**TITLE PAGE**

**Title: Blueberry anthocyanin intake attenuates the postprandial cardiometabolic effect of an energy-dense food challenge: results from a double blind, randomized controlled trial in metabolic syndrome participants**

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**ABSTRACT**

*Background & Aims.* Whilst the cardioprotective effects of blueberry intake have been shown in prospective studies and short-term randomized controlled trials (RCTs), it is unknown whether anthocyanin-rich blueberries can attenuate the postprandial, cardiometabolic dysfunction which follows energy-dense food intakes; especially in at-risk populations. We therefore examined whether adding blueberries to a high-fat/high-sugar meal affected the postprandial cardiometabolic response over 24h.

*Methods.* A parallel, double-blind RCT (n=45; age 63.4±7.4 years; 64% male; BMI 31.4±3.1 kg/m$^2$) was conducted in participants with metabolic syndrome. After baseline assessments, an energy-dense drink (969 Kcals, 64.5g fat, 84.5g carbohydrate, 17.9g protein) was consumed with either 26g (freeze-dried) blueberries (equivalent to 1 cup/150g fresh blueberries) or 26g isocaloric matched placebo. Repeat blood samples (30, 60, 90, 120, 180, 360 min and 24h), a 24h urine collection and vascular measures (at 3, 6, and 24h) were performed. Insulin and glucose, lipoprotein levels, endothelial function (flow mediated dilatation (FMD)), aortic and systemic arterial stiffness (pulse wave velocity (PWV), Augmentation Index (AIx) respectively), blood pressure (BP), and anthocyanin metabolism (serum and 24h urine) were assessed.

*Results.* Blueberries favorably affected postprandial (0 to 24 h) concentrations of glucose ($p<0.001$), insulin ($p<0.01$), total cholesterol ($p=0.04$), HDL-C, large HDL particles (L-HDL-P) (both $p<0.01$), extra-large HDL particles (XL-HDL-P; $p=0.04$) and Apo-A1 ($p=0.01$), but not LDL-C, TG, or Apo-B. After a transient higher peak glucose concentration at 1h after blueberry intake ([8.2 mmol/L, 95%CI: 7.7, 8.8] vs placebo [6.9 mmol/L, 95%CI: 6.4, 7.4]; $p=0.001$), blueberries significantly attenuated 3h glucose ([4.3 mmol/L, 95%CI: 3.8, 4.8] vs placebo [5.1 mmol/L, 95%CI: 4.6, 5.6]; $p=0.03$) and insulin concentrations (blueberry: [23.4

pmol/L, 95%CI: 15.4, 31.3] vs placebo [52.9 pmol/L, 95%CI: 41.0, 64.8]; p=0.0001). Blueberries also improved HDL-C ([1.12 mmol/L, 95%CI: 1.06, 1.19] vs placebo [1.08 mmol/L, 95%CI: 1.02, 1.14]; p=0.04) at 90 min and XL-HDLP levels ([0.38 x10-6, 95%CI: 0.35, 0.42] vs placebo [0.35 x10-6, 95%CI: 0.32, 0.39]; p=0.02) at 3h. Likewise, significant improvements were observed 6h after blueberries for HDL-C ([1.17 mmol/L, 95%CI: 1.11, 1.24] vs placebo [1.10 mmol/L, 95%CI: 1.03, 1.16]; p<0.001), Apo-A1 ([1.37 mmol/L, 95%CI: 1.32, 1.41] vs placebo [1.31 mmol/L, 95%CI: 1.27, 1.35]; p=0.003), L-HDLP ([0.70 x10-6, 95%CI: 0.60, 0.81] vs placebo [0.59 x10-6, 95%CI: 0.50, 0.68]; p=0.003) and XL-HDLP ([0.44 x10-6, 95%CI: 0.40, 0.48] vs placebo [0.40 x10-6, 95%CI: 0.36, 0.44]; p<0.001). Similarly, total cholesterol levels were significantly lower 24h after blueberries ([4.9 mmol/L, 95%CI: 4.6, 5.1] vs placebo [5.0 mmol/L, 95%CI: 4.8, 5.3]; p=0.04). Conversely, no effects were observed for FMD, PWV, AIx and BP. As anticipated, total anthocyanin-derived phenolic acid metabolite concentrations significantly increased in the 24h after blueberry intake; especially hippuric acid (6-7-fold serum increase, 10-fold urinary increase). In exploratory analysis, a range of serum/urine metabolites were associated with favorable changes in total cholesterol, HDL-C, XL-HDLP and Apo-A1) (R=0.43 to 0.50).

*Conclusions.* For the first time, in an at-risk population, we show that single-exposure to the equivalent of 1 cup blueberries (provided as freeze-dried powder) attenuates the deleterious postprandial effects of consuming an energy-dense high-fat/high-sugar meal over 24h; reducing insulinaemia and glucose levels, lowering cholesterol, and improving HDL-C, fractions of HDL-P and Apo-A1. Consequently, intake of anthocyanin-rich blueberries may reduce the acute cardiometabolic burden of energy-dense meals.

Clinical Trial Registry: NCT02035592 at www.clinicaltrials.gov

**KEYWORDS**

Metabolic syndrome

Postprandial assessment

Energy-dense meal challenge

Blueberry Anthocyanins

Flavonoids

Cardiovascular disease risk

**ABBREVIATIONS**

AIx, Augmentation index; Apo-A1, apolipoprotein A1; Apo-B, apolipoprotein B; BP, Blood pressure; cfPWV, Carotid to femoral pulse wave velocity; CRF, clinical research facility; CV, Cardiovascular; CHD, coronary heart disease; FDR, false discovery rate; FFQ, food frequency questionnaire; FMD; flow mediated dilatation; HbA1c, glycosylated haemoglobin, type A1c; HDL-P, n, high density lipoprotein particle number; L-HDL-P, n, large, high density lipoprotein particle number; MetS, metabolic syndrome; NMR, nuclear magnetic resonance; RCT, randomised controlled trial; TG, triglycerides; XL-HDL-P, n, extra large, high density lipoprotein particle number.

## INTRODUCTION

The cardioprotective benefits of blueberry intake, or their constituent (poly)phenols (particularly anthocyanins), have been previously shown to reduce cardiovascular (CV) disease and all-cause mortality [1, 2], CV incidence and type 2 diabetes [3-8]. These associations are substantiated by population-based studies observing that higher intakes of blueberries are associated with improvements in intermediate markers of CV risk; including insulin resistance [9], hypertension [10], arterial stiffness, and central blood pressure [11]. Likewise, meta-analyses of randomized controlled trials (RCTs), have shown that chronic intake of anthocyanin-rich foods or extracts improve endothelial function (*n*=9 RCTs [12]), reduce fasting and 2h postprandial glucose, HbA1c, total cholesterol and LDL-C (*n*=32 RCTs [13]) and reduce insulin resistance (*n*=19 RCTs [14]); whilst blueberry RCTs (up to 6-months [15]) reduced endothelial dysfunction [15, 16] and systemic arterial stiffness [15, 17]. Yet despite these positive findings for chronic intake, the effectiveness of blueberry intake on postprandial health remains understudied in those at elevated risk of CV disease; especially in relation to negating the health burden of high-fat/high-sugar and energy dense meals, which continue to be widely consumed.

The assessment of postprandial responses replicates the predominant, non-fasted state experienced throughout daily waking-hours and, consequently, is of particular public health relevance. Moreover, postprandial responses to meal-induced metabolic perturbations have also been shown to predict future adverse CV events [18]. To date, whilst adding blueberries to a high-carbohydrate/low-fat meal challenge improved endothelial function [19], the same study had no effect on postprandial serum concentrations of triglyceride (TG), total cholesterol, LDL-C or HDL-C [19]; and glycemic responses were also unaltered in studies when blueberries accompanied high-fat [20, 21] or carbohydrate-rich challenge meals [22-24]. Currently, no studies have concurrently fed high-fat/high-sugar meals and the available

data are further limited to predominantly young, healthy subjects with a healthy BMI (i.e. ≤25 kg/m$^2$) [19-27]. Whilst we know there is wide inter-individual variability in anthocyanin metabolism [28] and some evidence to support a role for phenolic metabolites on acute (2-24h) CV health biomarkers [29, 30], few studies have concurrently measured metabolite profiles along with cardiometabolic health endpoints for an extended period of time. Finally, although anthocyanin metabolite concentrations peak between 6 and 24h after intake [31], postprandial assessments of heath related endpoints have been traditionally assessed prior to this timeframe; at 2h [22, 23], 4h [30], or 6h after blueberry intake [19, 25]. Postprandial assessments of health biomarkers, which coincide with maximum metabolite concentrations at 24h [31], are required to determine the associations between metabolite status and health.

Therefore, in participants with metabolic syndrome, we investigated whether adding blueberries (freeze-dried) to an energy-dense, high-fat/high-sugar challenge meal, affected 24h acute postprandial glycaemic and insulinaemic control, vascular function and lipoprotein status. Additionally, we examined the extent to which anthocyanin and phenolic metabolite concentrations were associated with significant postprandial changes in the broadest portfolio of metabolic health markers conducted to date.

## MATERIALS AND METHODS

### Study Design and Participant Population

A 24 h, postprandial sub-study was conducted in adults with metabolic syndrome (MetS) (meeting ≥3 metabolic syndrome criterion [32], BMI ≥25 kg/m$^2$, aged 50-75 yr) that were enrolled in a 6- month double-blind, placebo-controlled, parallel designed intervention study [15]. The data presented, was collected on day 1 of the aforementioned 6- month study; after a 21 d run-in period of dietary restrictions (as previously described [15]). In this acute sub-study, all baseline assessments were made prior to the intake of an energy-dense meal

challenge (which resembled a thick milkshake) which also contained the first intervention sachet for those randomized to the 26 g freeze-dried blueberry (equivalent to 1 cup /150 g fresh blueberries / d) or the 26 g isocaloric placebo powder groups. The primary endpoints were repeated measures assessments of glucose and insulin levels (0 to 6 h after the energy-dense, test meal) as postprandial indicators related to insulin resistance. Postprandial lipid and lipoprotein responses (0 to 6 h), changes in vascular function (BP, FMD, AIx and cfPWV; 0 to 24 h) and blueberry metabolite responses (0-24 h), were secondary outcomes.

Key participant exclusion criteria included history of diabetes, vascular disease and cancer; current or recent past smoking history (within 6- month); use of hormone replacement therapy, hypoglycaemic or vasodilator medications (e.g. Viagra). In this pragmatic trial, statin or anti-hypertensive therapies (or a combination) were permitted after habituation (i.e. statins, ≥3 month; anti-hypertensive medication, ≥ 6- month). The study inclusion / exclusion criteria are further described elsewhere [15]. Randomization to study treatment in the main study was conducted using adaptive random-sequence allocation software (AR2007 software [33]), with four between group balancing strata incorporated (i.e. sex, number of MetS criteria, age and statin/BP medication use); see [15]. All participants in the 1 cup blueberries /d and placebo groups were invited to 'opt-in' to the postprandial sub-study; which involved consecutive and extended assessment days at the clinical facility (~10 h on day 1, ~3 h on day 2) and continuous urine collection (total 24 h urine) and blood pressure monitoring. Forty-five participants accepted the invitation to enrol in the sub-study (n = 23, 1 cup blueberry; n = 22, placebo; **Figure 1A**).

The study was approved by the National Research Ethics Committee (East of England), conducted at the NHS Clinical trials facility, University of East Anglia, UK and completed between January 2014 and November 2016. The study was registered at

www.clinicaltrials.gov (NCT02035592), followed the principles of the Declaration of Helsinki of 1975, as revised in 1983, and participants gave written consent before enrolment.

**Intervention Products and the Energy-dense Challenge Meal**

A full description of the blueberry and placebo intervention products, treatment blinding and the daily intake regimen and study compliance (in the chronic study) have been reported previously [15]. In brief, the 26 g freeze-dried blueberry powder (provided by the U.S. Highbush Blueberry Council, USA) was the equivalent of 1 cup fresh blueberries (~150 g fresh weight; containing 364 mg anthocyanin and 879 mg phenolics). The placebo was commercially produced (The National Food Lab, USA) and was an isocaloric and carbohydrate matched (glucose 31%, fructose 30%, sucrose 0%; 0 mg anthocyanin or phenolics) purple colored powder, of similar appearance (i.e. texture) and taste, to the milled freeze-dried blueberries.

The energy-dense challenge meal was commercially prepared (NIZO food research B.V., The Netherlands) as a 500 g milkshake-like emulsion with Protifar; formulated from a combination of milk protein, glucose, oil and vanilla flavouring. Each 500 g serving provided 969 kcals, 64.5 g fat (25.8 g saturates, 30.3 g monounsaturates, 8.15 g polyunsaturates), 84.5 g carbohydrate (83.5 g glucose and 1.0 g fructose) and 17.85 g protein. For comparative purposes, the energy provided by our challenge meal was similar to a widely consumed large burger, fries and cola meal [34]. Product stability was checked annually and no significant change in macronutrient composition was observed during the study. The addition of the 26 g matched blueberry or placebo powder provided a further 104 kcal, 0.6 g fat, 23.6 g carbohydrate (7.3 g glucose, 7.1 g fructose) and 1.1 g protein.

The combination of the treatment (i.e. blueberries or placebo) and the 500 g challenge meal was prepared by staff not involved in data collection or analysis. Briefly, 26 g of

intervention material was weighed and added to an opaque shaker bottle. The 500 g emulsion (challenge meal) was decanted into the bottle, with residual emulsion further liberated with 50 g water. The resultant mixture was vigorously shaken and refrigerated until required. A 15- minute target was set for consumption, which was verified by an observer; two rinses of the shaker were performed (50 g water, *per* time) to ensure complete intake compliance. The timing of test-food initiation was used to coordinate all future biological sampling and cardiometabolic measures. No further food was consumed until the 6 h cardiometabolic measurements and blood samples were taken.

**Dietary and lifestyle restrictions and standardized food intake**

For 21 d prior to the postprandial assessment, the intake of blueberries, and foods containing blueberries, was restricted. The frequency of intake for anthocyanin-rich fruits and vegetables, dark chocolate, oily fish, red wine, tea / coffee and alcohol were also restricted, as previously described [15]. To further limit the influence of background diet on fasted and postprandial analyses, nitrate/nitrite-rich foods, caffeine and alcohol were avoided for 24 h prior to and during the 24 h assessment. Low nitrate/nitrite bottled water (Buxton) also replaced all drinks during this period. The provided low flavonoid / low nitrate-nitrite meals also included; 1) identical standardized evening meals (i.e. macaroni cheese, bread rolls, rice pudding) which were consumed the night before and on the evening of the assessment day (when rice pudding was provided for men only), and 2) a standardized lunch of cream cheese filled bread rolls (1 for women, 2 for men) and Greek yoghurt with honey (fat free for women; full fat for men) (**Figure 1B)**, which was consumed after the final blood draw and the completion of the cardiometabolic assessments on the assessment day. Marginal differences in meal provisions, by sex, were implemented to balance the proportion of energy provided (by the standardized meals) relative to sex differences in daily energy requirements. Strenuous exercise was avoided for 48 h before each assessment visit. A validated food

frequency questionnaire (FFQ) [35] was assessed at the beginning of the study to confirm dietary intakes.

**Assessment of glycaemic control and biomarkers of cardiometabolic health**

After an overnight fast (≥10 h), participants were cannulated at the clinical facility and venous blood collected prior to, and then 30, 60, 90, 120, 180 and 360 min after the energy-dense meal challenge. No other food was consumed during this time (0 to 6 h) and the principal repeated measures assessment of glucose, insulin, lipid and lipoprotein levels was performed over this time frame. A further single blood sample was taken at 24 h (day 2; follow-up).

The determination of fasting and postprandial glucose, total cholesterol, HDL-C and triglyceride (TG), was conducted at the Norfolk & Norwich University Hospital, by clinical chemistry autoanalyzer (ARCHITECT c; Abbott Laboratories, Abbott Park, USA) and the Friedewald equation [36] was used to calculate LDL-C. Further aliquots of the blood samples were centrifuged, and the resultant serum and plasma aliquots stored at -80 $^{o}$C for future biomarker analysis and the quantification of anthocyanin/phenolic metabolites (as previously described [15]). Serum insulin was measured in duplicate by ELISA (Mercodia, Uppsala, Sweden) according to manufacturer's instructions. Nuclear Magnetic Resonance (NMR) spectroscopy (Nightingale Health, Helsinki, Finland) was used to further assess postprandial lipid and lipoprotein responses. The reproducibility of laboratory assessments has been previously reported [15].

Vascular function was assessed prior to- and then 3, 6 and 24 h after consuming the energy-dense challenge meal, using methodologies previously described [15]. In brief, triplicate office BP assessments (Omron 705IT, Omron Healthcare Co., Kyoto, Japan) followed supine rest (15- min) in a quiet, temperature monitored room. Thereafter, flow

mediated dilatation (FMD) was assessed by ultrasound (Philips iE33; 11-3MHz linear transducer, Philips, Surrey, UK) with electrocardiogram gating, with the maximum vessel dilation in the 5-min post-hyperemia phase (induced by 220 *mmHg* sphygmomanometric cuff inflation for 5-min) compared against the pre-occlusion diameter (1-min baseline phase); $\%FMD = (diameter_{max} - diameter_{baseline}) / diameter_{baseline} \times 100$. Commercial analysis software was used to acquire and then determine brachial artery vessel diameters (Vascular Imager, and Brachial Analyzer V5, respectively; Medical Imaging Applications LLC, Coralville, USA). Aortic distensibility and systemic arterial stiffness were assessed via carotid-femoral pulse wave velocity (cfPWV) and augmentation index (AIx; standardized to 75 bpm), respectively (both Vicorder, Smart medical, UK), with a target mean of ≤10% CV. Twenty-four hour ambulatory BP (ABP) was assessed (ultralite monitor model 90217A-1, Spacelabs healthcare, UK), with default monitoring frequencies of 3 /h (06:00 to 22:00) and 1 /h (22:00 to 06:00); see supporting information file.

Measures of body weight, height, waist and hip circumference were taken in duplicate and the BMI was subsequently calculated.

**Anthocyanin-derived Phenolic Metabolite Analysis**

At baseline (pre-test meal), a fasting serum sample and a complete urine void were collected before the energy-dense challenge meal. Thereafter, postprandial bloods (at 30, 60, 90, 120, 180, 360 min and 24 h) and the total urine passed over 24 h were collected; with timings synchronized to the initiation of the test meal. Serum was separated from blood samples, acidified using 52.5 µL/mL formic acid (>95% reagent grade, Sigma, Dorset UK), and stored at -80 °C until later analysis. Each urine passed within the research facility was; measured for volume, time-stamped, homogenised, sub-aliquoted and acidified with 32 µL/mL formic acid, then stored at -80 °C prior to each sample being analyzed separately. The

serum analysis was performed using a SCIEX UPLC coupled 6500+ Q-trap (SCIEX, Framingham, MA) electrospray ionization tandem mass spectrometer (ESI-MS/MS), with advanced scheduled multiple-reaction monitoring (ADsMRM). This method quantified *n* = 170 phenolic metabolites. Similarly, *n* = 61 urinary metabolites were quantified, as previously reported [15, 37], across three scheduled multiple reaction monitoring methods on an 1200 Agilent HPLC coupled to a SCIEX 3200 Q-trap ESI-MS/MS (Sciex, Warrington, UK). An overview of the metabolites in the MS methodology can be found in **Supplemental Table 1** in the supporting information file.

**Statistical analysis**

This postprandial study was a secondary analysis of the main study [15] and, accordingly, a retrospective power calculation was established; demonstrating that the time-by-treatment interaction for the primary outcome measure, insulin, had 76% power with 22 participants per group (GLIMMPSE software https://glimmpse.samplesizeshop.org/#). Between-group differences in participant characteristics at baseline were assessed by t-test or chi-squared test. The effect of the intervention on cardiometabolic endpoints, serum metabolite levels and total urinary levels of metabolites was analysed using a constrained linear mixed effect model where *timepoint* and the *treatment*-by- *timepoint* interaction were included as fixed factors, *participants* included as random effects, and *age*, *sex* and *BMI* included as covariates. In this baseline corrected model, *treatment* is omitted as a fixed factor; consequently baseline means, across both groups, were assumed to be equal [38]. Non-normally distributed data (assessed using the Skewness-Kurtosis test) were analysed using a generalized linear model with a link log function. In analyses of lipid, glucose and insulin levels, the data were additionally adjusted for the use of statins. Likewise, vascular assessments were adjusted for the use of statins and blood pressure medications. Serum metabolite and total urinary metabolite models were adjusted for the analysis plate number to account for batch effects. To account for

multiple testing in our principal analysis for the time (across 0 – 24 h) x treatment interactions, we separately calculated false discovery rate (FDR)-adjusted P values using the Benjamini–Hochberg procedure (reported as Q values) from the vascular, metabolite and lipid, glucose and insulin analyses. Where significant time by treatment effects were observed, indicated by a significant q value, linear combination of coefficients between groups were explored at each time point. These targeted post-hoc analyses, in already q value adjusted data, were not further adjusted and are presented as p-values.

In additional analysis, the incremental area under the curve (iAUC) was calculated using the trapezoid rule by deducting the area below baseline. Differences in iAUC were compared between treatments using a linear regression model with treatment group as the predictor and relevant confounders as described above.

For recovery of urinary metabolites (*mg*), the total void collection data was pooled into time-bins (0 to 3 h, 3 to 6 h, 6 to 24 h) and the cumulative mean recovery masses calculated. For each time bin the recovered metabolite mass in the pre-test urine collection was subtracted to adjust for baseline levels; negative values indicate a decrease in metabolite mass, compared with pre-intervention levels. The values used in the analysis of the individual metabolites were the cumulative values within each time bin minus the pre-dose value plus the value from the previous time-bin. The data were analysed using a linear mixed effect model where *treatment*, *timepoint* and the *treatment*-by- *timepoint* interaction were included as fixed factors and *participants* included as random effects.

Ambulatory blood pressure data (see **Supplemental Table 2** in the supporting information file) were analysed using a linear regression with treatment group as the predictor and age, sex, BMI and use of blood pressure or statin medications as covariates.

An exploratory correlation analysis was performed in the blueberry group (only), between analytes displaying a significant time-by-treatment interaction after accounting for multiple testing. Consequently, the iAUC of the metabolites in urine and serum (both, 0 to 24 h), which differed between intervention groups, was assessed against glucose, insulin, total cholesterol, HDL-C, large HDL particles (L-HDLP), extra-large HDL particles (XL-HDLP), and Apo-A1. Spearman's Rank Order (non-parametric) correlation was used to determine a correlation coefficient (R=).

Data were analysed using Stata version 15 (Stata Corp., College Station, USA). P-values and q-values (FDR adjusted) <0.05 were considered statistically significant.

**RESULTS**

45 participants completed the postprandial study (n=23 blueberry, n=22 placebo; see **Figure 1**). Participants were predominately male (64%), with an average BMI of 31kg/m², and aged 63yr (**Table 1**; baseline characteristics). Statin and anti-hypertensive medications were prescribed to 44% and 38% respectively (blueberry and placebo combined).

Between 0 and 24h, postprandial insulin and glucose levels differed by intervention group ($q<0.01$ and $q<0.001$ respectively; **Figure 2**). In post-hoc analysis of these primary endpoints, glucose levels were initially higher 1h after blueberry intake (8.2mmol/L, 95%CI: 7.7, 8.8) relative to placebo (6.9 mmol/L, 95%CI: 6.4, 7.4) ($p=0.001$), but from 2h onwards glucose and insulin levels tended to be lower after blueberries; significantly so at 3h (glucose: $p=0.03$; insulin: $p=0.0001$) (see Figure 2).

**Figure 1: Flow chart of study participants and intervention conduct.**

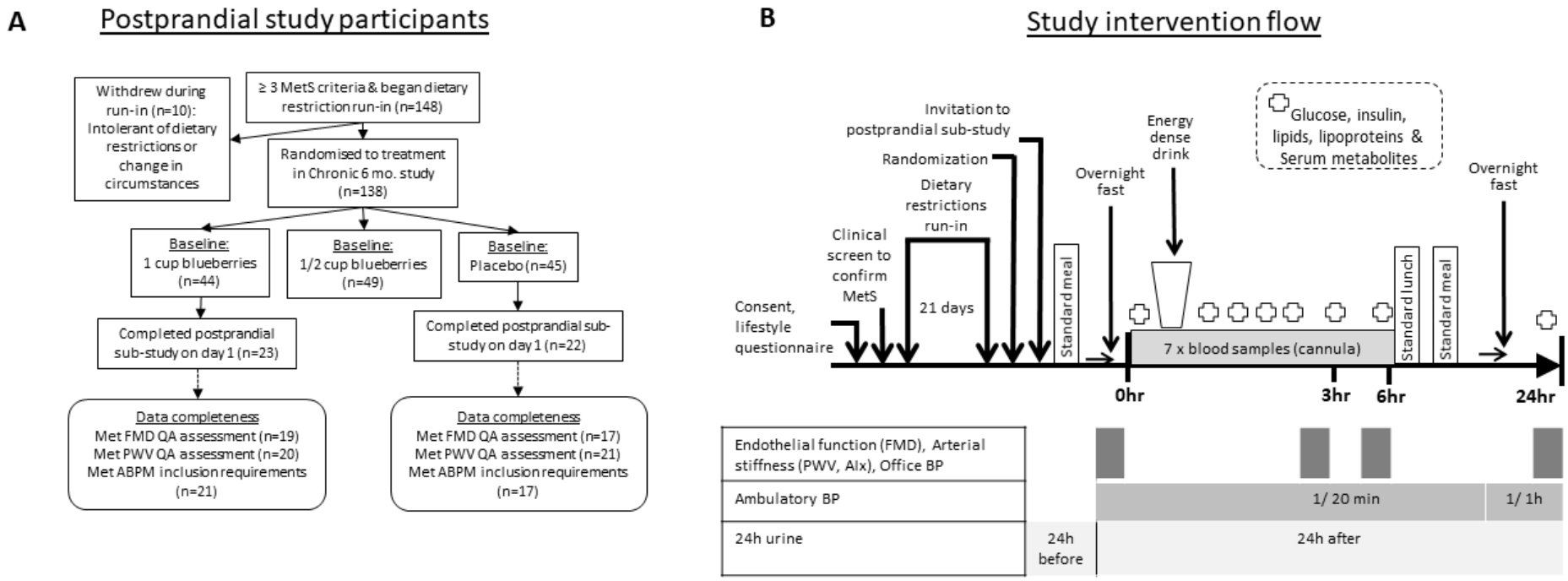


**Figure 1: A**. shows the recruitment and retention in the study; **B**. shows a schematic of the preparation for the acute postprandial study and the timings for the biological sampling and cardiometabolic assessments. BP, blood pressure; MetS, metabolic syndrome; FMD, flow mediated dilatation: QA, quality assurance assessment; PWV, pulse wave velocity; ABPM ambulatory blood pressure monitoring; AIx, augmentation index.

**Figure 2: Postprandial plasma glucose and insulin concentrations following an energy-dense meal challenge accompanied by the equivalent of 1 cup blueberries, or placebo.**

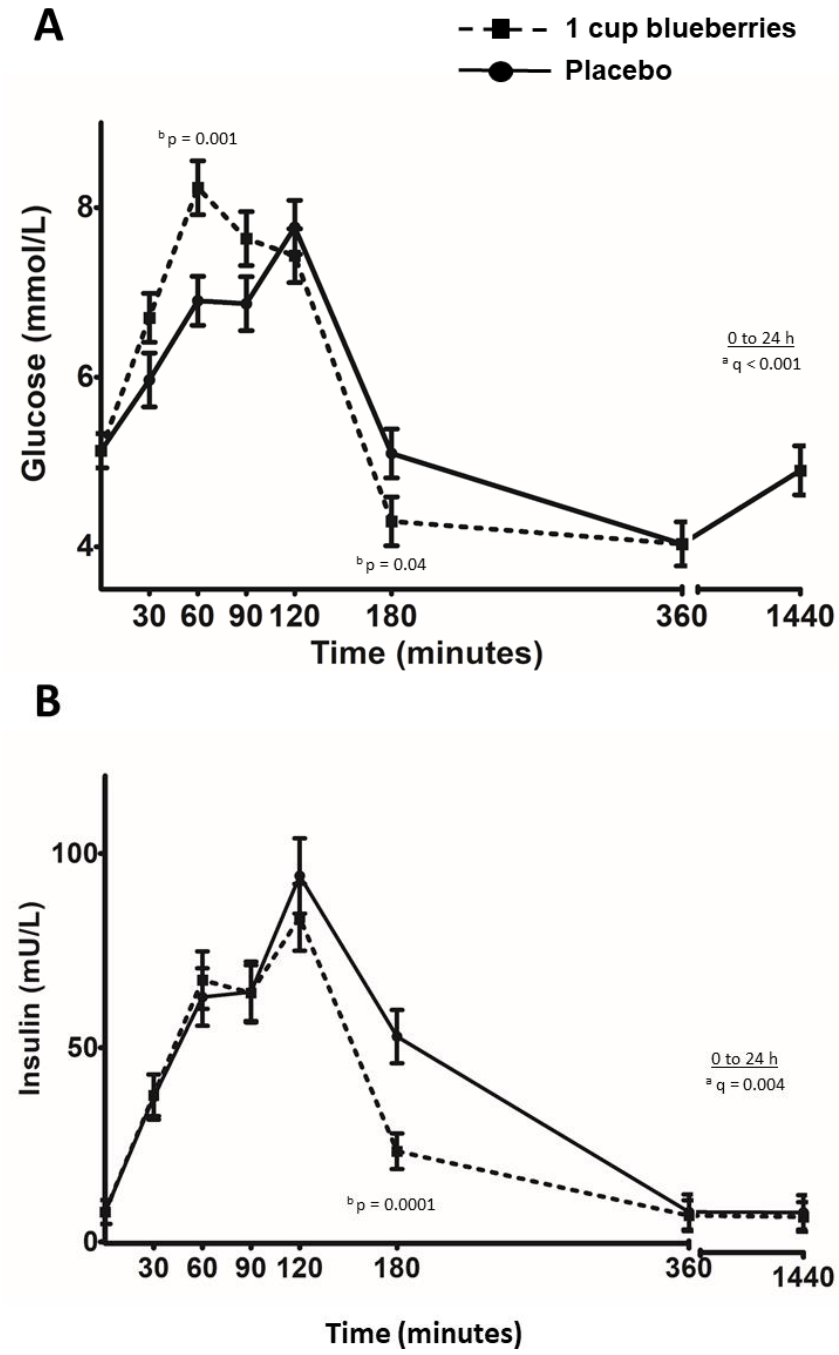


**Figure 2:** The plasma concentrations from 0 to 24 h are reported by intervention group, for; **A**. glucose (mmol/L); **B**. insulin (mU/L). Values are mean (95% CI) adjusted for age, sex, BMI, and use of statins. Adjustment for the baseline values of outcome variables were made by constraining the treatment variable in the model therefore assuming group means were equal at baseline. *P* = p values for the timepoint x treatment interaction calculated using a constrained linear mixed-effect model; [a]

indicates the repeated measures *q* value (false discovery rate (FDR) adjusted), [b] indicates a significant difference between groups, at a specific time point. Participant numbers: **A**, 1 cup (*n* = 22), placebo (*n* = 22); **B**, 1 cup (*n* = 23), placebo (*n* = 22). In panel **A**, glucose concentration was the same for both treatments at 1440 min. (4.9mmol/L; 4.4, 5.4).

Across the 24h period, concentrations of total cholesterol, HDL-C, XL-HDLP, L-HDLP and Apo-A1 significantly differed by treatment group (q=0.04; q<0.01; q=0.04; q<0.01; q=0.01, respectively) (see **Table 2**). The inclusion of blueberries attenuated the increase in total cholesterol concentrations observed in the placebo group (0 – 24h), and cholesterol levels were significantly lower at 24h specifically (p=0.04)). Similarly, blueberries attenuated the postprandial decline in HDL-C, L-HDLP, XL-HDLP and Apo-A1 concentrations observed in the placebo group; with post-hoc analyses identifying specific benefits of blueberries between 90 min and 6h (see Table 2).

Over the 24 h assessment period, no effect of the intervention on FMD, PWV, AIX or blood pressure (within clinic (**Table 3**) or via ABPM assessment (see **Supplemental Table 2** in the supporting information file)) were observed. There were also no beneficial effects observed for LDL-C, TG or Apo-B. As expected, however, there were significantly greater amounts of total anthocyanin and phenolic metabolites in serum and urine over the 24h which followed blueberry intake (both p<0.001); with higher concentrations in serum at 6 ([14.56 *uM*, 95%CI: 13.0, 16.1] vs placebo [12.02 *uM*, 95%CI: 10.5, 13.6]; p=0.01) and 24h ([15.36 *uM*, 95%CI: 13.8, 16.9] vs placebo [12.4 *uM*, 95%CI: 10.9, 14.0]; p<0.001), and urinary recovery at 6-24h ([144057 *mg*, 95%CI: 114625, 173489] vs placebo [70938 *mg*, 95%CI: 53154, 88723]; p <0.01) (see **Supplemental Tables 3 and 4** in the supporting information file). When each metabolite was assessed individually (time by treatment interaction; repeated measures, with false discovery rate adjustment), 10 serum (by concentrations) and 12 recovered urinary metabolites (by mass) differed significantly between groups during the

0 to 24h assessment period (see **Table 4** and **Table 5,** respectively). Notably, differences in serum metabolite concentrations occurred as early as 2h (see Table 4), whereas urinary metabolites differed from 6h onwards (see Table 5). Of the 4 serum metabolites that were elevated at 6h after blueberry intake, *hippuric acid and hippuric acid sulfate,* remained elevated at 24h and were 6-7-fold higher than the placebo group (Table 4). Similarly, along with *3-hydroxyhippuric acid*, the most abundant urinary metabolite recovery was *hippuric acid* which was ~10-fold higher (total recovery by mass) after blueberry intake (cumulative 0-24 h assessment) compared to placebo. Other urinary metabolites, that were significantly higher after blueberries (cumulative mass, 0-24h), included *4-hydroxy-3,5-dimethoxybenzoic acid*, *4-methoxybenzoic acid-3-glucuronide* (*isovanillic acid-glucuronide)*, *3-O-caffeoylquinic acid*, *3,4-dihydroxybenzoic acid* (*protocatechuic acid),* and *3,4-dihydroxyphenylacetic acid (DOPAC*) (Table 5).

In exploratory correlation analyses in the blueberry group only, which assessed iAUCs of the significantly changed variables (metabolites and cardiometabolic outcomes), a number of potentially favourable associations were identified across the 0-24 h data (see **Supplemental Tables 5** in the supporting information file). In serum, the iAUC of *3-methoxyphenylacetic acid-4-sulfate* was positively correlated with Apo-A1 (R= 0.44, p=0.04) and the iAUC of *hippuric acid* and *hippuric acid-sulfate* were positively correlated with iAUC XL-HDLP concentrations (R= 0.43, p=0.04; and R= 0.45, p=0.03, respectively). In urine, the iAUC for *benzoylglutamic acid* was positively correlated with iAUC of HDL-C (R=0.50; p=0.02) and negatively associated with the iAUC of total cholesterol (R=-0.48; p=0.03). Likewise, *benzoic acid-4-sulfate* was similarly negatively correlated with iAUC total cholesterol (R=-0.49; p=0.03).

## DISCUSSION

In the most comprehensive acute blueberry RCT to date in 'at risk' participants, we found that adding the equivalent of 1 cup of blueberries to an energy-dense, high-fat/high-sugar meal, reduced postprandial insulinaemia and glucose levels (primary endpoints) and attenuated the deleterious lipid and lipoprotein perturbations experienced by metabolic syndrome (MetS) participants in the placebo group. Notably, the postprandial improvements in HDL-C (including larger HDL particle fractions) and Apo-A1 paralleled our previous 6-month observations [15]. These findings are noteworthy because: (i) elevated postprandial glucose / impaired glucose tolerance increases CV disease risk [39, 40], (ii) MetS, and it's component criteria, are associated with postprandial dysregulation of glucose homeostasis and lipid profiles [41]; (iii) energy dense, high-fat/high-sugar foods are increasingly consumed [42, 43] and predispose to MetS [44]; and (iv) discernable cardiometabolic benefits followed a single, 1-cup portion of anthocyanin and hydroxycinnamic acid-rich blueberries.

Previously, oral glucose tolerance test (OGTT; 75g glucose load) data have shown that 2h glucose concentrations are associated with diabetes development and CV events [45] and predict CV disease deaths more accurately than fasting glucose [46]. Similarly, elevated postprandial insulinemia is positively associated with CHD risk [47]. In our at-risk population, blueberries attenuated postprandial glucose and insulin levels after a high-fat/high-sugar meal (from 2h), which was significant at 3h. Given that high-fat, high-energy foods slow gastric emptying [48, 49], our 3h favorable glucose and insulin levels may convey clinical benefit; and lower insulin levels sustained from 2 to 24h (compared with placebo), may be particularly important for the MetS population studied. As previously shown [20], peak glucose concentrations were significantly higher after blueberries, without an exaggerated insulin response (thus, not indicative of reactive hypoglycaemia); from ≥ 2 h thereafter, glucose remained within '*normal*' anticipated range for non-diabetic populations,

i.e. ≤7.8 mmol/L (140 mg/dL) at 2h [50] and at 4.0 to 5.9 mmol/L between meals. Whilst previous '*meal-challenge*' studies with blueberries showed no postprandial glucose [20-24, 26] or insulin [21, 23] improvements; younger, healthy participants have generally been assessed (mean; age range, 22-47y; BMI range, 21.9–23.8 kg/m$^2$), over a shorter duration (median 2.75h), after markedly lower energy intakes (mean; 167 – 853Kcal) [20-24, 26]). In support of our findings, other polyphenol-rich foods (i.e. pomegranate, epicatechin, orange pomace, black tea, strawberries and raspberries [51-56]) have reported improved postprandial glucose [53-56] and insulin [51, 52, 55-57] concentrations; many of which were in overweight/obesity or MetS participants [51, 52, 54-57]. Together, these data emphasise specific benefits of adding flavonoids to energy-dense meals for those at elevated CV risk.

Although the underpinning mechanisms for the postprandial glucose homeostasis observed are currently unknown, it has recently been suggested that anthocyanins favorably effect; 1) insulin-dependent pathways e.g. cellular redox status, cell signalling paths affecting insulin synthesis and secretion, or peripheral tissue insulin sensitivity via Phosphoinositide 3-kinases (PI3K) / Protein kinase B (AKT) or Peroxisome proliferator-activated receptor gamma (PPAR-) activation) and, 2) insulin-independent pathways e.g. AMP- activated protein kinase in tissues increasing energy sensing, carbohydrate digestion and glucose via α-amylase and/or α-glucosidase activity inhibition or interference with glucose transport [58]. Tangentially, we propose that a pro-insulin-resistant state was exacerbated by high-fat/high-sugar intake, and the favorable attenuation of postprandial reductions in HDL-C (including large and extra-large HDL-particle density) and Apo-A1 concentrations, mediated the severity of this response. In support of this theory, high-fat/high-energy intakes have previously reduced insulin sensitivity, whereas insulin sensitivity has been positively associated with HDL-C and Apo-A1 concentrations [59]. The lower postprandial insulin concentrations, sustained following blueberry intake, provide support for this reasoning.

High-fat meals have been shown previously (in the GOLDN study [60]) to substantially reduce HDL-C levels until 6h after intake [60]. In our study, only the blueberry group approached baseline levels at the 6h assessment; suggesting that in the absence of blueberries, MetS further suppresses the decline in postprandial HDL-C response to high-fat, high-sugar meals. Whilst the clinical relevance of maintaining higher postprandial HDL-C (and sub-fractions) is unclear, the role of the extracellular cholesterol acceptors HDL-C and Apo-A1 have been well described in relation to cardioprotection via reverse cholesterol transport [61]; in part, by being a significant determinant of macrophage cholesterol efflux capacity [62]. Whilst we observed no effect on triglycerides, total cholesterol was significantly reduced which provides further evidence of enhanced postprandial lipid removal. In similar 'at-risk' populations, mixed effects on postprandial lipoprotein levels have followed flavonoid-rich food intake; with cocoa flavanols significantly increasing HDL-C [63], while dried cranberries having no effect [64]. Further studies are needed to mechanistically identify how flavonoid-rich foods facilitate an improved lipid and lipoprotein environment; including the regulation of cholesterol removal from arterial walls and the functionality of HDL-C particles.

Unlike RCTs in healthy, young participants, which have reported vascular benefits after feeding blueberries with water [25, 65] or a high carbohydrate/low-fat meal [19], we found that endothelial function was suppressed in both groups, at all timepoints, after the high-fat/high-sugar meal. The reason for our lack of vascular response is likely to be an additive consequence of several factors. Firstly, our test meal contained 65.1g of fat (including 25.8g of saturated fat), thus it is perhaps unsurprising that endothelial function was blunted in comparison with previous '*meal-free*' or lower-fat assessments of acute blueberry intake [19, 25, 65]. In support of this rationale, it has been widely observed that meal consumption *per se* significantly reduces FMD response (assessed in meta-analysis, across

*n*=78 studies [66]) and that postprandial vascular function is significantly impaired following high-fat intakes (i.e. 36-80g), especially when the contribution of saturated fat is high (assessed across *n*=12 studies; [67]). Previously, a transient period of postprandial stress, characterised by inflammation and oxidative stress which constrains vascular responses, has been attributed to high-fat and energy dense meal intakes [68]. Yet this phenomenon alone is unlikely to have completely accounted for the lack of FMD response we observed; as other, single-dose anthocyanin-based food interventions i.e. Acai fruit smoothie [69] and Queen Garnet plum juice [68], attenuated the high-fat meal induced endothelial dysfunction in healthy, but comparatively overweight/obese participants. Likewise, intervention with quercetin rich apples [70] and enzymatically modified isoquercitrin [71], in adults with at least 1 health risk indicator (i.e. elevated BP, moderately elevated blood sugar level, raised fasting cholesterol or elevated waist circumference), resulted in greater postprandial FMD between 1 and 2h after a comparatively lower energy and fat content meal. It has previously been shown that elevated CV disease risk profiles or cardio-metabolic disorders (such as MetS) supresses postprandial FMD response [66, 67] and we consider it likely that the multimorbidity of MetS, and the compounding effect of each additional MetS criterion on cardiometabolic dysfunction, exacerbated the vascular burden of our study test meal moreso than other studies. As an indicator of the relative extent of endothelial dysfunction between studies, our MetS population's mean peak FMD at baseline of 2.1%, was notably lower than the 4% to 7.2% range of the studies which showed significant attenuation of the decline in postprandial endothelial function [68-71]. It is noteworthy that at 3h, our blueberry intervention group had a 0.5%FMD higher response than placebo, but our primary assessment of responses over 0-24 h was not significantly different. In further support of a lack of endothelial function effect (on microvascular function and FMD) in those with existing vascular dysfunction, it has been shown that acute intervention with both apple

extract and Montmorency tart cherry was ineffective in hypertensive / borderline hypertensives [72, 73]. Our data also showed no effect on 24h postprandial blood pressures, which is consistent with meta-analysis of chronic studies [74].

By assessing metabolite concentrations and health endpoints for a 24h postprandial period, which was confirmed in our previous 13-C tracer study [31] as the timing of peak anthocyanin derived metabolite concentrations, we have significantly strengthened the capacity to determine metabolite-health endpoint interactions beyond the 2-6h observations previously reported [19, 29, 30, 75]. In doing so, our data confirmed the sheer scope in the increase of metabolites from likely gut microbial origin (i.e., hippuric, benzoic, (phenyl)propanoic and phenylacetic acids) after blueberry intake, which was significantly greater in urine samples by 6h and remained elevated at 24h. The most abundant being *hippuric acid* (up to 10-fold higher than placebo at 24h). Through exploratory correlation analysis, we identified a series of metabolites which were associated with favorable lipid and lipoprotein profiles (i.e. *3-methoxyphenylacetic acid-4-sulfate, hippuric acids, benzoic acid-4-sulfate* and *benzoylglutamic acid)* and further well-controlled studies are required to a) confirm whether / how these functionally align with health, and b) to what extent the associations observed are attributable to microbial *versus* endogenous generation to the circulating pools of these metabolites (e.g. *benzoic acids* and *hippuric acids).*

To our knowledge, we present the first 24h postprandial assessment which has confirmed that the co-ingestion of freeze-dried blueberries can alleviate metabolic disturbances in glucose homeostasis and elements of dyslipidaemia (i.e. low HDL-C and elevated cholesterol), which occur following the intake of an energy-dense, high-fat/high-sugar meal. Further studies are now required to elucidate the effect of mastication, the role of the enteric nervous system (with regards to the gut-brain axis) and food matrix attributable

differences on postprandial responses, when providing whole blueberries as fresh *versus* a pragmatically selected stable, homogeneous freeze-dried powder. Despite recruiting more males, and those able to commit to extended assessments (who '*opted-in*' to this sub-study), our data provides clear evidence how polyphenol-rich foods can benefit those at elevated CV risk. Our metabolite data suggests that blueberry phytochemicals are readily, and extensively metabolized by the gut microbiome and our NMR analysis of postprandial lipoprotein kinetics, identified that attenuating the decline in HDL-C related lipoproteins (i.e. Apo-A1, HDL particles) is a key driver in recovering postprandial, cardiometabolic function. In our study, the benefits of freeze-dried blueberries, equivalent to a 1 cup fresh portion size (i.e. 150g), were evident over a 24h period demonstrating that adding anthocyanin-rich foods (in a variety of forms) to energy-dense, high-fat/high-sugar meals, should be advocated to lessen the postprandial burden in populations with multi-factorial disease risk profiles.

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## CONFLICT OF INTEREST

AC and ERB both act as advisors to the USHBC grant committee. All other authors declare no relevant conflicts of interest. The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the manuscript.

## AUTHOR CONTRIBUTIONS

Designed research; AC, EBR, PC. Conducted research; PC, LB and VvdV. Laboratory analysis of insulin; VvdV. Metabolite analyses conducted under the guidance of CK by $PC^2$ (serum) and LH (urine). Performed statistical analysis; AJ. Wrote paper; PC and AC, with contributions from CK, AJ. AC had primary responsibility for final content. All authors read and approved the final version of the manuscript.

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