.27	0.81	6	5.28	0.89	0.75	0.59
	HIGH fat	7	5.70	0.83	0.76		7	5.14	0.81	0.20		6	5.32	0.58	0.43	
Cholesterol (mmol/L)	LOW fat	5	5.58	0.64	-0.22	0.64	6	5.60	0.81	-0.02	0.37	6	5.37	0.80	-0.27	1.00
	HIGH fat	7	5.70	0.83	0.13		7	5.41	0.91	-0.16		7	5.33	1.13	-0.27	
HDLC (mmol/L)	LOW fat	5	1.64	0.25	0.03	0.05	7	1.54	0.25	-0.06	0.54	6	1.51	0.28	-0.12	0.49
	HIGH fat	7	1.18	0.20	-0.03		7	1.13	0.19	-0.08		6	1.05	0.18	-0.15	
LDLC (mmol/L)	LOW fat	3	2.97	0.35	0.00	0.38	5	3.18	0.54	-0.06	0.33	4	2.80	0.59	-0.30	0.91
	HIGH fat	6	4.15	0.89	0.10		6	3.88	0.78	-0.17		5	3.82	0.81	-0.34	
Triglycerides (mmol/L)	LOW fat	3	1.17	0.20	0.15	0.71	5	1.10	0.33	0.12	0.66	4	1.46	0.55	0.52	0.91
	HIGH fat	6	1.44	0.39	0.13		6	1.42	0.43	0.11		5	1.81	0.61	0.53	

CONTINUED - Table 6.5: Placebo volunteers mean change from baseline for serum cardiometabolic markers in those in the 'lower fat' test meal *versus* those in the 'higher fat test meal '

		360MIN					24HR					48HR				
		N	Mean	SD	BL Δ	P	N	Mean	SD	BL Δ	P	N	Mean	SD	BL Δ	P
Glucose (mmol/L)	LOW fat	7	4.43	0.16	-0.07	**0.04***	7	4.41	0.25	-0.09	0.71	6	4.15	0.25	-0.27	0.45
	HIGH fat	7	4.44	0.34	-0.50		7	4.83	0.17	-0.11		7	4.77	0.10	-0.17	
Cholesterol (mmol/L)	LOW fat	6	5.47	0.84	-0.15	0.63	6	5.71	0.83	0.08	0.84	5	5.68	0.85	0.06	0.53
	HIGH fat	7	5.49	1.09	-0.09		7	5.74	1.10	0.17		7	5.60	1.13	0.03	
HDLC (mmol/L)	LOW fat	7	1.48	0.27	-0.12	0.46	7	1.55	0.21	-0.04	0.32	6	1.56	0.25	-0.02	0.84
	HIGH fat	7	1.05	0.20	-0.16		7	1.22	0.25	0.01		7	1.20	0.24	0.00	
LDLC (mmol/L)	LOW fat	6	3.15	0.74	-0.23	0.13	6	3.67	0.78	0.08	0.82	5	3.63	0.87	0.04	0.53
	HIGH fat	6	3.55	0.84	-0.50		6	4.12	0.74	0.07		7	3.80	1.02	0.03	
Triglycerides (mmol/L)	LOW fat	6	1.48	0.48	0.49	0.07	7	1.07	0.23	0.06	0.23	6	1.08	0.20	0.02	0.63
	HIGH fat	6	2.52	1.04	1.21		6	1.50	0.44	0.20		7	1.37	0.37	0.09	

Mean and BL Δ for glucose, cholesterol, HDLC, LDLC and triglycerides is shown in millimoles per litre (mmol/L). Statistical differences between the higher fat test meal group and the lower fat test meal group were established using a Mann Whitney U test. A P value of < 0.05 was considered statistically significant and indicated with a * above. Abbreviations LDLC (low density lipoprotein cholesterol), HDLC (high density lipoprotein cholesterol), BL (baseline) SD (standard deviation), Δ (change), min (minimum) max (maximum).

Table 6.1 outlines the baseline descriptive characteristics of all volunteers (*n* =14) who completed the placebo intervention arm and were retrospectively categorised into two groups based on absolute fat content of their personalised test meal. In the 'lower' and 'higher' fat groups, the mean fat contents were 47.27g and 62.61g respectively. Those consuming the lower fat test meal were female (higher fat group had 6 males and 1 female, p = 0.01), had higher body fat (36.46%, compared with 27.71% in the higher fat group, p = 0.03) and a smaller waist circumference (85.86cm, compared with 98.14cm in the higher fat group, p = 0.01).

As shown in table 6.2 there were no between group differences, at any timepoint, for peak FMD, low flow-mediated constriction (LFMC), high flow-mediated constriction (HFMC) or time to peak (TTP). Though no differences were detected, TTP at increased less at each time point in the higher fat group than the lower fat group. Though the mean TTP in the lower fat group was lower at baseline than the higher fat group, the opposite was true at 1.5, 6 and 48 hours (table 6.2).

Table 6.3 shows heart rate (HR) had increased from baseline measurements significantly at 3 hours (p = 0.02), 6 hours (p = 0.04) and 48 hours (p = 0.02) in the lower fat group compared with the higher fat group. Though not significant, systolic BP decreased in both groups at each timepoint and had not returned to baseline by 48 hours (table 6.3). This is similar for diastolic BP with the exception of the 3-hour timepoint (table 6.3). Table 6.4 shows ambulatory blood pressure monitor (ABPM) results which found similar results to resting BP with systolic BP, diastolic BP, HR, pulse pressure (PP) and mean arterial pressure (MAP) all having decreased from baseline levels on day 1 and day 2.

Table 6.5 shows the difference in serum glucose, lipids and cholesterol between the higher and lower fat groups. Glucose had decreased significantly in the higher fat group (p = 0.04) at 360mins (6 hours) compared with the lower fat group. Triglycerides increased from baseline at all timepoints (not significant) and had not returned to fasting levels at 48 hours in both groups (table 6.5).

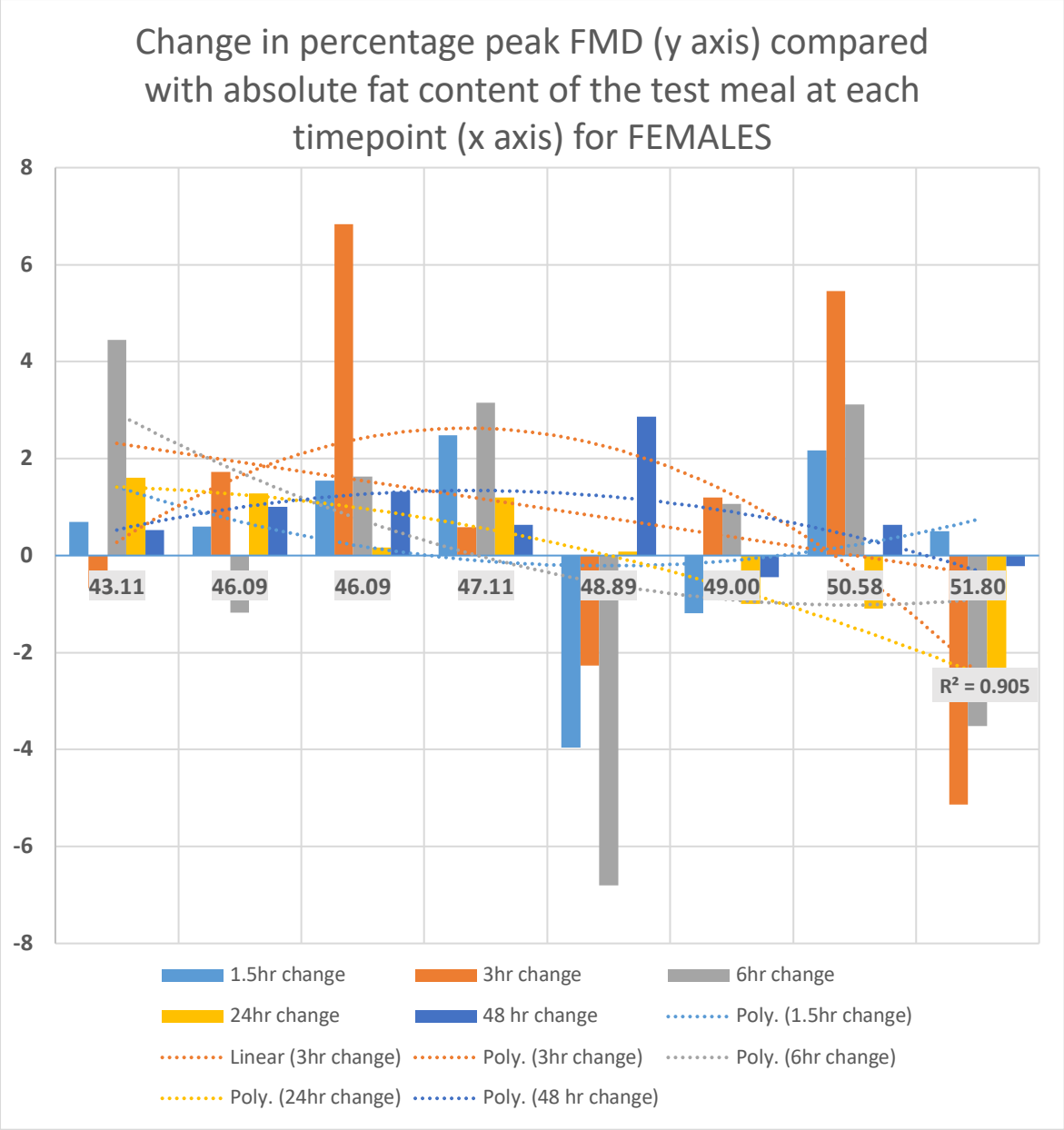


Figure 6.1: Change in percentage peak flow-mediated dilation, for each participant, at each timepoint compared with absolute fat content of test meal for females only. Data includes female participants who completed the placebo intervention week. Y axis displays percentage change in peak FMD from baseline, x axis displays absolute fat content of the test meal, with each cluster representing a different participant. Lines were applied to assess for correlations between absolute fat content and change in percentage peak FMD. At 1.5hr, 3hr, 6hr and 48hr there was a weak correlation with R^2 <0.30, at 24 hours there was a strong negative correlation with R^2 =0.91.

Table 6.1 shows differences between the lower fat and high-fat groups for sex therefore male and female change in percentage FMD was plotted for absolute grams of fat, for each individual at each time point (figure 6.1 and 6.2). Statistical testing was not performed however correlation (R) was computed for best fit curves (which represent a change in peak FMD at specific time points). For females, the change data between baseline and 1.5, 3, 6 and 48 hours all showed a weak correlation on trend lines, with R^2 values <0.30 (figure 6.1).

At 24 hours there was a strong negative correlation between absolute fat and change in percentage peak FMD in females (R^2 =0.91).

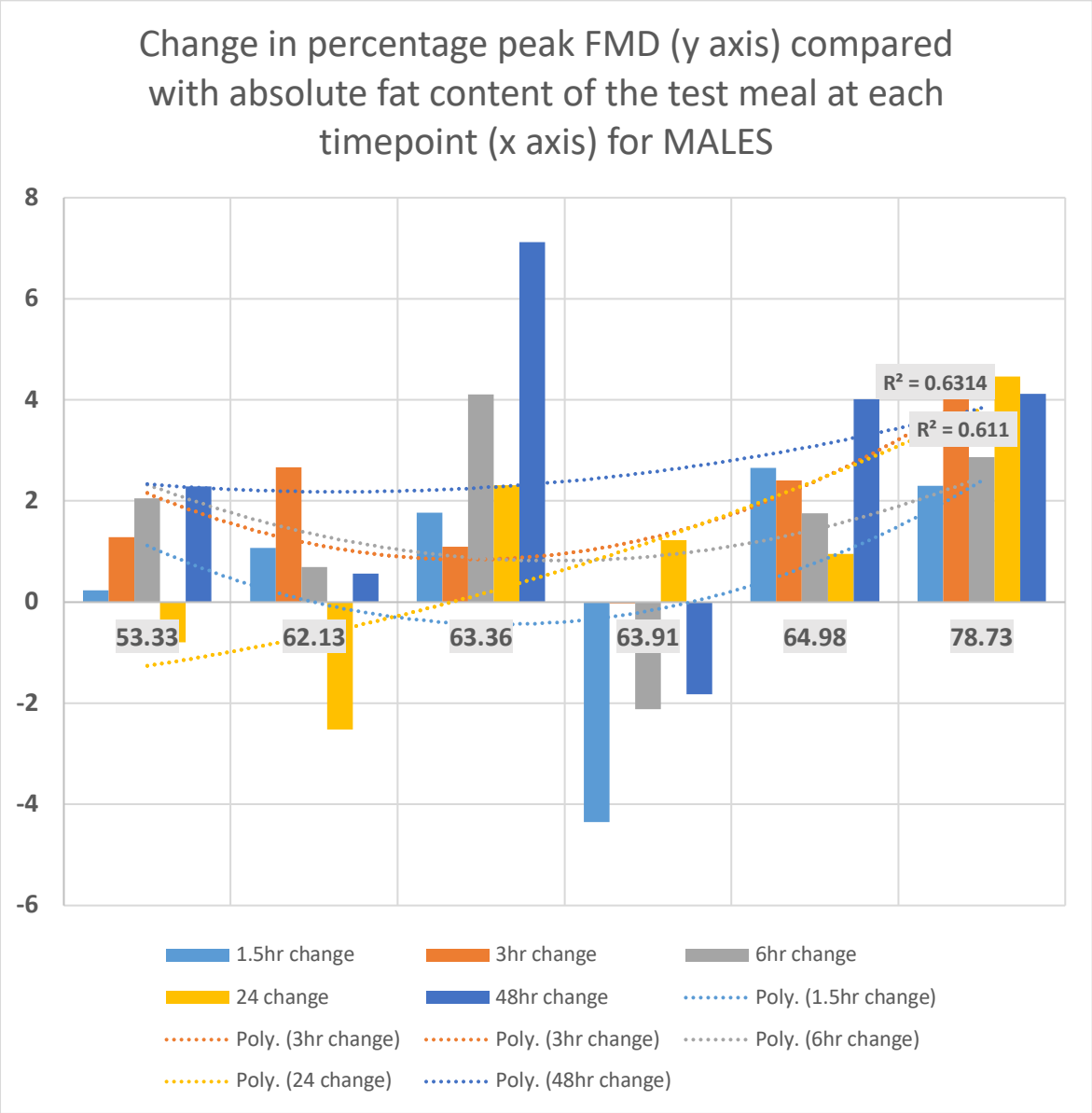


Figure 6.2: Change in percentage peak flow-mediated dilation, for each participant, at each timepoint compared with absolute fat content of test meal for males only. Data includes male participants who completed the placebo intervention week. Y axis displays percentage change in peak FMD from baseline, x axis displays absolute fat content of the test meal, with each cluster representing a different participant. Lines were applied to assess for correlations between absolute fat content and change in percentage peak FMD. At 1.5hr, 6hr and 48hr there was a weak correlation with R^2 <0.30, at 3hr (R^2 =0.61) and 24 hours (R^2 =0.63) there was a moderate positive relationship.

For males the best fit curves which represented change from baseline to 1.5, 6 and 48 hours showed a weak correlation with R^2 values <0.30 (figure 6.2). At 3hr (R^2 =0.61) and 24 hours (R^2 =0.63) there was a moderate positive relationship between absolute fat and change in percentage peak FMD in males.

6.4.2 Exploring the impact of composite health (measured by QRISK3 score) on postprandial cardiometabolic responses: Placebo intervention participants only, retrospectively separated into 'lower' and 'higher' QRISK3 groups

Table 6.6: Characteristics of placebo volunteers who were assessed by a 'higher' or 'lower' mean QRISK3 score

	Lower QRISK3 Score					Higher QRISK3 Score					P Value
	N	Mean	SD	Min	Max	N	Mean	SD	Min	Max	
QRISK3 score (%)	7	5.74	2.78	2.00	8.40	7	10.94	1.53	8.90	13.50	**0.00**
Test meal fat content (g)	7	54.62	12.14	43.11	78.73	7	55.25	8.62	46.09	64.98	0.71
Age (y)	7	56.86	5.34	51.00	65.00	7	61.86	5.55	53.00	67.00	0.10
Sex (male)	7	2.00	/	/	/	7	4.00	/	/	/	0.29
BMI (kg/m^2)	7	29.86	3.26	25.60	34.90	7	27.24	1.23	25.90	29.00	0.10
Statin (YES)	7	1.00	/	/	/	7	0.00	/	/	/	0.30
Metaboliser (HIGH)	7	5.00	/	/	/	7	4.00	/	/	/	0.58
Body fat %	7	33.91	8.50	21.40	46.60	7	30.26	8.10	20.30	40.60	0.54
Trunk fat %	7	31.79	8.83	22.90	46.60	7	29.89	6.67	20.30	39.10	1.00
Hip (cm)	7	108.56	4.89	102.00	114.65	7	105.08	3.67	100.85	110.30	0.17
Waist (cm)	7	92.69	11.50	79.75	112.15	7	91.31	8.23	74.00	99.45	1.00
Peak FMD %	7	6.43	4.60	0.22	11.73	7	2.70	2.34	-0.33	6.21	0.13
HFMC %	7	-0.76	1.41	-2.56	1.09	7	-1.89	1.80	-5.02	0.43	0.26
LFMC %	7	-0.16	1.38	-2.19	1.90	7	-1.12	1.66	-3.50	1.68	0.26
TTP (seconds)	6	41.00	12.59	25.00	56.00	7	46.71	18.11	23.00	78.00	0.53
PWV (m/s)	7	9.88	1.19	8.20	11.73	6	10.03	0.65	9.07	11.10	0.95
AIx (%)	7	24.31	4.20	16.67	30.33	7	30.25	8.76	19.25	44.33	0.17
SBP (mmHg)	7	138.57	12.52	119.50	157.00	7	142.43	12.73	124.00	162.00	0.62
DBP (mmHg)	7	84.14	6.42	76.50	94.00	7	86.71	13.17	65.00	104.00	0.54
HR (BPM)	6	64.17	9.18	52.00	75.50	7	62.64	6.45	53.00	72.50	0.95
ABPM SBP (mmHg)	7	128.71	7.57	122.00	142.00	6	132.17	10.11	117.00	143.00	0.53
ABPM DBP (mmHg)	7	80.43	7.76	70.00	94.00	6	80.67	8.82	64.00	89.00	0.53
ABPM MAP (mmHg)	7	96.29	6.16	88.00	108.00	6	97.83	7.19	85.00	105.00	0.53
ABPM PP (mmHg)	7	48.00	6.76	39.00	60.00	6	51.17	5.23	44.00	58.00	0.30
ABPM HR (BPM)	7	74.00	8.27	65.00	88.00	6	73.83	8.75	63.00	82.00	0.84
Glucose (mmol/L)	7	4.67	0.45	4.10	5.30	7	4.77	0.28	4.20	5.10	0.81
Cholesterol (mmol/L)	7	5.31	0.94	3.90	6.80	6	5.72	0.81	4.80	7.10	0.63
LDLC (mmol/L)	7	3.36	0.90	2.10	4.90	6	3.87	0.84	2.90	5.30	0.23
HDLC (mmol/L)	7	1.43	0.30	1.16	1.96	7	1.37	0.29	1.02	1.72	0.62
Triglyceride (mmol/L)	7	1.14	0.30	0.70	1.54	7	1.15	0.34	0.66	1.60	0.81

Statistical differences between those with a higher QRISK3 score and those with a lower QRISK3 score was established using a Mann Whitney U test, for all nominal data a Chi square test was performed. A P value of < 0.05 was considered statistically significant. Abbreviations: SD (standard deviation), min (minimum), max (maximum), BMI (body mass index), FMD (flow-mediated dilation), HFMC (high flow-mediated dilation), LFMC (low flow-mediated dilation), TTP (time to peak), PWV (pulse wave velocity), AIx (augmentation index), SBP (systolic blood pressure), DBP (diastolic blood pressure), HR (heart rate), ABPM SBP (ambulatory blood pressure monitor systolic blood pressure), ABPM DBP (ambulatory blood pressure monitor diastolic blood pressure), ABPM MAP (ambulatory blood pressure monitor mean arterial pressure), ABPM PP (ambulatory blood pressure monitor pulse pressure), ABPM HR (ambulatory blood pressure monitor heart rate), LDLC (low density lipoprotein cholesterol) and HDLC (high density lipoprotein cholesterol).

Table 6.7: Placebo volunteers mean change from baseline for markers of endothelial function in those in with a 'higher' or 'lower' mean QRISK3 score

		BASELINE					1.5HR					3HR				
		N	Mean	SD	Min	Max	N	Mean	SD	BL Δ	P Value	N	Mean	SD	BL Δ	P Value
Peak FMD %	LOW QRISK3	7	6.43	4.60	0.22	11.73	7	7.18	3.39	0.75	0.54	7	7.17	4.06	0.74	0.54
	HIGH QRISK3	7	2.70	2.34	-0.33	6.21	7	2.88	0.77	0.18		7	4.77	2.28	2.08	
LFMC %	LOW QRISK3	7	-0.16	1.38	-2.19	1.90	7	0.73	1.27	0.88	0.54	7	0.17	1.26	0.33	0.81
	HIGH QRISK3	7	-1.12	1.66	-3.50	1.68	7	0.74	1.28	1.85		7	0.18	1.76	1.30	
HFMC %	LOW QRISK3	7	-0.76	1.41	-2.56	1.09	7	0.37	0.91	1.14	0.62	7	-0.40	1.02	0.37	0.32
	HIGH QRISK3	7	-1.89	1.80	-5.02	0.43	7	-0.14	0.98	1.75		7	-0.14	2.14	1.75	
TTP (seconds)	LOW QRISK3	6	41.00	12.59	25.00	56.00	6	43.50	8.41	2.50	0.18	6	45.00	9.27	4.00	0.73
	HIGH QRISK3	7	46.71	18.11	23.00	78.00	7	72.00	47.54	25.29		7	52.57	11.56	5.86	

		6HR					24HR					48HR				
		N	Mean	SD	BL Δ	P Value	N	Mean	SD	BL Δ	P Value	N	Mean	SD	BL Δ	P Value
Peak FMD %	LOW QRISK3	7	7.00	3.09	0.57	0.81	7	6.58	3.26	0.15	0.54	7	7.73	4.41	1.30	0.71
	HIGH QRISK3	7	3.74	1.72	1.05		7	3.29	2.22	0.59		7	4.62	1.66	1.93	
LFMC %	LOW QRISK3	7	-0.05	1.68	0.11	0.32	7	0.69	1.65	0.84	1.00	7	0.83	1.59	0.99	0.62
	HIGH QRISK3	7	0.03	1.53	1.15		7	-0.07	0.53	1.05		7	0.20	0.54	1.31	
HFMC %	LOW QRISK3	7	-0.77	1.69	0.00	0.46	7	-0.27	1.46	0.49	0.90	7	-0.22	1.09	0.54	0.32
	HIGH QRISK3	7	-0.93	1.46	0.96		7	-1.05	0.65	0.84		7	-0.53	0.54	1.36	
TTP (seconds)	LOW QRISK3	6	39.83	5.74	-1.17	0.84	6	45.00	10.20	4.00	1.00	6	42.50	8.98	1.50	0.95
	HIGH QRISK3	7	41.71	7.50	-5.00		7	54.29	14.56	7.57		7	48.71	14.35	2.00	

Mean BL Δ for TTP is shown in seconds. Statistical differences between those with a higher QRISK3 score and those with a lower QRISK3 score was established using a Mann Whitney U test. A P value of < 0.05 was considered statistically significant however no results indicated differences between the groups at any timepoint. Abbreviations: FMD (flow-mediated dilation), HFMC (high flow-mediated dilation), LFMC (low flow-mediated dilation), TTP (time to peak), BL (baseline) SD (standard deviation), Δ (change), min (minimum) max (maximum). Mean and BL Δ for FMD, LFMC and HFMC are shown in percentage.

Table 6.8: Placebo volunteers mean change from baseline for measures of blood pressure and arterial stiffness in those in with a 'higher' or 'lower' mean QRISK3 score

		BASELINE					1.5hr					3HR				
		N	Mean	SD	Min	Max	N	Mean	SD	BL Δ	P	N	Mean	SD	BL Δ	P
PWV (m/s)	LOW QRISK3	7	9.88	1.19	8.20	11.73						7	10.04	1.23	0.16	0.34
	HIGH QRISK3	6	10.03	0.65	9.07	11.10						5	9.79	1.17	-0.23	
AIx (%)	LOW QRISK3	7	24.31	4.20	16.67	30.33						7	21.41	5.63	-2.90	0.53
	HIGH QRISK3	7	30.25	8.76	19.25	44.33						6	25.35	9.97	-3.50	
SBP (mmHg)	LOW QRISK3	7	138.57	12.52	119.50	157.00	7	132.57	8.46	-6.00	0.32	7	132.64	9.00	-5.93	0.17
	HIGH QRISK3	7	142.43	12.73	124.00	162.00	7	128.64	7.84	-13.79		7	128.93	11.48	-13.50	
DBP (mmHg)	LOW QRISK3	7	84.14	6.42	76.50	94.00	7	79.43	5.62	-4.71	0.01*	7	81.71	5.95	-2.43	0.02*
	HIGH QRISK3	7	86.71	13.17	65.00	104.00	7	75.07	10.52	-11.64		7	77.00	11.03	-9.71	
HR (BPM)	LOW QRISK3	6	64.17	9.18	52.00	75.50	6	71.58	6.25	7.42	0.95	6	69.92	5.46	5.75	0.37
	HIGH QRISK3	7	62.64	6.45	53.00	72.50	7	69.93	7.77	7.29		7	66.36	10.75	3.71	
		6HR					**24HR**					**48HR**				
		N	Mean	SD	BL Δ	P	N	Mean	SD	BL Δ	P	N	Mean	SD	BL Δ	P
PWV (m/s)	LOW QRISK3	5	9.95	1.29	0.25	0.18	7	9.65	0.98	-0.23	0.73	6	9.43	0.94	-0.48	0.49
	HIGH QRISK3	6	9.88	0.91	-0.14		6	9.84	1.18	-0.19		6	9.57	0.31	-0.46	
AIx (%)	LOW QRISK3	7	22.69	5.89	-1.62	0.90	7	25.84	5.30	1.53	0.38	7	24.79	5.15	0.48	0.71
	HIGH QRISK3	7	29.08	8.37	-1.17		7	30.44	9.27	0.19		7	31.10	9.61	0.85	
SBP (mmHg)	LOW QRISK3	7	138.07	11.37	-0.50	0.46	7	128.64	5.36	-9.93	1.00	7	132.71	8.34	-5.86	0.71
	HIGH QRISK3	7	136.14	11.24	-6.29		7	133.29	6.97	-9.14		7	136.00	7.44	-6.43	
DBP (mmHg)	LOW QRISK3	7	86.86	5.79	2.71	0.05	7	81.07	6.53	-3.07	0.62	7	83.21	5.62	-0.93	0.38
	HIGH QRISK3	7	84.00	11.30	-2.71		7	82.57	9.83	-4.14		7	83.57	9.31	-3.14	
HR (BPM)	LOW QRISK3	6	67.92	9.23	3.75	0.53	6	64.50	6.03	0.33	0.84	6	65.42	7.26	1.25	0.73
	HIGH QRISK3	7	64.14	9.14	1.50		7	61.93	5.33	-0.71		7	62.14	8.88	-0.50	

Mean and BL Δ for PWV is shown in meter per second squared (m/s^2), AIx is shown in percentage (%) HR are shown in beats per minute (BPM). Statistical differences between those with a higher QRISK3 score and those with a lower QRISK3 score was established using a Mann Whitney U test. A P value of < 0.05 was considered statistically significant and indicated with a * above. Abbreviations: PWV (pulse wave velocity), AIx (augmentation index), SBP (systolic blood pressure), DBP (diastolic blood pressure), HR (heart rate), BL (baseline) SD (standard deviation), Δ (change), min (minimum) max (maximum). Mean and BL Δ for SBP and DBP are shown in millimetres of mercury (mmHg).

Table 6.9: Placebo volunteers mean change from baseline for ambulatory blood pressure measurements in those in with a 'higher' or 'lower' mean QRISK3 score

		BASELINE (DAY 0)					DAY 1					DAY 2				
		N	Mean	SD	Min	Max	N	Mean	SD	BL Δ	P	N	Mean	SD	BL Δ	P
ABPM SBP (mmHg)	LOW QRISK3	7	128.71	7.57	122.00	142.00	7	125.86	5.96	-2.86	0.23	7	125.71	6.26	-3.00	0.04*
	HIGH QRISK3	6	132.17	10.11	117.00	143.00	6	126.17	11.69	-6.00		6	123.50	6.83	-8.67	
ABPM DBP (mmHg)	LOW QRISK3	7	80.43	7.76	70.00	94.00	7	79.00	5.80	-1.43	0.30	7	79.00	5.94	-1.43	0.01*
	HIGH QRISK3	6	80.67	8.82	64.00	89.00	6	77.00	11.42	-3.67		6	75.00	8.22	-5.67	
ABPM HR (BPM)	LOW QRISK3	7	74.00	8.27	65.00	88.00	7	74.43	9.25	0.43	0.01*	7	74.29	7.78	0.29	0.01*
	HIGH QRISK3	6	73.83	8.75	63.00	82.00	6	69.50	8.07	-4.33		6	70.33	7.31	-3.50	
ABPM PP (mmHg)	LOW QRISK3	7	48.00	6.76	39.00	60.00	7	46.86	7.40	-1.14	0.37	7	46.71	5.94	-1.29	0.73
	HIGH QRISK3	6	51.17	5.23	44.00	58.00	6	48.67	4.84	-2.50		6	48.67	4.37	-2.50	
ABPM MAP (mmHg)	LOW QRISK3	7	96.29	6.16	88.00	108.00	7	94.29	4.99	-2.00	0.07	7	94.43	5.13	-1.86	0.01
	HIGH QRISK3	6	97.83	7.19	85.00	105.00	6	92.83	8.66	-5.00		6	91.50	5.24	-6.33	

Mean and BL Δ for ABPM SBP, ABPM DBP ABPM MAP and ABPM PP are shown in millimetres of mercury (mmHg). Mean BL Δ for ABPM HR is shown in beats per minute (BPM). Statistical differences between those with a higher QRISK3 score and those with a lower QRISK3 score was established using a Mann Whitney U test. A P value of < 0.05 was considered statistically significant and indicated with a * above. Abbreviations: ABPM SBP (ambulatory blood pressure monitor systolic blood pressure), ABPM DBP (ambulatory blood pressure monitor diastolic blood pressure), ABPM MAP (ambulatory blood pressure monitor mean arterial pressure), ABPM PP (ambulatory blood pressure monitor pulse pressure), ABPM HR (ambulatory blood pressure monitor heart rate), BL (baseline) SD (standard deviation), Δ (change), min (minimum) max (maximum).

Table 6.10: Placebo volunteers mean change from baseline for serum cardiometabolic markers in those in with a 'higher' or 'lower' mean QRISK3 score

		BASELINE					20MIN					40MIN				
		N	Mean	SD	Min	Max	N	Mean	SD	BL Δ	P	N	Mean	SD	BL Δ	P
Glucose (mmol/L)	LOW QRISK3	7	4.67	0.45	4.10	5.30	4	6.78	0.78	1.95	0.02*	5	6.56	1.05	1.70	0.76
	HIGH QRISK3	7	4.77	0.28	4.20	5.10	7	5.63	0.74	0.86		7	6.44	1.17	1.67	
Cholesterol (mmol/L)	LOW QRISK3	7	5.31	0.94	3.90	6.80	4	6.08	0.74	0.28	0.05	5	5.58	0.90	0.13	0.33
	HIGH QRISK3	6	5.72	0.81	4.80	7.10	7	6.44	0.86	0.53		6	6.33	1.01	0.30	
HDLC (mmol/L)	LOW QRISK3	7	1.43	0.30	1.16	1.96	4	1.61	0.43	0.08	0.79	5	1.50	0.40	0.02	0.43
	HIGH QRISK3	7	1.37	0.29	1.02	1.72	7	1.44	0.32	0.07		7	1.44	0.33	0.07	
LDLC (mmol/L)	LOW QRISK3	7	3.36	0.90	2.10	4.90	4	3.90	0.90	0.15	0.06	4	3.78	0.87	0.03	0.20
	HIGH QRISK3	6	3.87	0.84	2.90	5.30	5	4.22	0.95	0.40		4	4.20	0.99	0.23	
Triglycerides (mmol/L)	LOW QRISK3	7	1.14	0.30	0.70	1.54	4	1.23	0.35	0.11	0.91	4	1.30	0.53	0.18	0.56
	HIGH QRISK3	7	1.15	0.34	0.66	1.60	5	1.26	0.33	0.08		5	1.30	0.33	0.12	

		60MIN					90MIN					180MIN				
		N	Mean	SD	BL Δ	P	N	Mean	SD	BL Δ	P	N	Mean	SD	BL Δ	P
Glucose (mmol/L)	LOW QRISK3	5	5.72	0.65	1.04	0.76	7	4.99	1.04	0.31	0.71	5	5.18	0.85	0.56	0.76
	HIGH QRISK3	7	5.60	0.83	0.83		7	4.93	0.77	0.16		7	5.39	0.67	0.61	
Cholesterol (mmol/L)	LOW QRISK3	5	5.72	0.65	0.18	0.70	7	5.26	0.80	-0.06	0.45	5	4.98	1.13	-0.27	0.73
	HIGH QRISK3	7	5.60	0.83	-0.22		7	5.76	0.85	-0.13		7	5.61	0.74	-0.27	
HDLC (mmol/L)	LOW QRISK3	5	1.39	0.35	0.01	0.43	7	1.38	0.33	-0.05	0.26	5	1.37	0.40	-0.10	0.15
	HIGH QRISK3	7	1.36	0.32	-0.01		7	1.28	0.28	-0.09		7	1.21	0.28	-0.16	
LDLC (mmol/L)	LOW QRISK3	4	3.48	1.00	-0.05	0.02*	6	3.43	0.71	-0.13	0.79	4	3.15	1.01	-0.38	0.56
	HIGH QRISK3	5	3.98	0.92	0.16		5	3.72	0.84	-0.10		5	3.54	0.80	-0.28	
Triglycerides (mmol/L)	LOW QRISK3	4	1.47	0.39	0.22	0.11	6	1.33	0.46	0.18	0.13	4	1.59	0.65	0.53	0.73
	HIGH QRISK3	5	1.25	0.32	0.06		5	1.21	0.37	0.03		5	1.70	0.58	0.52	

CONTINUED - Table 6.10: Placebo volunteers mean change from baseline for serum cardiometabolic markers in those in with a 'higher' or 'lower' mean QRISK3 score

		360MIN					24HR					48HR				
		N	Mean	SD	BL Δ	P	N	Mean	SD	BL Δ	P	N	Mean	SD	BL Δ	P
Glucose (mmol/L)	LOW QRISK3	7	4.41	0.27	-0.26	0.46	7	4.56	0.31	-0.11	1.00	6	4.37	0.37	-0.25	0.63
	HIGH QRISK3	7	4.46	0.26	-0.31		7	4.69	0.30	-0.09		7	4.59	0.36	-0.19	
Cholesterol (mmol/L)	LOW QRISK3	7	5.17	0.94	-0.14	0.84	7	5.40	0.86	0.09	0.95	6	5.27	1.00	0.00	0.59
	HIGH QRISK3	7	5.79	0.88	-0.08		7	6.06	0.96	0.18		7	5.96	0.90	0.08	
HDLC (mmol/L)	LOW QRISK3	7	1.29	0.34	-0.14	0.81	7	1.40	0.28	-0.03	0.81	6	1.37	0.30	-0.01	0.95
	HIGH QRISK3	7	1.24	0.31	-0.13		7	1.37	0.30	0.00		7	1.37	0.32	0.00	
LDLC (mmol/L)	LOW QRISK3	6	3.20	0.82	-0.37	1.00	6	3.67	0.65	0.10	0.59	6	3.33	1.02	0.00	1.00
	HIGH QRISK3	6	3.50	0.79	-0.37		7	4.06	0.86	0.05		7	4.06	0.73	0.07	
Triglycerides (mmol/L)	LOW QRISK3	6	1.94	0.83	0.80	1.00	6	1.21	0.36	0.07	0.53	6	1.28	0.31	0.06	0.95
	HIGH QRISK3	6	2.06	1.12	0.90		7	1.32	0.44	0.17		7	1.19	0.36	0.04	

Mean and BL Δ for ABPM SBP and ABPM DBP are shown in millimetres of mercury (mmHg). Mean and BL Δ for glucose cholesterol, HDLC, LDLC and triglycerides is shown in millimoles per litre (mmol/L). Statistical differences between those with a higher QRISK3 score and those with a lower QRISK3 score was established using a Mann Whitney U test. A P value of < 0.05 was considered statistically significant and indicated with a * above. Abbreviations LDLC (low density lipoprotein cholesterol), HDLC (high density lipoprotein cholesterol), BL (baseline) SD (standard deviation), Δ (change), min (minimum) max (maximum).

The mean QRISK3 score (percent risk of CVD event within the next 10 years) was 5.74% for the lower QRISK3 group and 10.94% for the higher QRISK3 group (table 6.6). No other baseline descriptive characteristics were significantly different between either group (table 6.6). Regarding the primary assessment in this thesis, the group with the lower QRISK3 score had a higher average peak FMD (6.43%, SD = 4.60) compared with the higher QRISK3 group (2.70%, SD = 2.34) (table 6.6). This was, however, not significant and no postprandial differences in endothelial function were detected (table 6.7).

As shown in table 6.8, diastolic BP had decreased significantly more in the higher QRISK3 group at 1.5 hours (baseline change = -11.64mmHg, p = 0.01) and (baseline change = -9.71mmHg, p = 0.02) compared with the lower QRISK3 group. No other results were statistically significant though it should be noted at each timepoint systolic BP and diastolic BP had decreased from their baseline measurements in both groups (table 6.8). HR, as measured by ABPM, had significantly decreased from baseline on day 1 (p = 0.01) and day 2 (p = 0.01) in the higher QRISK3 group compared with the lower QRISK3 group (table 6.9). Systolic BP and diastolic BP had decreased from baseline for both groups on day 2 (p = 0.04 for systolic BP, p = 0.01 for diastolic BP), but had decreased more in the higher QRISK3 group (table 6.9). At 48 hours LDLC had increased from baseline in the higher QRISK3 group, compared with the lower QRISK3 group (table 6.10).

6.5 Discussion

This chapter aimed to assess the importance of two methodological considerations on assessments of vascular function and intervention effectiveness; namely: 1) the variation in absolute fat content in the test meal personalised for BMR and physical activity, and 2) the holistic health of a participant assessed by the composite 10-year CVD risk score, (QRISK3). Our results showed that those in the lower fat content test meal tended to be female ($p = 0.01$), with a higher body fat percentage ($p = 0.03$) and waist circumference ($p = 0.01$) (table 6.1). To account for sex differences in absolute fat content, we plotted percentage peak FMD change against fat content individually for females (figure 6.1) and males (figure 6.2). A negative correlation was observed with increasing absolute fat content resulting in a negative change in percentage peak FMD for females at 24 hours ($R^2 = 0.91$) (figure 6.1). The exploration of the impact of the QRISK3 score suggested a potential influence on changes in systolic BP and diastolic BP in participants taking the placebo after an energy-dense meal (tables 6.8 and 6.9).

Previously, postprandial anthocyanin studies have not accounted for potential variation of cardiometabolic effects after a standardised meal. Addressing this limitation in the protocol design has resulted in varying absolute levels of fat intake (in g / test meal) among study participants. Considering the evidence, which has shown fat intake (especially saturated fat) impairs endothelial function when measured by FMD (when fat is given as a standardised dose to study populations) [36], [130], [223], this chapter intended to evaluate whether body-size adjusted fat intakes had a similar effect to standardised fat intake. To do this, cardiometabolic response following the placebo treatment week was assessed. The findings suggested that greater fat intakes *per se* was associated with a lower heart rate at 3 hours ($p= 0.02$), 6 hours ($p = 0.04$) and 48 hours ($p = 0.02$) (table 6.3). The percentage peak FMD also increased in both groups, at most timepoints but these findings did not reach statistical significance. This differs from previous findings suggesting high-fat meals (with fat content standardised and ranging 41-65.2g per meal) impair endothelial function [36], [130], [223]. It is noteworthy that in our analysis there was a strong skewing of the participant population, towards lower fat intakes in females – with all 7 of the lowest fat consumers being women. The reason for this is due to the use of the Henry equation [218], used to

calculate BMR, which has different equations for the sexes. They provide a higher BMR to a male even if weight and age is the same as a female due to sex differences in body composition and metabolism. When determining a volunteer's physical activity factor, for similar occupational and non-occupational activity males would be given a number due to differences in body composition (see Appendix B.1) [172], [224].

To account for the potential impact of sex differences in the analysis of fat (g) intake and vascular function, a sub-group assessment was performed. Change in percentage peak FMD was compared against absolute fat for each volunteer, at each timepoint separately for males and females (figure 6.1 and 6.2). Percentage peak FMD was chosen as it was the primary endpoint of this study (see chapter 3). From these data, there was between person variation for females. However, at 24 hours, increasing absolute fat strongly correlated with a reduction in percentage change FMD (R^2 =0.91) (figure 6.1). For the males, the correlations were weaker overall (R^2 <0.70). However, at 3 hours (R^2 =0.61) and 24 hours (R^2 =0.63) there was a moderate positive relationship between absolute fat and change in percentage peak FMD (figure 6.2).

There are several considerations when interpreting the trends in these data. Firstly, FMD scores with differences of >1% (for peak FMD, LFMC or HFMC) or >10 seconds (for TTP) between reviewer values were not validated with a dual-researcher analysis to identify the source of disparity (which was initially intended). Table 3.4, in Chapter 3 shows the large percentage change from the values used for this thesis and values decided after a joint review. Two independent sonographers carrying out analysis is recommended [100] and being done in published nutrition interventions [65]. Secondly when we consider studies where subjects did not experience endothelial dysfunction (measured by FMD) after a high-fat meal, it has been previously shown that habitually active individuals can attenuate the consequences of a high-fat meal on endothelial function when compared with those who are less active [225]. However, the physical activity factor for men (mean = 1.57, minimum 1.5 and maximum 1.7) and women (mean = 1.55, minimum 1.4 and maximum 1.6) remained almost identical indicating light-moderate daily activity (see Appendix B1).

Lastly, it is important to consider that despite there being no differences in BMI between the sexes, there were notable differences in body composition. Females in the 'lower fat intake (g)' group had a higher body fat and trunk fat percentage than the 'higher fat intake (g)' group, which was predominantly comprised of males (table 6.1). These differences may be clinically important, as increased trunk fat, even within the 'healthy' BMI range, is associated with an elevated CVD risk in men and post-menopausal women and this may have impacted how the females handled the energy-dense meal [226], [227]. Increased energy intake and low energy expenditure can lead to excessive energy being stored as triglycerides in subcutaneous abdominal tissue, which in turn can lead to dysregulation and insulin resistance [228]. As the women in our study are likely to be post-menopausal this can also lead to the dysregulation of adipocyte lipolysis due to decreasing oestrogen levels [228]. Although the two groups differed in waist circumference in absolute terms (in cm), all the participants had waist circumferences above the sex-adjusted cut-offs for increased CVD risk (i.e. >80cm women, >94cm men) [229]. Across both sexes, excess visceral fat (implicated by larger waist circumference measurements) can lead to differences in lipid metabolism, affect the body's ability to cope with excessive caloric or fat consumption and put the individual at risk of metabolic syndrome [227]. Interestingly, despite being provided with variable amounts of dietary fat in the test meal (lower *versus* higher fat contents), we did not observe any consistent differences in LDLC or triglycerides concentrations during the postprandial phase between the groups (table 6.5).

In this analysis of the placebo arm participants, heart rate (HR) increased from baseline at all timepoints in the all-female, lower fat group (table 6.3). The higher fat group, predominantly male (*n* = 1 female), increased from baseline HR levels at 1.5 hours, but by 3 hours they had almost returned to baseline levels (table 6.3). A cross-over feeding study (with a fixed macronutrient test meal of 27.5g fat) previously reported that postprandial HR was significantly higher in females than males [230]. Though the heart rate changes in our study are not clinically significant (low fat group mean HR = <72bpm and mean change <10bpm across all timepoints), it suggests that there may be sex differences in the physiological responses, even with individualised meals. These sex specific differences should be considered when designing and interpreting future studies.

A criticism when utilising predictions of energy requirements, based on body size and physical activity level estimations, is that participants with obesity (BMI >30kg/m^2) may have their energy requirements over-estimated when incorporating estimations of activity factors. The British Dietetic Association Parenteral and Enteral Nutrition Group therefore recommend not to include a physical activity multiplying factor when estimating obese patients nutritional requirements [172]. We considered this recommendation on a participant-by-participant basis. In participants with a BMI in the borderline obese range (for example, a BMI of 30kg/m^2), their 3-day phase 1 food diary was analysed to estimate their average energy intake by a registered dietitian (CF). This aimed to gauge whether their intake was closer to their BMR or their BMR plus activity factor, their estimated energy requirements for the test meal were then chosen. This participant-centred approach of personalising each individual's test meal, accounted for the varying phenotypes within our study. A cross-over study, that is adequately powered and recruited (so that adjustments for sex and body fat could be made) would be the best design to apply this approach in future studies.

Our second research design question was whether background holistic health, measured by QRISK (even within a relatively narrow band of variation), mediated the extent to which participants experience postprandial health responses. QRISK3 indicates the probability of having a cardiovascular event within the next 10 years and NICE regards individuals with a QIRSK score above 10% as 'high risk' for developing CVD [231]. The QRISK score encompasses composite measures of health to assess absolute CVD risk, given CVD is multifactorial it may give a better overview than singular markers, such as those used in the inclusion criteria. We hypothesised that those with elevated global CVD risk would not perform as well in the acute cardiometabolic outcome assessments. The exploration of the QRISK3 score in our placebo arm participants (*n* = 14) characterises our study population and gives an insight into the extent to which QRISK3 score is predictive of postprandial cardiometabolic responses. Notably the QRISK score for our population ranged from 2.00% to 13.5% (table 6.6), and the classification of groups (low *versus* high QRISK score) reflects numerical rather than clinical values. However, it still indicates a potential varying degree of holistic / composite cardiovascular health in our study population, despite no significant differences in singular characteristic data being detected (table 6.6).

We observed that mean percentage peak FMD was higher in the low QRISK3 group (mean FMD = 6.43%), indicating greater endothelial function, and this was supported by less pronounced LFMC and HFMC (mean LFMC = -0.16% and HFMC = -0.76%) compared with the group with higher QRISK3 score (mean FMD = 2.70%, LFMC = -1.12% and HFMC = -1.89%) (table 6.6). These findings are consistent with the existing literature, which has shown that LFMC is positively associated with poorer endothelial function [134], [135]. Although evidence comparing peak FMD and QRISK scores is lacking, a meta-analysis has suggested that a 1% improvement in endothelial function, as measured by FMD, may reduce CVD risk by 13% [76].

The QRISK3 score suggested a potential impact of an energy-dense meal on changes in systolic BP and diastolic BP in the placebo arm participants (tables 6.8 and 6.9). In terms of BP, assessed through resting BP and ABPM measurements, both systolic BP and diastolic BP reduced after the postprandial challenge in the group with higher QRISK3 scores. Whilst the decrease from baseline levels (shown in tables 6.8 and 6.9) did not meet the definition of postprandial hypotension (i.e. a decrease of 20mmHg systolic BP), it was notable that the reduction in systolic BP of 14mmHg at 1.5 hours, observed in the higher QRISK3 score group, was more pronounced than the lower score QRISK3 score group (mean change in systolic BP = -6.00mmHg). It has been shown previously, that postprandial hypotension tends to be diagnosed in older aged adults (>65 years) and is associated with increased hospital admissions and neurodegenerative conditions [232], [233]. It usually occurs within 2 hours after a meal and should be considered in future feeding studies with older adults. We speculate that worsening QRISK3 score may also increase the likelihood of developing postprandial hypotension and its associated health consequences.

Limitations of this analysis include the imbalance of sex in the exploration of the impact of the energy-dense meals, with females tending to receive less fat and calories that their male counterparts which means definitive conclusions cannot be made. If more study volunteers were recruited, with adequate statistical power, we may have been able to examine the postprandial response by sex. This is particularly important for comparisons of absolute fat content of the meal and peak FMD, in both men and women (figure 6.1 and 6.2). Other

limitations include the small sample size used in exploring the fat content of the test meal and the QRISK score. Lastly, there was a relatively low diversity of QRISK3 scores (dictated by our stringent inclusion criteria) making it more difficult to detect any differences in cardiometabolic response.

Many nutrition studies provide a test meal with a fixed amount of macronutrients which ignores the differences in metabolism based on sex, age and body fat composition. Future studies should investigate the effects of individualised test meals *versus* fixed test meals on cardiometabolic outcomes. This is of particular importance in mixed sex studies where women may have altered lipid clearance due to hormones (particularly if pre-menopausal), body fat and insulin resistance though the clear pathophysiological difference in lipid metabolism between the sexes require further research [234]. The exploratory QRISK analysis observed an association between QRISK3 score and some postprandial cardiometabolic assessments (BP) and some fasting assessments (FMD). This highlights the importance of adjusting for covariates in human research which Chapters 4 and 5 of this thesis were not able to do due to small study numbers.

CHAPTER 7: General discussion and future directions

7.1 Thesis overview

7.1.1 Summary

The overarching goal of this thesis was to examine whether a novel blueberry anthocyanin metabolism profile was associated with a favourable cardiometabolic response after an energy-dense meal challenge. The metabolism profile (or 'metabotype') was created following the results of a previous 6-month chronic blueberry intervention which indicated that improvements in endothelial function coincided with elevated levels of a combination of specific urinary metabolites. In this study, the same metabotype profile of specific urinary metabolites was identified in 48-hour urine collections following the intake of a 'blueberry challenge'. The relative concentrations of blueberry metabolites (over the 48-hour excretion period) were ranked, and those participants in the extremes of the anthocyanin metabolism distribution (i.e. lowest, and highest) progressed to the randomised controlled trial. We hypothesised that (i) a high concentration of late-stage anthocyanin metabolites (HIGH group) reflected greater microbial-host metabolism interactions and (ii) that these late-stage, small molecular weight anthocyanin metabolites, would have greater bioactivity. This hypothesis was built on similar metabolite observations for other flavonoids (i.e. daidzein to equol; ellagatannins to urolithins).

The primary focus of my research was the cardiometabolic response, and specifically endothelial function, in the extended postprandial phase when anthocyanin metabolites have been shown to still be circulating (i.e. 0 to 48 hours). As described extensively, COVID-19 resulted in the abandonment of my study. This, however, presented me with an opportunity to re-focus my thesis plan and I was able to additionally look at QRISK3 scores and individualised test meals in the partial dataset that I had collected.

This chapter presents a discussion of the overall thesis findings and makes a series of recommendations, and considerations, for future research into blueberry anthocyanins and human health. It also identifies study limitations and reflects on the research, the challenges that I experienced and the learnings I have gained through the process.

7.1.2 Novel blueberry anthocyanin metabotype

As described in Chapter 1, cardiovascular disease (CVD) is the primary cause of death worldwide [1]. Equally, it is well established that increased consumption of fruit and vegetables is associated with a reduced incidence of CVD [25], [26]. Although the direct mechanism for this relationship is not fully understood, data from large epidemiological studies suggest that increased consumption of specific dietary flavonoid subclasses (ubiquitous in fruits and vegetables) may drive a proportion of the inverse association with CVD [27]–[29]. Anthocyanins in particular have been consistently inversely associated with coronary heart disease [27]–[29], [53]. These observational associations are supported by randomised controlled trials (RCTs) which have assessed the short and medium-term effectiveness of anthocyanins against intermediate markers of CVD risk including endothelial function [61], [64], [68], [71], [72]. That said, the evidence for cardio-protection is not unequivocal. Several studies reported no effect of anthocyanins on key markers of CVD risk including for example blood pressure [60], [61], [64], [67], [69]. High interindividual variability in polyphenol metabolism has been proposed as a possible explanation for variability in response to anthocyanin interventions. Several authors speculated that the metabolism of anthocyanins, and other flavonoids, may be key for potential cardiometabolic benefits [81], [91], [92]. This thesis tested whether a newly proposed anthocyanin metabolism profile, identified as potentially cardioprotective by our research group (recently reported in the thesis of Laura Haag, PhD, UEA) influenced cardiometabolic function after a single dose of blueberry anthocyanins within an energy-dense meal challenge (Chapters 3, 4 and 5).

Postprandial responses for a wide range of intermediate cardiometabolic endpoints were explored; including endothelial function (measured by FMD), blood pressure (BP), arterial stiffness and augmentation index, and concentrations of glucose, cholesterol and triglycerides. When comparing these cardiometabolic markers in those classified as HIGH and LOW metabolisers of anthocyanin (*n* =8 in total), some potentially interesting findings were observed. These included apparent reduction in BP in the 'LOW' group. This finding was consistent when BP was measured at rest in clinic, and during ambulatory BP monitoring. Clinically relevant (>6mmHg) reductions in resting BP after blueberry intake in the LOW group were seen for both systolic BP and diastolic BP and consistent over 24- and 48-hour follow-up. This was supported by similar albeit smaller (>4mmHg) reductions in systolic BP and diastolic BP at 48 hours when assessed by ambulatory BP monitoring. Given that even small increases in systolic BP, of just 2mmHg, can increase the risk of heart disease [11], the sustained reductions observed over 48 hours may be important. No consistent differences were observed between the HIGH and LOW groups for the primary endpoint (endothelial function as measured by FMD), arterial stiffness and augmentation index, nor for concentrations of glucose, cholesterol and triglycerides.

Although the HIGH / LOW metaboliser analysis was substantially under-powered (and thus no formal statistical comparisons were conducted), it was of interest to examine how the two metaboliser profile groups differed in their characteristics. As expected, considering the determinants for 'HIGH' or 'LOW' classification, the two groups exhibited differences in metabolite response to a blueberry challenge (Phase 1 of the study). Specifically, those in the 'HIGH' group had a greater 'increase' in the metabolites of interest above the levels found in their habitual, pre-challenge baseline samples. This baseline-adjusted calculation was intended to separate 'responders' from 'non-responders' for the metabolism profile.

When reviewing the pre-challenge baseline concentrations of the four key metabolites in the metaboliser profile (described in Chapter 5) it was apparent that the LOW group had substantially higher habitual levels. This potentially indicates a greater habitual adherence to a more 'health conscious' diet which generates substantially more microbial derived gut metabolites. Although our sample from Phase 2 was small (*n* = 8), a similar trend in metabolite profile was seen in all Phase 1 completers (*n* = 119) – with 'LOW' participants having the highest pre-challenge levels, but consequently not increasing levels as much as those who had the lowest pre-challenge levels (the 'HIGH' group). The habitual dietary intake data, from a self- reported validated food frequency questionnaire (FFQ), demonstrated that LOW metabolisers tended to consume more wholegrains (median; LOW = 84.29g/d, HIGH = 32.14g/d) and tea (median; LOW = 665.00g/d, HIGH = 488.57g/d), and less fat (median; LOW = 62.99g/d, HIGH = 104.25g/d) and beer (median; LOW = 41.43g/d, HIGH = 124.29g/d) than HIGH metabolisers. These findings also support the hypothesis that LOW metabolisers (as defined by the baseline-adjusted calculation) may have been the more habitually health-conscious of the two groups and that this may have influenced their gut microbiota. Taken together, our findings suggest that more habitually health-conscious eaters (i.e. the LOW group) potentially gained more vascular benefits, including reduced BP, when blueberries were included in an energy-dense meal.

7.1.3 Endothelial Function

The primary endpoint of the study described in Chapter 3 was endothelial function. This was assessed via flow-mediated dilation (FMD); which required the candidate (CF) to develop knowledge and image acquisition and analytical skills to reproducibly determine endothelial function / dysfunction. No improvement in peak FMD was observed following blueberry intake (Chapter 4) however we note that this study was considerably underpowered.

Alternate endpoints of the FMD assessment were analysed including low flow-mediated constriction (LFMC), high flow-mediated constriction (HFMC) and time

to peak (TTP). To our knowledge this is the first time they have been applied to a blueberry anthocyanin RCT. We found no effect of the blueberry intervention on LFMC, HFMC or TTP in either populations studied (Chapters 2 and 4).

We did however find that HFMC was less pronounced in statin users, potentially indicating one of the underlying mechanisms of how statins reduce the cardiometabolic burden. Accordingly, it has been previously widely reported that statin therapy is associated with higher percentage peak FMD and sustained improvements on endothelial function [155]–[160]. We also found that a greater constriction in LFMC was associated with greater constriction in HFMC (R^2= -0.75, $p < 0.01$), something that has not previously been reported. These findings may give insight into the interpretation of HFMC and LFMC, both relatively new endpoints, in future studies.

The exploratory analysis of TTP indicated that sex had an impact; with females taking significantly longer to reach peak FMD, than males. This is an interesting finding as sex differences in FMD are mainly reported in pre-menopausal women, and the average age of the females in this study was 64 years. Thus it is likely oestrogen, and its protective effects, have declined potentially putting these female participants at a disadvantage as longer TTP is associated with those who tend to have a greater CVD risk [126], [142], [144]. These exploratory analyses provide novel insights into the use of an extended range of FMD parameters and how some endpoints differed between participants.

7.1.4 Personalised test meal and QRISK3 score

In Chapter 6, we firstly explored the impact of personalised test meal (by comparing the absolute fat content of the individualised test meals) on acute cardiometabolic response. Secondly, we examined the potential impact of the baseline QRISK3 score on cardiometabolic response. Within our study design, we personalised the energy-dense test meal for each individual using a basal

metabolic rate equation with the addition of a physical activity factor. While the absolute fat content varied, the percentage of calories from fat within the test meal remained the same, at 40%. We compared those with lower absolute fat (g) and those with higher absolute fat (g) in the test meal and their acute cardiometabolic responses (of those who completed the placebo arm only, *n* =14). However, it became apparent that there were sex differences, with those in the lower fat group all being female, and those in the higher fat group being predominantly male (*n* = 1 female). A sub-group assessment was performed (figure 6.1 and 6.2) where changes in percentage peak FMD were compared by absolute fat content for each individual, and separately for males and females. Within females there was a strong correlation between increasing absolute fat and declining change in peak FMD at 24 hours (R^2 = 0.91). This was not observed in the males. Potential reasons for this included metabolic sex differences and differences in body fat percentage (with the females having more body and trunk fat than men), highlighting the importance of adequate statistical power and adjustment for sex in future studies.

The application of the QRISK 3 score in Chapter 6 explored whether composite cardiovascular measures of health might influence acute cardiometabolic response in the placebo participants. This was of interest as previous nutrition interventions have recruited participants on the basis of a certain QRISK score thresholds [94], [222]. The range of QRISK3 score for our study population was narrow, 2.00-13.5%. Participants were compared by 'lower' QRISK3 score and 'higher' QRISK3 score. We observed that mean percentage peak FMD at baseline was higher in the low QRISK3 group (mean FMD = 6.43%), compared with the higher QRISK3 group (mean FMD = 2.70%). This data confirmed that those with lower CVD risk scores had significantly greater endothelial function. This is potentially considered important as FMD was the main endpoint of our study in Chapters 3 and 4.

7.2 Limitations

There were some limitations experienced when completing this thesis which have affected the extent to which the primary hypothesis could be tested. These limitations are highlighted hereafter, in order to identify areas for future studies to address and improve upon.

The timing of the COVID-19 pandemic directly impacted our ability to complete the AMP study (Chapter 3) and collect trial data, with significant repercussions on my analysis plan. Study numbers were greatly reduced, and most participants completed only one crossover arm (*n* = 22). Comparative statistical tests based on the HIGH / LOW metabotypes were deemed inappropriate due to the low numbers completing both arms of the cross-over (*n* = 8) with imbalanced groups (3 LOW, 5 HIGH). Due to these limitations, the hypothesis that an individual's anthocyanin metabotype will influence the effect of a single dose of blueberry anthocyanins on vascular and metabolic function remains untested.

To maximise the data from the volunteers recruited those that completed at least one crossover arm (*n* = 22) were included in a *post hoc* parallel comparison of blueberry and placebo responses (presented in Chapters 4, 5 and 6). As described, the study was not designed for this purpose and consequently this resulted in comparative groups that were imbalanced. The key disparity was sex, and is likely to have affected the between-group comparisons, as men and women have different cardiometabolic health profiles - with pre-menopausal women tending to have better endothelial function [190]. After the menopause the sex difference begins to diminish with timing dependent on the individual, but considered to be between 60-69 years of age [154], [190]. It is therefore important to ensure adequate power and that adjustment for sex is made in statistical testing – which we were unable to do.

A further limitation is a disparity between the methodology used to determine metaboliser profile (using chronic 6-month data), and the application of this to a single-dose, postprandial blueberry study. The metabolites associated with vascular changes over 6-months duration relate to chronic metabolite changes following daily blueberry intake

which may have promoted longer-term changes in the gut microbiome and the abundance of specific bacterial species. Re-colonisation of microbial species has not been reported following single exposures to food, and thus microbe dependent metabolite production may not be possible in those who have not developed these ecologies through chronic dietary exposure.

A further limitation was that the metabolites included in the HIGH / LOW metabotype were not specific to blueberries and can also be produced from ingesting other foods (such as wholegrains). Though we controlled background diet prior to both phase 1 and phase 2 and provided low flavonoid foods to consume for two days before and throughout the 48-hour assessment period, it is still possible that changes in vascular function could be influenced from other, non-flavonoid foods, with common metabolism to the same four key metabolites.

7.3 Research gaps and future directions

This thesis aimed to address the current research gaps identified in the literature surrounding anthocyanins and cardiometabolic health (Chapter 1). As the study was stopped before reaching our recruitment target of *n* = 74, it was substantially underpowered to test the primary hypothesis of whether a novel blueberry anthocyanin metabolism profile, was associated with a favourable cardiometabolic response after an energy-dense meal challenge. Therefore, this question remains unanswered. Despite the small numbers and sample size limitations, some interesting findings emerged from our novel blueberry anthocyanin metabotype data. Resting and ambulatory blood pressure was lower at 24 and 48 hours in the LOW metabotype group. Additionally, the LOW group had higher baseline levels of the four metabolites in the metabotype profile and consumed more wholegrains and tea, and less beer and fat than the HIGH group (Chapter 5). Similar dietary intake trends between the HIGH and LOW metabolisers were recently described by our research group (Laura Haag UEA PhD 2021 – pending corrections) in a larger sample (*n* = 119). Taken together, these findings suggest that different responses to a blueberry intervention may be driven by an individual's gut health and background diet.

7.3.1 Understanding LOW metabolisers

With our data suggesting LOW metabolisers have habitually higher levels of the four key metabolites a potential next step could be to confirm the vascular effect in a multi-arm cross-over RCT. This could help establish the contribution of background diet when testing blueberry anthocyanins. Such an RCT should recruit healthy older adults and assess habitual metabolite levels in a 24-hour urine sample (to confirm levels of key metabolites as per LOW metabolisers in Phase 1) without the need for a dietary challenge. An FFQ could be collected to associate dietary patterns with the concentration of the metabolites in the LOW metaboliser profile). From the analysis of FFQ data in the present study, the following treatments arms are proposed (each lasting a week):

1. **Controlled diet + placebo:** Diet controlled for flavonoids, anthocyanins, wholegrains, tea, beer and fat with the addition of placebo powder.
2. **Controlled diet + blueberry anthocyanins:** Diet controlled for flavonoids, anthocyanins, wholegrains, tea, beer and fat with the addition of a single dose of blueberry anthocyanins.
3. **'Healthy' diet + placebo:** As per FFQ, we would recommend increased wholegrains, tea and lower levels of fat and beer with the addition of placebo powder.
4. **'Healthy' diet + blueberry anthocyanins:** As per FFQ, we would recommend increased wholegrains, tea and lower levels of fat and beer with the addition of a single dose of blueberry anthocyanins

Cardiometabolic function should be tested over a 48-hour period to understand whether beneficial vascular improvements are associated with a 'healthier' diet or anthocyanins or both. This would facilitate an understanding of what makes a LOW metaboliser unique, and how one might be able to make any individual a LOW metaboliser.

Whilst constructing a study such as this it gives a unique opportunity to explore the mechanism of vascular action *in vivo*, something we were not able to do in this thesis. An important consideration is that nitric oxide (NO), which is often put forward as the main component of vaso-relaxation, does not explain the entire dilatory response [114]. This raises the question about what other vasoactive substances are involved in the vascular

response and remains unexplored such as hydrogen sulphide, nitrosopersulfide (an intermediate product of NO and hydrogen sulphide) and prostacyclin.

To better understand the vascular changes that are potentially observed we would suggest taking a blood sample, at the time of key vascular measurements at key timepoints, to investigate vasoactive substances that may be involved. Although costly, it would be valuable to take the samples at key time points, for example blood pressure measurements at 24 and 48 hours, and to then bank the samples. During data analysis if there are specific endpoints and timepoints of interest, these banked samples can be analysed at a later date to give insight into the *in vivo* mechanism of action.

7.3.2 Altering an individual's metabotype

Anthocyanins are present in many fruits and vegetables in the UK, for example blueberries, and could be recommended to be included into the diet by public health officials as a simple dietary CVD prevention initiative. At present the UK government recommendation of 5 fruit and vegetables a day doesn't specify any particular types and has poor adherence with only 27% of the UK population meeting the recommended amount [30]. Ultimately, the purpose of research into anthocyanins and cardiometabolic health outcomes is to understand their therapeutic potential in CVD prevention, and potential inclusion in dietary guidance. Individual variation in response to anthocyanins in RCTs has led some investigators to question their efficacy. Thus, further understanding of sex-specific differences and inter- and intra-individual variations of metabolism is required. If future research into LOW metabolisers demonstrates preferential cardiometabolic outcomes the next step would be to understand if it is possible for any individual to become a LOW metaboliser.

As described in Chapter 5, individual components in the diet, such as wholegrain intake, are known to influence and manipulate the gut microbiota and bacteria and produce similar metabolites to those included in the HIGH / LOW metabotype. There is a clear need for longer-term (for example 6 months) habitual diet interventions to examine changes in gut microbiota and metabolite levels. Ideally, faecal samples, urine samples and an FFQ could be taken (for example monthly) to ascertain any changes from baseline and provide insights

into whether the duration of exposure is critical in transforming the capacity to produce key metabolites. As well as cardiometabolic measurements determined to be of interest in the study described in section 7.3.1. If the study described in 7.3.1 confirmed anthocyanins to be the mechanism for vascular change (and a healthy habitual diet is the necessary constituent), then it would also be informative to test vascular function responses after a single dose of blueberry anthocyanins at baseline (0 months) and at the end of the study (6 months).

In conclusion, despite obvious limitations due to the COVID-19 pandemic disruptions, this thesis provides new insights into the potential interindividual metabolism of blueberry anthocyanins. Future research should examine the impact of habitual diet on metaboliser profile and the extent to which the gut microbiome mediates this association to inform a more targeted approach for CVD prevention

Appendices

Appendix A

A1 Scientific contributions and acknowledgements for retrospective analysis (chapter 2)

The CIRCLES study was designed and completed prior to my start at UEA. The Flow-mediated dilation (FMD) images were captured and analysed by Dr Peter Curtis and Dr Lindsey Berends. I was given permission by the Chief investigator (Professor Aedin Cassidy) to retrospectively analyse the FMD images with methods not used in the previous analysis (low flow-mediated constriction, high flow-mediated constriction and time to peak). I completed the methodology, the new FMD analysis and statistically analysed the data presented.

A2 Scientific contributions and acknowledgements for the AMP study (chapters 3, 4, 5 and 6)

The AMP study (described in chapter 3) consisted of several different team members who were part of the study for varying amounts of time. Table A2.1 describes each AMP team member and their role in the study. Table A2.2 reports the specific tasks performed by each team member. I completed all of the analysis of the data presented in chapters 4, 5 and 6 with supporting supervision from my supervisors.

Table A2.1: AMP study job role and description for each team member

Name	ROLE & JOB DESCRIPTION
Caoimhe Flynn	PhD student tasked with organising and running phase 2 of the study. Present from start of protocol formation (December 2017) until study stopped due to lockdown (March 2020). Continued with data organisation, data analysis and statistical analysis for thesis write-up.
Laura Haag	PhD student tasked with organising and running phase 1 of the study. Present from start of protocol formation (December 2017) until leaving the study (February 2020). Continued with phase 1 data analysis for thesis write-up.
Peter Curtis	Principal Investigator at UEA (based in Norwich, Norfolk) and primary supervisor of CF & LH. Present from start of protocol formation (December 2017) until study stopped due to lockdown (March 2020). Main role was on-site study coordinator (for any immediate issues arising & communication with various partners e.g. GP surgeries), volunteer consent and phase 2 study visit days.
Nicky Bondonno	Visiting post-doc from Perth, Australia who worked part-time on the study June 2019 – March 2020. Main role was volunteer consent and phase 1 & 2 lab work.
Faith Ndungwani	Medical student who completed a 3-month summer internship (June-August 2019) and then completed *ad hoc* days January – March 2020. Main role was volunteer communication and recruitment.
Chris Benwell	PhD internship student from Quadram Institute Bioscience (Norwich) who was involved with the study between September – December 2019. Main role was volunteer communication and phase 1 lab work.
Aedin Cassidy	Chief Investigator of the study (based at Queens University, Belfast, Northern Ireland) and lead grant fund holder. Final sign off on protocol and ethical application. Offered assistance and guidance to UEA PI on issues as they arose.
Research Nurses	NHS research nurses present at the clinical research facility to carry out the general health screen (prior to phase 1) and take repeated blood samples (during phase 2).
Clinical Advisor (Dr Jane Ewing)	Confirmed clinical eligibility to participate and signed off the clinical biochemistry results. On a case-by-case basis, gave clinical guidance regarding issues which arose during the conduct of the study.

Role and job descriptions of the AMP team members. Abbreviations: CF (Caoimhe Flynn), LH (Laura Haag), PI (principal investigator) and UEA (University of East Anglia).

Table A2.2: AMP study tasks performed by each team member

STUDY TASKS	CAOIMHE FLYNN	LAURA HAAG	PETER CURTIS	NICKY BONDONNO	FAITH NDUNGWANI	CHRIS BENWELL	AEDIN CASSIDY	RESEARCH NURSES
Protocol formation	✓	✓	✓				✓	
Ethics approval	✓	✓	✓				✓	
Review of volunteer suitability from HLQ data	✓	✓			✓	✓		
Volunteer communications	✓	✓		✓	✓	✓		
Taking volunteer consent	✓	✓	✓	✓				
General health screen visits	✓	✓		✓	✓	✓		✓
Phase 1 – visit days	✓	✓		✓	✓	✓		
Phase 1 – lab sample work		✓		✓	✓	✓		
Phase 1 – metabotype analysis		✓		✓				
Phase 2 – individualised 4-day diet and energy-dense meal	✓							
Phase 2 – pre-visit meeting	✓							
Phase 2 - visit days	✓		✓					
Phase 2 – flow-mediated dilation assessment	✓		✓					
Phase 2 – pulse wave velocity & augmentation index assessment	✓		✓					
Phase 2 – anthropometrics	✓		✓					
Phase 2 – blood sampling								✓
Phase 2 – lab sample work		✓		✓	✓	✓		
Phase 2 - flow-mediated dilation analysis	✓		✓					
Phase 2 - data organisation	✓							
Phase 2 - statistical analysis	✓							

Phase 1 refers to the blueberry challenge and phase 2 refers to the cross-over intervention study. Abbreviations: HLQ (health and lifestyle questionnaire)

Appendix B

B1 Physical activity factor

CRF 6. Physical Activity Level (PAL) Assessment

☐ Male ☐ Female

Section a. Occupational Activity (work activity)

Confirm which of the following best describes the participant's *occupational* activity level. Only **tick ONE** form of *occupational* activity:

Non-Active and Light	**Moderately Active**	**Very Active**
Predominately sedentary job – for example desk based, office work, market place seller,	Regular work-related physical tasks – for example delivering post, household decorator	Very active job - for example labourer, farm worker, landscape gardener

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Section b. Non-Occupational Activity (Leisure)

Confirm which of the following best describes the participant's *non-occupational* activity level. Only **tick ONE** form of *non-occupational* activity:

Non-Active (or light activity)	**Moderately Active** (30 minutes x 5 a week)	**Very Active** (75 minutes a week)
• Always drives or takes public transport • Minimal household and garden activities • No active recreation • Most leisure time spent reading, watching tv, using the computer etc.	• Regular active commuting on foot or by bicycle • Regular household and garden activities • Regular active recreation or social sport at a ***moderate intensity*** (see table 1)	• Regular active commuting on foot or by bicycle • Regular household and garden activities • Regular active recreation or social sport at a ***vigorous intensity*** (see table 1)

Table 1. Defining Light, moderate and vigorous activity (adapted from department of health, 2004)

Activity	Intensity
Ironing	Light
Cleaning	Light
Walking – strolling, 2mph	Light
Painting / decorating	Moderate
Walking 3 mph	Moderate
Hoovering	Moderate
Golf – walking, pulling clubs	Moderate
Badminton – social	Moderate
Tennis – doubles	Moderate
Walking – brisk, 4mph	Moderate
Mowing Lawn – walking using power mower	Moderate
Cycling – 10-12mph	Moderate
Aerobic dancing	Vigorous
Cycling – 12-14mph	Vigorous
Swimming – slow crawl, 50 yards per minute	Vigorous
Tennis – singles	Vigorous
Running – 6-8mph (7.5-10min/mile)	Vigorous

Name of scientist / research nurse (PRINT): ……..…….

Signature of the scientist / research nurse: …….

Date: **18 Mar 2020**

COMMENTS:

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Section c. Determining an Individual's PAL

Based on the boxes you have ticked in section a and b, please **circle the appropriate number**.

Non- occupational activity	**Occupational activity *non-active/light***		**Occupational activity *moderate***		**Occupational activity *vigorous***	
	Male	Female	Male	Female	Male	Female
Non-active	1.4	1.4	1.6	1.5	1.7	1.5
Moderately active	1.5	1.5	1.7	1.6	1.8	1.6
Very active	1.6	1.6	1.8	1.7	1.9	1.7

PAL number = _____

CRF 7. Participant menu for Phase 2

What type of milk do you prefer (please circle)? skimmed / semi-skimmed / whole

What type of bread do you prefer (please circle)? white / wholemeal

	DAY 1	DAY 2	DAY 3	DAY 4
Breakfast				
Cornflakes with milk			**Test Meal Day**	
Rice Krispies with milk				
Toast with butter or honey *(circle one or both)*				
Lunch				
Chicken sandwich			**Test Meal Day**	
Cheese sandwich				
Chicken soup with bread and butter				
Evening meal				
Cheese pizza				
Chicken and gravy pie				
Steak and kidney pie				
Macaroni and cheese				

Meal accompaniments	***Do you like:***
Garlic bread	
Plain rice	
Bread with butter	
Peas	
Sweetcorn	

Snacks	***Do you like:***
Babybel cheese	
Custard cream biscuits	
Cheese scone	
Toffee yoghurt	
Mini cheddars	
Crumpets	
Melon	

Drinks	***Do you like:***
Still water	
Soda water	
Malted milk drink	
Milk	

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

We are interested in finding out about the kinds of physical activities that people do as part of their everyday lives. The questions will ask you about the time you spent being physically active in the **last 7 days**. Please answer each question even if you do not consider yourself to be an active person. Please think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport.

Think about all the **vigorous** and **moderate** activities that you did in the **last 7 days**. **Vigorous** physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. **Moderate** activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal.

PART 1: JOB-RELATED PHYSICAL ACTIVITY

The first section is about your work. This includes paid jobs, farming, volunteer work, course work, and any other unpaid work that you did outside your home. Do not include unpaid work you might do around your home, like housework, yard work, general maintenance, and caring for your family. These are asked in Part 3.

1. Do you currently have a job or do any unpaid work outside your home?

☐ Yes

☐ No → ***Skip to PART 2: TRANSPORTATION***

The next questions are about all the physical activity you did in the **last 7 days** as part of your paid or unpaid work. This does not include traveling to and from work.

2. During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, digging, heavy construction, or climbing up stairs **as part of your work**? Think about only those physical activities that you did for at least 10 minutes at a time.

_____ **days per week**

☐ No vigorous job-related physical activity → ***Skip to question 4***

3. How much time did you usually spend on one of those days doing **vigorous** physical activities as part of your work?

_____ **hours per day**
_____ **minutes per day**

4. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** physical activities like carrying light loads **as part of your work**? Please do not include walking.

_____ **days per week**

☐ No moderate job-related physical activity → ***Skip to question 6***

5. How much time did you usually spend on one of those days doing **moderate** physical activities as part of your work?

_____ **hours per day**
_____ **minutes per day**

6. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time **as part of your work**? Please do not count any walking you did to travel to or from work.

_____ **days per week**

☐ No job-related walking → ***Skip to PART 2: TRANSPORTATION***

7. How much time did you usually spend on one of those days **walking** as part of your work?

_____ **hours per day**
_____ **minutes per day**

PART 2: TRANSPORTATION PHYSICAL ACTIVITY

These questions are about how you traveled from place to place, including to places like work, stores, movies, and so on.

8. During the **last 7 days**, on how many days did you **travel in a motor vehicle** like a train, bus, car, or tram?

_____ **days per week**

☐ No traveling in a motor vehicle → ***Skip to question 10***

9. How much time did you usually spend on one of those days **traveling** in a train, bus, car, tram, or other kind of motor vehicle?

_____ **hours per day**
_____ **minutes per day**

Now think only about the **bicycling** and **walking** you might have done to travel to and from work, to do errands, or to go from place to place.

10. During the **last 7 days**, on how many days did you **bicycle** for at least 10 minutes at a time to go **from place to place**?

_____ **days per week**

☐ No bicycling from place to place → ***Skip to question 12***

11. How much time did you usually spend on one of those days to **bicycle** from place to place?

_____ **hours per day**
_____ **minutes per day**

12. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time to go **from place to place**?

_____ **days per week**

☐ No walking from place to place → ***Skip to PART 3: HOUSEWORK, HOUSE MAINTENANCE, AND CARING FOR FAMILY***

13. How much time did you usually spend on one of those days *walking* ***from place to place?***

_____ **hours per day**
_____ **minutes per day**

PART 3: HOUSEWORK, HOUSE MAINTENANCE, AND CARING FOR FAMILY

This section is about some of the physical activities you might have done in the **last 7 days** in and around your home, like housework, gardening, yard work, general maintenance work, and caring for your family.

14. Think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, chopping wood, shoveling snow, or digging **in the garden or yard**?

_____ **days per week**

☐ No vigorous activity in garden or yard → ***Skip to question 16***

15. How much time did you usually spend on one of those days doing **vigorous** physical activities in the garden or yard?

_____ **hours per day**
_____ **minutes per day**

16. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** activities like carrying light loads, sweeping, washing windows, and raking **in the garden or yard**?

_____ **days per week**

☐ No moderate activity in garden or yard → ***Skip to question 18***

17. How much time did you usually spend on one of those days doing **moderate** physical activities in the garden or yard?

_____ **hours per day**
_____ **minutes per day**

18. Once again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** activities like carrying light loads, washing windows, scrubbing floors and sweeping **inside your home**?

_____ **days per week**

☐ No moderate activity inside home → ***Skip to PART 4: RECREATION, SPORT AND LEISURE-TIME PHYSICAL ACTIVITY***

19. How much time did you usually spend on one of those days doing **moderate** physical activities inside your home?

_____ **hours per day**
_____ **minutes per day**

PART 4: RECREATION, SPORT, AND LEISURE-TIME PHYSICAL ACTIVITY

This section is about all the physical activities that you did in the **last 7 days** solely for recreation, sport, exercise or leisure. Please do not include any activities you have already mentioned.

20. Not counting any walking you have already mentioned, during the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time **in your leisure time**?

_____ **days per week**

☐ No walking in leisure time → ***Skip to question 22***

21. How much time did you usually spend on one of those days **walking** in your leisure time?

_____ **hours per day**
_____ **minutes per day**

22. Think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **vigorous** physical activities like aerobics, running, fast bicycling, or fast swimming **in your leisure time**?

_____ **days per week**

☐ No vigorous activity in leisure time → ***Skip to question 24***

23. How much time did you usually spend on one of those days doing **vigorous** physical activities in your leisure time?

_____ **hours per day**
_____ **minutes per day**

24. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** physical activities like bicycling at a regular pace, swimming at a regular pace, and doubles tennis **in your leisure time**?

_____ **days per week**

☐ No moderate activity in leisure time → ***Skip to PART 5: TIME SPENT SITTING***

25. How much time did you usually spend on one of those days doing **moderate** physical activities in your leisure time?
_____ **hours per day**
_____ **minutes per day**

PART 5: TIME SPENT SITTING

The last questions are about the time you spend sitting while at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading or sitting or lying down to watch television. Do not include any time spent sitting in a motor vehicle that you have already told me about.

26. During the **last 7 days**, how much time did you usually spend **sitting** on a **weekday**?

_____ **hours per day**
_____ **minutes per day**

27. During the **last 7 days**, how much time did you usually spend **sitting** on a **weekend day**?

_____ **hours per day**
_____ **minutes per day**

This is the end of the questionnaire, thank you for participating.

Appendix C

C1 Chapter 4 supplementary data

Table 4.8: Characteristics of study participants who completed at least one arm of the intervention

	Blueberry					Placebo					
Descriptive	n	Mean	SD	Min	Max	n	Mean	SD	Min	Max	p value
Age (y)	10	58.10	5.82	51.00	66.00	12	60.75	5.74	54.00	71.00	0.21
Sex (male)	10	25% (3)	/	/	/	12	75% (9)	/	/	/	**0.04**
BMI (kg/m^2)	10	29.21	2.97	25.60	34.90	12	28.09	2.20	25.20	32.50	0.18
Statin	10	11.11	/	/	/	12	0.00	/	/	/	0.26
Body fat %	10	35.09	6.83	26.20	46.60	12	27.03	7.85	18.70	43.50	**0.02**
Trunk fat %	10	33.37	7.10	25.30	46.60	12	27.31	6.54	18.70	40.80	0.06
Hip (cm)	10	107.49	4.53	102.00	114.65	12	107.12	5.93	100.85	122.25	0.67
Waist (cm)	10	92.43	9.28	79.75	112.15	12	95.54	10.01	74.00	112.75	0.26
Peak FMD %	10	5.87	3.95	0.22	11.73	12	4.06	2.41	1.03	8.18	0.35
HFMC %	10	-1.08	1.42	-2.60	1.09	11	-0.38	1.24	-2.10	1.29	0.35
LFMC %	10	-0.40	1.50	-2.19	1.90	12	0.13	1.42	-2.21	2.71	0.47
TTP (seconds)	9	39.00	11.47	23.00	51.00	12	46.67	10.38	26.00	69.00	0.20
PWV (m/s)	9	10.05	0.91	8.20	11.73	12	10.71	1.66	8.47	13.30	0.42
AIx (%)	10	27.50	4.95	22.50	38.67	12	24.65	5.50	17.67	35.00	0.18
SBP (mmHg)	10	143.85	12.60	119.50	162.00	12	139.79	10.18	126.00	165.00	0.16
DBP (mmHg)	10	88.10	8.47	76.50	104.00	12	83.13	7.11	72.00	99.00	0.14
HR (BPM)	9	63.17	8.50	52.00	75.50	12	57.92	7.75	43.00	73.00	0.25
ABPM SBP (mmHg)	10	131.60	8.66	122.00	143.00	12	130.58	10.71	117.00	149.00	0.77
ABPM DBP (mmHg)	10	82.40	6.90	70.00	94.00	12	78.58	8.05	68.00	96.00	0.20
ABPM MAP (mmHg)	10	98.50	5.87	88.00	108.00	12	95.58	6.54	89.00	110.00	0.23
ABPM PP (mmHg)	10	48.90	6.84	39.00	60.00	12	51.83	8.05	38.00	67.00	0.42
ABPM HR (BPM)	10	73.30	8.64	63.00	88.00	12	68.08	8.18	53.00	81.00	0.18
Glucose (mmol/L)	10	4.68	0.42	4.10	5.30	12	4.74	0.33	4.40	5.50	0.82
Cholesterol (mmol/L)	9	5.41	0.66	4.40	6.80	12	5.61	1.56	2.70	8.50	0.65
LDLC (mmol/L)	9	3.48	0.67	2.60	4.90	12	3.65	1.33	1.50	5.60	0.60
HDLC (mmol/L)	10	1.43	0.31	1.02	1.96	12	1.35	0.33	0.83	2.03	0.58
Triglyceride (mmol/L)	10	1.16	0.30	0.70	1.60	12	1.32	0.37	0.77	2.02	0.31

Statistical differences between the blueberry and placebo group were established using a Mann Whitney U test, for all nominal data a Chi square test was performed. A p value of <0.05 was considered statistically significant.
Abbreviations: SD (standard deviation), BMI (body mass index), FMD (flow-mediated dilation), HFMC (high flow-mediated dilation), LFMC (low flow-mediated dilation), TTP (time to peak), PWV (pulse wave velocity), AIx (augmentation index), SBP (systolic blood pressure), DBP (diastolic blood pressure), HR (heart rate), ABPM SBP (ambulatory blood pressure monitor systolic blood pressure), ABPM DBP (ambulatory blood pressure monitor diastolic blood pressure), ABPM MAP (ambulatory blood pressure monitor mean arterial pressure), ABPM PP (ambulatory blood pressure monitor pulse pressure), ABPM HR (ambulatory blood pressure monitor heart rate), LDLC (low density lipoprotein cholesterol), HDLC (high density lipoprotein cholesterol), Min (minimum), Max (maximum).

Table 4.9: Participants who completed one arm of the intervention - mean change from baseline for endothelial function after dietary intervention

		BASELINE					1.5HR					3HR				
		N	Mean	SD	Min	Max	N	Mean	SD	BL Δ	p value	N	Mean	SD	BL Δ	p value
Peak FMD %	BLUEBERRY	12	4.06	2.41	1.03	8.18	10	3.62	2.78	-0.22	0.82	11	4.46	3.03	0.33	0.78
	PLACEBO	10	5.87	3.95	0.22	11.73	10	5.85	3.54	-0.02		10	6.33	3.77	0.46	
LFMC %	BLUEBERRY	11	0.13	1.42	-2.21	2.71	9	-0.14	1.13	0.00	0.41	10	-0.26	3.39	-0.51	0.50
	PLACEBO	10	-0.40	1.50	-2.19	1.90	10	0.53	1.33	0.93		10	-0.25	1.18	0.16	
HFMC %	BLUEBERRY	11	-0.38	1.24	-2.10	1.29	9	-0.36	1.27	0.28	0.29	9	0.18	1.92	0.47	0.68
	PLACEBO	10	-1.08	1.42	-2.60	1.09	10	0.08	1.09	1.16		10	-0.71	0.91	0.37	
TTP (seconds)	BLUEBERRY	12	46.67	10.38	26.00	69.00	10	54.20	16.36	7.50	0.33	11	55.91	14.36	11.27	0.82
	PLACEBO	9	39.00	11.47	23.00	51.00	9	43.44	8.56	4.44		9	47.56	9.41	8.56	

		6HR					24HR					48HR				
		N	Mean	SD	BL Δ	p value	N	Mean	SD	BL Δ	p value	N	Mean	SD	BL Δ	p value
Peak FMD %	BLUEBERRY	12	4.23	2.97	0.17	0.74	11	2.97	1.77	-1.22	0.14	10	3.26	2.69	-1.45	0.07
	PLACEBO	10	6.16	2.99	0.30		10	5.91	3.10	0.04		10	6.96	3.94	1.09	
LFMC %	BLUEBERRY	11	-0.07	1.38	-0.21	0.26	11	-0.60	1.67	-0.80	0.06	10	-0.19	1.61	-0.23	0.17
	PLACEBO	10	0.06	1.84	0.46		10	0.58	1.39	0.98		10	0.75	1.31	1.15	
HFMC %	BLUEBERRY	11	-0.57	1.28	-0.19	0.67	11	-1.02	1.60	-0.66	0.29	10	-0.81	1.64	-0.63	0.14
	PLACEBO	10	-0.80	1.78	0.28		10	-0.61	1.31	0.47		10	-0.22	0.90	0.86	
TTP (seconds)	BLUEBERRY	12	48.13	8.42	1.46	0.78	12	54.25	14.88	6.36	0.68	10	54.00	15.98	5.10	0.98
	PLACEBO	9	39.33	5.27	0.33		9	43.78	8.57	4.78		9	45.67	13.86	6.67	

Mean BL Δ for TTP is shown in seconds. Statistical differences between the blueberry and placebo group were established using a Kruskal Wallis test. A p value of < 0.05 was considered statistically significant however no results indicated differences between the groups at any timepoint. Mean and BL Δ for FMD, LFMC and HFMC are shown in percentage. Abbreviations: FMD (flow-mediated dilation), HFMC (high flow-mediated dilation), LFMC (low flow-mediated dilation), TTP (time to peak), BL (baseline) SD (standard deviation), Δ (change), min (minimum) max (maximum).

Table 5.7: Baseline characteristics of all participants *(n* = 22) taking part in at least one arm of the intervention study analysed by blueberry or placebo intervention groups

	Blueberry					Placebo					
Descriptive	n	Mean	SD	Min	Max	n	Mean	SD	Min	Max	P Value
Age (y)	10	58.10	5.82	51.00	66.00	12	60.75	5.74	54.00	71.00	0.21
Sex (male)	10	25% (3)	/	/	/	12	75% (9)	/	/	/	**0.04**
BMI (kg/m^2)	10	29.21	2.97	25.60	34.90	12	28.09	2.20	25.20	32.50	0.18
Test meal fat content (g)	10	11.11	/	/	/	12	0.00	/	/	/	0.26
Statin (NO)	10	35.09	6.83	26.20	46.60	12	27.03	7.85	18.70	43.50	**0.02**
Body fat %	10	33.37	7.10	25.30	46.60	12	27.31	6.54	18.70	40.80	**0.06**
Trunk fat %	10	107.49	4.53	102.00	114.65	12	107.12	5.93	100.85	122.25	0.67
Hip (cm)	10	92.43	9.28	79.75	112.15	12	95.54	10.01	74.00	112.75	0.26
Waist (cm)	10	5.87	3.95	0.22	11.73	12	4.06	2.41	1.03	8.18	0.35
Peak FMD %	10	-1.08	1.42	-2.60	1.09	11	-0.38	1.24	-2.10	1.29	0.35
HFMC %	10	-0.40	1.50	-2.19	1.90	12	0.13	1.42	-2.21	2.71	0.47
LFMC %	9	39.00	11.47	23.00	51.00	12	46.67	10.38	26.00	69.00	0.20
TTP (seconds)	9	10.05	0.91	8.20	11.73	12	10.71	1.66	8.47	13.30	0.42
PWV (m/s)	10	27.50	4.95	22.50	38.67	12	24.65	5.50	17.67	35.00	0.18
AIx (%)	10	143.85	12.60	119.50	162.00	12	139.79	10.18	126.00	165.00	0.16
SBP (mmHg)	10	88.10	8.47	76.50	104.00	12	83.13	7.11	72.00	99.00	0.14
DBP (mmHg)	9	63.17	8.50	52.00	75.50	12	57.92	7.75	43.00	73.00	0.25
HR (BPM)	10	131.60	8.66	122.00	143.00	12	130.58	10.71	117.00	149.00	0.77
ABPM SBP (mmHg)	10	82.40	6.90	70.00	94.00	12	78.58	8.05	68.00	96.00	0.20
ABPM DBP (mmHg)	10	98.50	5.87	88.00	108.00	12	95.58	6.54	89.00	110.00	0.23
ABPM MAP (mmHg)	10	48.90	6.84	39.00	60.00	12	51.83	8.05	38.00	67.00	0.42
ABPM PP (mmHg)	10	73.30	8.64	63.00	88.00	12	68.08	8.18	53.00	81.00	0.18
ABPM HR (BPM)	10	4.68	0.42	4.10	5.30	12	4.74	0.33	4.40	5.50	0.82
Glucose (mmol/L)	9	5.41	0.66	4.40	6.80	12	5.61	1.56	2.70	8.50	0.65
Cholesterol (mmol/L)	9	3.48	0.67	2.60	4.90	12	3.65	1.33	1.50	5.60	0.60
LDLC (mmol/L)	10	1.43	0.31	1.02	1.96	12	1.35	0.33	0.83	2.03	0.58
Triglyceride (mmol/L)	10	1.16	0.30	0.70	1.60	12	1.32	0.37	0.77	2.02	0.31

Statistical differences between the blueberry and placebo group were established using a Mann Whitney U test, for all nominal data a Chi square test was performed. A p value of <0.05 was considered statistically significant. Abbreviations: SD (standard deviation), BMI (body mass index), FMD (flow-mediated dilation), HFMC (high flow-mediated dilation), LFMC (low flow-mediated dilation), TTP (time to peak), PWV (pulse wave velocity), AIx (augmentation index), SBP (systolic blood pressure), DBP (diastolic blood pressure), HR (heart rate), ABPM SBP (ambulatory blood pressure monitor systolic blood pressure), ABPM DBP (ambulatory blood pressure monitor diastolic blood pressure), ABPM MAP (ambulatory blood pressure monitor mean arterial pressure), ABPM PP (ambulatory blood pressure monitor pulse pressure), ABPM HR (ambulatory blood pressure monitor heart), LDLC (low density lipoprotein cholesterol), HDLC (high density lipoprotein cholesterol), Min (minimum), Max (Maximum).

Table 5.8: Participants who completed one arm of the intervention - mean change from baseline for measures of arterial stiffness and blood pressure after dietary intervention

		BASELINE					1.5hr					3HR				
		N	Mean	SD	Min	Max	N	Mean	SD	BL Δ	P Value	N	Mean	SD	BL Δ	P Value
PWV (m/s)	BLUEBERRY	12	10.71	1.66	8.47	13.30						12	10.37	1.41	-0.34	0.17
	PLACEBO	9	10.05	0.91	8.20	11.73						8	10.14	1.10	0.08	
AIx (%)	BLUEBERRY	12	24.65	5.50	17.67	35.00						12	21.31	5.74	-3.34	0.34
	PLACEBO	10	27.50	4.95	22.50	38.67						9	22.47	4.59	-3.79	
SBP (mmHg)	BLUEBERRY	12	139.79	10.18	126.00	165.00	12	132.33	9.53	-7.46	0.45	12	131.83	8.37	-7.96	0.64
	PLACEBO	10	143.85	12.60	119.50	162.00	10	131.70	8.44	-12.15		10	132.85	9.24	-11.00	
DBP (mmHg)	BLUEBERRY	12	83.13	7.11	72.00	99.00	12	77.13	7.32	-6.00	0.32	12	78.50	5.91	-4.63	0.72
	PLACEBO	10	88.10	8.47	76.50	104.00	10	79.25	6.16	-8.85		10	81.90	7.23	-6.20	
HR (BPM)	BLUEBERRY	12	57.92	7.75	43.00	73.00	12	65.42	9.94	7.50	0.83	12	63.73	9.46	5.82	0.67
	PLACEBO	9	63.17	8.50	52.00	75.50	10	70.45	6.58	7.00		10	68.55	7.78	5.28	
		6HR					24HR					48HR				
		N	Mean	SD	BL Δ	P Value	N	Mean	SD	BL Δ	P Value	N	Mean	SD	BL Δ	P Value
PWV (m/s)	BLUEBERRY	12	10.58	1.37	-0.13	0.40	12	10.56	1.31	-0.20	0.91	12	10.27	1.27	-0.45	0.46
	PLACEBO	7	10.13	1.07	0.13		9	9.84	0.72	-0.23		8	9.65	0.72	-0.47	
AIx (%)	BLUEBERRY	12	24.87	5.93	0.22	0.09	12	24.65	6.70	-0.32	0.83	12	24.83	5.86	0.06	0.62
	PLACEBO	10	25.41	5.64	-2.09		10	28.07	5.60	0.57		10	27.42	5.36	-0.08	
SBP (mmHg)	BLUEBERRY	12	137.75	15.00	-2.04	0.84	12	134.33	14.74	-5.32	0.12	12	135.79	10.93	-4.00	0.07
	PLACEBO	10	139.10	12.27	-4.75		10	132.35	6.95	-11.50		10	134.50	9.14	-9.35	
DBP (mmHg)	BLUEBERRY	12	83.29	7.84	0.17	0.62	12	80.13	10.05	-2.73	0.06	12	78.92	7.79	-4.14	0.72
	PLACEBO	10	87.80	8.56	-0.30		10	83.80	7.83	-4.30		10	84.95	7.39	-3.15	
HR (BPM)	BLUEBERRY	12	60.88	8.61	2.96	0.59	12	57.92	7.76	0.18	0.70	12	57.00	8.42	-1.73	0.06
	PLACEBO	10	67.75	8.80	4.22		10	62.80	4.42	-0.61		10	64.50	7.58	1.56	

Mean and BL Δ for PWV is shown in meter per second squared (m/s^2), AIx is shown in percentage (%) HR are shown in beats per minute (BPM). Mean and BL Δ for SBP and DBP are shown in millimetres of mercury (mmHg). Statistical differences between the blueberry and placebo group were established using a Kruskal Wallis test. A p value of < 0.05 was considered statistically significant and indicated with a * above. Abbreviations: PWV (pulse wave velocity), AIx (augmentation index), SBP (systolic blood pressure), DBP (diastolic blood pressure), HR (heart rate), BL (baseline) SD (standard deviation), Δ (change), min (minimum) max (maximum).

Table 5.9: Participants who completed one arm of the intervention - mean change from baseline for ambulatory blood pressure after dietary intervention

		BASELINE (DAY 0)					DAY 1					DAY 2				
		N	Mean	SD	Min	Max	N	Mean	SD	BL Δ	P value	N	Mean	SD	BL Δ	P Value
ABPM SBP (mmHg)	BLUEBERRY	12	130.58	10.71	117.00	149.00	12	127.33	10.50	-3.25	0.97	12	123.83	11.78	-6.75	0.53
	PLACEBO	10	131.60	8.66	122.00	143.00	10	127.40	8.24	-4.20		10	125.90	6.26	-5.70	
ABPM DBP (mmHg)	BLUEBERRY	12	78.58	8.05	68.00	96.00	12	76.58	6.24	-2.00	0.55	12	75.58	8.35	-3.00	0.92
	PLACEBO	10	82.40	6.90	70.00	94.00	10	79.90	6.06	-2.50		10	78.60	5.60	-3.80	
ABPM H (BPM)	BLUEBERRY	12	68.08	8.18	53.00	81.00	12	65.58	6.97	-2.50	0.22	11	68.09	7.85	0.45	0.29
	PLACEBO	10	73.30	8.64	63.00	88.00	10	72.30	9.26	-1.00		10	72.30	8.21	-1.00	
ABPM PP (mmHg)	BLUEBERRY	12	51.83	8.05	38.00	67.00	12	50.83	9.45	-1.00	0.92	11	49.55	9.32	-3.09	0.39
	PLACEBO	10	48.90	6.84	39.00	60.00	10	47.20	6.78	-1.70		10	47.30	5.27	-1.60	
ABPM MAP (mmHg)	BLUEBERRY	12	95.58	6.54	89.00	110.00	12	93.25	5.55	-2.33	0.55	11	91.55	7.05	-4.55	0.48
	PLACEBO	10	98.50	5.87	88.00	108.00	10	95.10	5.34	-3.40		10	94.20	4.80	-4.30	

Mean and BL Δ for ABPM SBP, ABPM DBP ABPM MAP and ABPM PP are shown in millimetres of mercury (mmHg). Mean BL Δ for ABPM HR is shown in beats per minute (BPM). Statistical differences between the blueberry and placebo group were established using a Kruskal Wallis test. A P value of < 0.05 was considered statistically significant however no results indicated differences between the intervention. Abbreviations ABPM SBP (ambulatory blood pressure monitor systolic blood pressure), ABPM DBP (ambulatory blood pressure monitor diastolic blood pressure), ABPM MAP (ambulatory blood pressure monitor mean arterial pressure), ABPM PP (ambulatory blood pressure monitor pulse pressure), ABPM HR (ambulatory blood pressure monitor heart rate), BL (baseline) SD (standard deviation), Δ (change), min (minimum) max (maximum).

Table 5.11: Characteristics of study participants who completed the cross-over intervention

	N	Mean / %	SD	Minimum	Maximum
Age (y)	8	61.75	5.39	52.00	67.00
Sex (male)	8	3	/	/	/
BMI (kg/m^2)	8	28.50	2.81	26.10	34.90
Statin (NO)	8	8	/	/	/
Body fat %	8	31.64	9.27	20.30	42.90
Trunk fat %	8	29.94	8.05	20.30	41.00
Hip (cm)	8	106.12	4.81	100.85	113.00
Waist (cm)	8	90.55	8.67	74.00	99.45

Abbreviations: BMI (body mass index), SD (standard deviation).

Table 5.12: Characteristics of study participants, for each intervention week, who completed the cross-over study for measures of arterial stiffness, blood pressure and serum cardiometabolic markers

	Blueberry Week					**Placebo Week**					
	N	Mean	SD	Min	Max	N	Mean	SD	Min	Max	p
FMD %	7	5.06	4.47	1.03	13.83	7	3.50	4.02	-0.33	11.73	0.46
HFMC %	6	-0.54	1.01	-2.30	0.42	6	-1.07	1.24	-2.60	0.43	0.38
LFMC %	7	-0.06	1.41	-1.29	2.71	7	-0.92	1.71	-3.50	1.68	0.70
TTP (seconds)	7	46.29	9.32	33.00	61.00	7	49.29	17.82	23.00	78.00	0.81
PWV (m/s)	8	9.92	1.67	8.23	13.30	8	9.66	0.96	8.20	11.10	0.80
AIx %	8	27.88	7.07	17.67	36.00	8	28.16	9.39	16.67	44.33	0.96
SBP (mmHg)	8	138.44	7.84	128.00	151.00	8	140.44	11.56	124.00	162.00	0.65
DBP (mmHg)	8	85.25	8.17	72.00	98.00	8	85.75	12.56	65.00	104.00	0.88
HR (BPM)	8	62.00	4.38	56.00	70.00	8	63.69	7.06	52.00	72.50	0.72
ABPM SBP (mmHg)	7	127.14	5.87	116.00	132.00	7	128.57	8.30	117.00	140.00	0.81
ABPM DBP (mmHg)	7	79.29	5.50	68.00	83.00	7	80.00	8.08	64.00	89.00	0.62
ABPM MAP (mmHg)	7	95.43	4.12	89.00	100.00	7	96.14	6.49	85.00	105.00	0.81
ABPM PP (mmHg)	7	47.71	5.82	39.00	58.00	7	48.14	5.46	39.00	54.00	0.81
ABPM HR (BPM)	7	72.14	6.04	67.00	81.00	7	75.00	7.33	66.00	82.00	0.46
Glucose (mmol/L)	8	4.56	0.20	4.30	4.90	8	4.60	0.35	4.10	5.00	0.72
Cholesterol (mmol/L)	7	5.41	0.88	4.40	6.80	7	5.41	1.06	3.90	7.10	0.78
HDLC (mmol/L)	8	1.50	0.25	1.01	1.80	8	1.48	0.30	1.03	1.96	0.80

Statistical differences between the blueberry and placebo group were established using a Mann Whitney U test. A p value of < 0.05 was considered statistically significant however no results indicated differences between the intervention weeks. Abbreviations: SD (standard deviation), FMD (flow-mediated dilation), HFMC (high flow-mediated dilation), LFMC (low flow-mediated dilation), TTP (time to peak), PWV (pulse wave velocity), AIx (augmentation index), SBP (systolic blood pressure), DBP (diastolic blood pressure), HR (heart rate), ABPM SBP (ambulatory blood pressure monitor systolic blood pressure), ABPM DBP (ambulatory blood pressure monitor diastolic blood pressure), ABPM MAP (ambulatory blood pressure monitor mean arterial pressure), ABPM PP (ambulatory blood pressure monitor pulse pressure), ABPM HR (ambulatory blood pressure monitor heart rate) and HDLC (high density lipoprotein cholesterol), Min (minimum), Max (maximum).

Table 5.15: Serum cardiometabolic markers for those participants who completed the crossover intervention (blueberry vs placebo)

		BASELINE					20MIN					40MIN				
		N	Mean	SD	Min	Max	N	Mean	SD	BL Δ	P	N	Mean	SD	BL Δ	P
Glucose (mmol/L)	BLUEBERRY	8	4.56	0.20	4.30	4.90	5	6.04	0.36	1.44	0.22	6	5.57	1.09	0.95	0.13
	PLACEBO	8	4.60	0.35	4.10	5.00	5	5.56	0.93	0.98		6	6.65	0.64	2.00	
Glucose (mmol/L)	BLUEBERRY	8	5.63	1.01	4.40	7.10	4	6.03	0.77	0.30	0.49	4	5.50	0.97	0.04	0.29
	PLACEBO	7	5.41	1.06	3.90	7.10	4	6.45	0.79	0.48		4	5.85	1.27	0.23	
HDLC (mmol/L)	BLUEBERRY	8	1.50	0.25	1.01	1.80	5	1.57	0.32	0.06	0.69	6	1.49	0.31	0.02	0.09
	PLACEBO	8	1.48	0.30	1.03	1.96	5	1.64	0.40	0.07		6	1.58	0.35	0.07	
LDLC (mmol/L)	BLUEBERRY	7	3.37	1.00	2.40	5.20										
	PLACEBO	7	3.47	1.06	2.10	5.30										
Triglycerides (mmol/L)	BLUEBERRY	8	1.14	0.22	0.78	1.36										
	PLACEBO	8	1.08	0.28	0.66	1.60										

		60MIN					90MIN					180MIN				
		N	Mean	SD	BL Δ	P	N	Mean	SD	BL Δ	P	N	Mean	SD	BL Δ	P
Glucose (mmol/L)	BLUEBERRY	6	5.15	1.21	0.53	1.00	6	5.00	1.06	0.38	1.00	8	4.53	0.59	-0.04	**0.04***
	PLACEBO	6	5.40	0.64	0.75		6	4.95	0.87	0.30		8	5.31	0.79	0.71	
Glucose (mmol/L)	BLUEBERRY	5	5.42	1.13	-0.04	0.84	5	5.24	0.88	-0.22	0.15	6	5.22	0.85	0.03	0.18
	PLACEBO	5	5.24	0.56	-0.32		5	5.56	1.04	0.00		6	4.92	0.85	-0.22	
HDLC (mmol/L)	BLUEBERRY	6	1.50	0.31	0.03	0.82	6	1.40	0.29	-0.08	0.82	8	1.43	0.25	-0.08	0.07
	PLACEBO	6	1.52	0.36	0.00		6	1.46	0.34	-0.06		8	1.34	0.34	-0.14	
LDLC (mmol/L)	BLUEBERRY											4	2.80	0.78	-0.23	1.00
	PLACEBO											4	2.90	0.74	-0.30	
Triglycerides (mmol/L)	BLUEBERRY											4	1.94	0.62	0.87	0.49
	PLACEBO											4	1.52	0.44	0.59	

CONTINUED: Table 5.15: Serum cardiometabolic markers for those participants who completed the crossover intervention (blueberry vs placebo)

		360MIN					24HR					48HR				
		N	Mean	SD	BL Δ	P	N	Mean	SD	BL Δ	P	N	Mean	SD	BL Δ	P
Glucose (mmol/L)	BLUEBERRY	7	4.27	0.24	-0.33	0.90	8	4.44	0.29	-0.13	0.51	8	4.38	0.32	-0.19	0.96
	PLACEBO	7	4.39	0.21	-0.29		8	4.58	0.25	-0.03		8	4.43	0.37	-0.18	
Glucose (mmol/L)	BLUEBERRY	6	5.33	0.72	-0.22	0.13	7	5.50	0.86	0.09	0.90	7	5.40	0.82	0.01	1.00
	PLACEBO	6	5.57	1.08	-0.02		7	5.59	1.19	0.17		7	5.51	1.12	0.10	
HDLC (mmol/L)	BLUEBERRY	7	1.41	0.29	-0.10	0.81	8	1.50	0.27	0.00	0.88	8	1.48	0.26	-0.02	0.33
	PLACEBO	7	1.40	0.33	-0.12		8	1.49	0.27	0.01		8	1.51	0.28	0.02	
LDLC (mmol/L)	BLUEBERRY	5	3.34	0.90	-0.38	0.42	6	3.60	0.89	0.04	0.95	7	3.36	0.93	-0.01	0.81
	PLACEBO	5	3.64	0.72	-0.28		6	3.75	0.98	0.05		7	3.47	1.07	0.00	
Triglycerides (mmol/L)	BLUEBERRY	5	1.62	0.20	0.49	0.84	7	1.19	0.32	0.09	0.81	8	1.21	0.30	0.08	0.72
	PLACEBO	5	1.85	1.01	0.82		7	1.26	0.46	0.19		8	1.22	0.36	0.14	

Statistics calculated on mean change from baseline. Missing values for LDLC and triglycerides at 20, 40, 60 and 90mins resulted in too few data for statistical testing. Mean and BL Δ for glucose is shown in millimoles per litre (mmol/L). Cholesterol, HDL, LDL and triglycerides. Mean and BL Δ are shown in milligrams per decilitre (mg/dL). Statistical differences between the blueberry and placebo group were established using a Mann Whitney U test. A p value of <0.05 was considered statistically significant and indicated with a * above. Abbreviations LDLC (low density lipoprotein cholesterol), HDLC (high density lipoprotein cholesterol), BL (baseline) SD (standard deviation), Δ (change), min (minimum) max (maximum). Mean and BL Δ for ABPM SBP and ABPM DBP are shown in millimetres of mercury (mmHg).

Glossary

ABPM	Ambulatory blood pressure monitoring
AE	Adverse event
AIx	Augmentation index
BMI	Body mass index
BMR	Basal metabolic rate
BP	Blood pressure
C3G	Cyanidin-3-glucoside
CF	Caoimhe Flynn
cGMP	Cyclic guanosine monophosphate
CI	Confidence interval
CRP	C-reactive protein
CVD	Cardiovascular disease
ECs	Endothelial cells
eNOS	Endothelial nitric oxide synthase
FFQ	Food frequency questionnaire
FMD	Flow-mediated dilation
GTP	Guanosine triphosphate
HDLC	High density lipoprotein cholesterol
HFMC	High flow-mediated constriction
HR	Hazard ratio
HUVEC	Human umbilical vein endothelial cells
IPAQ	International physical activity questionnaire
LDLC	Low density lipoprotein cholesterol
LFMC	Low flow-mediated constriction
LH	Laura Haag
MAP	Mean arterial pressure
NADPH	Nicotinamide adenine dinucleotide phosphate
NICE	National Institute for Health and Clinical Excellence
NO	Nitric oxide
NOS	Nitric oxide synthase
OGTT	Oral glucose tolerance test
OLTT	Oral lipid tolerance test
PAL	Physical activity factor
PC	Peter Curtis
PP	Pulse pressure
PWV	Pulse wave velocity
RCT	Randomised controlled trial
RHI	Reactive hyperaemia index
ROS	Reactive oxygen species
RR	Relative risk
SAE	Serious adverse event
SD	Standard deviation
sGC	Soluble guanylyl cyclase
TTP	Time to peak

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