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Randomized Control Trials

The effects of Aronia berry (poly)phenol supplementation on arterial function and the gut microbiome in middle aged men and women: Results from a randomized controlled trial☆

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SUMMARY

Background and Aims: Berry (poly)phenol consumption has been associated with cardioprotective benefits, however little is known on the role the gut microbiome may play on such health benefits. Our objective was to investigate the effects of aronia berry (poly)phenol consumption on cardiometabolic health and gut microbiome richness and composition in prehypertensive middle-aged men and women.
Methods: A total of 102 prehypertensive participants were included in a parallel 12-week randomized double-blind placebo-controlled trial. Volunteers were randomly allocated to daily consume an encapsulated (poly)phenol-rich aronia berry extract (Aronia, n = 51) or a matched maltodextrin placebo (Control, n = 51). Blood pressure (BP) and arterial function (office and 24 h), endothelial function (measured as flow-mediated dilation), serum biochemistry (including blood lipids), plasma and urine (poly)phenol metabolites as well as gut microbiome composition through shotgun metagenomic sequencing were monitored over the study period. Relationships between vascular outcomes, (poly)phenol metabolites and gut microbiome were investigated using an integrated multi-levels approach.
Results: A significant improvement in arterial indices measured as augmentation index (AIx) and pulse wave velocity (PWV) was found in the Aronia compared to Control group (awake Δ PWV = −0.24 m/s; 95% CI: −0.79, −0.01 m/s, P < 0.05; 24 h peripheral Δ AIx = −6.8; −11.2, −2.3, %, P = 0.003; 24 h central Δ AIx = −3.3; −5.5, −1.0, %, P = 0.006). No changes in BP, endothelial function or blood lipids were found following the intervention. Consumption of aronia (poly)phenols led to a significant increase in gut microbiome gene richness and in the abundance of butyrate-producing species such as *Lawsonibacter asaccharolyticus* and *Intestinimonas butyriciproducens* species, compared to Control group. Results from an approach including metabolomic, metagenomic and clinical outcomes highlighted associations between aronia-derived phenolic metabolites, arterial stiffness, and gut microbiome.
Conclusions: Aronia berry (poly)phenol consumption improved arterial function in prehypertensive middle-aged individuals, possibly via modulation of gut microbiome richness and composition based on the associations observed between these parameters.
Clinical trial registry: The National Institutes of Health (NIH)-randomized trial records held on the NIH ClinicalTrials.gov website (NCT03434574). *Aronia Berry Consumption on Blood Pressure*.

Abbreviations: Augmentation index, AIx; Cardiovascular disease, CVD; Diastolic blood pressure, DBP; Flow-mediated dilation, FMD; International physical activity questionnaire, IPAQ; Intention to treat, ITT; Per protocol, PP; Pulse wave analysis, PWA; Pulse wave velocity, PWV; Randomized controlled trial, RCT; Systolic blood pressure, SBP.

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1. Introduction

Over the last decades, *Aronia melanocarpa,* or black chokeberry, has gained increased attention for its high content of (poly)phenols, and potential protection against chronic diseases such as cardiovascular disease and diabetes [1–3].

Limited randomized controlled trials (RCTs) have tested the impact of aronia berry consumption on cardiometabolic biomarkers, with positive effects found on blood pressure (BP) [4,5], blood lipids [5,6] and inflammatory markers [4,5], although results are mixed and some studies reported no changes [6,7]. A major limitation of these initial RCTs is the small number of participants, mainly at high cardiovascular risk [4–7]. We recently reported improved endothelial function after aronia berry (poly)phenol consumption in healthy young men [8]; however, whether such effects occur in other segments of the general population, such as women, middle-aged or older adults is currently unknown.

Recent evidence indicates that the gut microbiome may play a key role in modulating the effect that diet has on cardiometabolic risk [9–11]. (Poly)phenols are increasingly recognized as gut microbiome modulators, favoring abundance of beneficial bacteria and increasing gut microbial diversity [12,13]. The gut microbiome also enhances (poly)phenol bioavailability via extensive colonic metabolism producing a large array of smaller phenolic compounds that can be absorbed and may be more bioactive than the parent compound [14]. Little information exists though, on the interplay between (poly) phenols, gut microbiome and cardiometabolic health. We have previously reported that cocoa and aronia berry (poly)phenol consumption led to improvements in cardiometabolic biomarkers and modulated the gut microbiome in healthy individuals, with increases in *Bifidobacteria*, *Lactobacilli* and butyrate-producing bacteria *Anaerostipes*, and decreases in *Clostridia* populations [8,12]. In a large cohort (TwinsUK, n = 1810), we reported that (poly)phenol intake correlated with microbiome α-diversity, abundance of butyrate-producing bacteria (*Lachnospira*, *Faecalibacterium*) and lower odds of prevalent obesity, independently of fibre intake [13]. Studies investigating the relationship between (poly)phenols, gut microbiome and health outcomes are needed, in particular using shotgun metagenomics, a high-resolution sequencing technology. Furthermore, the mechanisms of action involved in the effects of (poly)phenols on cardiovascular health are not yet fully understood. Some evidence suggests that that phenolic metabolites may modulate signalling pathways and receptors to induce a response involving changes in vasodilation, anti-inflammation and antioxidant levels [15]. More specifically, aronia (poly)phenols could exert beneficial effects through the regulation of antioxidant enzyme activity and expression [16,17], the modulation of CVD risks factors such as TNF-α or NF-κB [16,17], or the enhancement of the nitric oxide synthesis, via the Akt and eNOS pathway [18].

Here, we tested the effects of 12-week daily consumption of an aronia berry extract on resting and 24 h ambulatory BP and arterial indices, including arterial stiffness measured as pulse wave velocity (PWV) and augmentation index (AIx), resting endothelial function, and blood lipids in a large sample of prehypertensive, otherwise healthy middle-aged men and women. Gut microbiome richness, function, and composition were examined using shotgun metagenomics and an integrated multi-omics analysis was adopted to test the relationships between circulating metabolites, gut microbiome and clinical outcomes.

2. Methods

2.1. Study population

Healthy prehypertensive (SBP 120–139 mmHg and/or DBP 80–89 mmHg) men and women aged 40–70 years were recruited in London (UK) over 17 months. Volunteers attended a screening visit during which their health was assessed by a routine clinical examination as well as a specific medical history questionnaire. Exclusion criteria included CVD, hypertension (≥140 mmHg systolic blood pressure (SBP) and/or ≥ 90 mmHg diastolic blood pressure (DBP), obesity (as BMI≥ 30 kg/m^2), acute inflammation, diabetes mellitus and metabolic syndrome, advanced renal failure, abnormal heart rate (<50 or >100 bpm) and malignancies. Additionally, volunteers were excluded if they had allergies to berries or other significant foods, were taking food supplements less than 1 month before inclusion and/or were taking treatment for hypertension, chronic antimicrobial or antiviral treatment, smoked an irregular daily number of cigarettes (e.g., 1 cigarette one day, 20 the next day) or were planning to quit smoking in the next 6 months. Participants were eligible only if their office BP was in the prehypertensive range at both screening and pre-visit 1.

2.2. Study design

A 2-arm, double-blind, parallel RCT was conducted. All participants gave informed consent during screening. During that visit, volunteers were asked specific medical questions about their health with BP checked for eligibility. Participants were instructed to stick to their usual dietary habits and maintain normal physical activity throughout the study, which was monitored via food and exercise questionnaires.

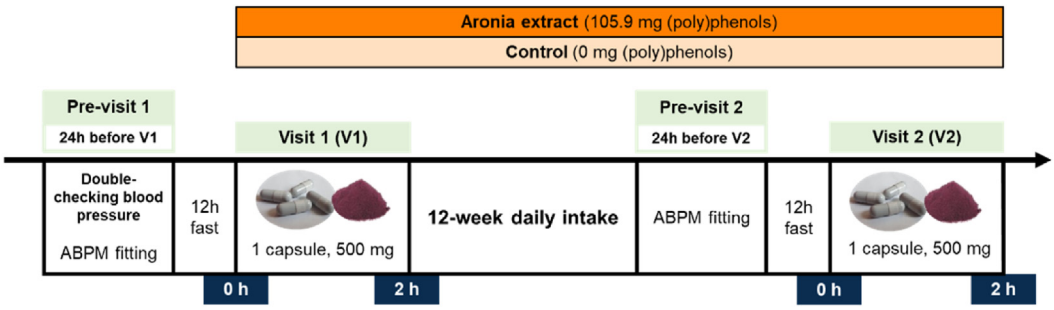
Once participants fulfilled screening criteria, they attended 4 visits as: pre-visit 1, visit 1, pre-visit 2 and visit 2 (Fig. 1).

Pre-visit 1 and visit 1 represent the baseline measurements while pre-visit 2 and visit 2 occurred after 12 weeks' daily consumption of the interventional products. Both pre-visits were short and were scheduled 24 h before visit 1 and 2. During pre-visits participants were given a urine collection kit with instructions on how to collect their urine for 24 h. In addition, 24 h ambulatory BP monitors (ABPM) were fitted on the non-dominant arm, and the first measurement was taken. Additionally, office BP was checked during pre-visit 1 to confirm consistency of the screening measurement. An independent investigator generated the randomized treatment allocation sequence using an arbitrary number generator. To capture habitual diet, and any changes occurring during the intervention, participants completed 7-day food diaries from the European Prospective Investigation of Cancer Study (EPIC Study) a week prior to each pre-visit. To assess physical activity, the validated International Physical Activity Questionnaire long-form was also filled by participants at visit 1 [19,20].

Participants were instructed to avoid caffeine, alcohol, strenuous exercise, and tobacco at least 1 h prior each visit, including the screening visit, and fast for 12 h before visit 1 and visit 2. Measurements of peripheral office BP, flow-mediated dilation (FMD), pulse-wave velocity (PWV), and augmentation index (AIx), as well as blood samples, were taken at baseline (0 h), then 2 h post-acute consumption of 1 capsule on visits 1 and 2. Fecal samples were self-collected at home by the participant, brought at both visits 1 and 2 and stored immediately at −80 °C.

Participants were instructed to take 1 capsule of the interventional product every morning with a glass of water, ideally with food, and were followed-up every month throughout the 12-weeks via email to boost compliance and to record any adverse event.

Outcomes for all measures are the differences between 0 h and 2 h at baseline ('acute effects'), or at 12 weeks ('acute on chronic effects'), or finally between 0 h at baseline and 0 h at 12 weeks ('chronic effects'), compared between aronia treatment and placebo arms as:



24-hour blood pressure collection	☑		☑	
Office blood pressure measurement	☑	☑	☑	☑
24-hour arterial stiffness collection	☑		☑	
Office arterial stiffness measurement	☑	☑	☑	☑
24-hour heart rate collection	☑		☑	
Office heart rate measurement	☑	☑	☑	☑
FMD and blood flow velocity measurement	☑	☑	☑	☑
Blood samples collection	☑	☑	☑	☑
24-hour urine sample collection	☑		☑	
Faecal sample collection	☑		☑	
7-day food diary collection	☑		☑	
Weight and body fat measurement	☑		☑	
IPAQ completion	☑			

Fig. 1. Study design. ABPM, Ambulatory blood pressure monitor; FMD, Flow mediated dilation; IPAQ, International physical activity questionnaire.

i. **Primary**: 24 h systolic and diastolic ABPM between 0 and 12 weeks.
ii. **Secondary**: office BP, heart rate, FMD, 24 h and office PWV and AIx, blood lipids (total, HDL and LDL cholesterol, triglycerides), blood cortisol levels, safety and tolerability.
ii. **Tertiary**: analysis of plasma and 24 h urine samples taken at all timepoints for aronia (poly)phenol metabolites and effects of the berry (poly)phenol extract on the gut microbiome measured using shotgun metagenomics.

The external independent investigator unblinded codes for active or placebo groups only after all study visits were completed and all data was analyzed. The trial was conducted under guidelines stated in the current revision of the Declaration of Helsinki and was approved by King's College London's Ethics Committee (RESCM-21/22–26721) with the trial registered at ClinicalTrials.gov NCT03434574. Data were collected on comprehensive case report forms from February 2018 to September 2019 in the Metabolic Research Unit of the Department of Nutritional Sciences at King's College London.

2.3. Aronia berry (poly)phenol extract and control capsules

The aronia berry extract, Aronox®, provided by Naturex SA (Avignon, France), was supplied in capsules. Active treatment capsules contained concentrated aronia berry (poly)phenol extract. One aronia berry-extract capsule contained 106 mg total (poly) phenols quantified by HPLC (200 mg gallic acid equivalent via Folin-Ciocalteu), The non-(poly)phenolic fraction of the treatment capsule contained fat, proteins, carbohydrates (sugar, dietary fibers) and minerals, all naturally coming from the berries used to manufacture the extract (Supplemental Table S1). Besides, the control capsules, identical in appearance to the active capsules, contained colored maltodextrin and no (poly)phenols. All capsules weighed 500 mg, were matched for carbohydrates, calories and fibers, and were stored in plastic bottles displaying the unique treatment allocation code and randomization number. Compliance to the intervention was measured by counting the capsules returned to the investigators by volunteers after completing the study. The (poly)phenol composition of the intervention capsule is detailed in Table 1.

2.4. Vascular measurements

ABPM24h was measured using an Arteriograph24™ (TensioMed, Budapest, Hungary) with 24-h heart rate, aortic PWV, AIx and BP_{ao} every 30 min over the 24 h between pre-visits and visits. Participants were asked to fill a 24-h activity record to track their actions throughout the day and assess their awake and asleep time. A minimum of 10 readings for awake time and 4 for asleep time were required to be included for analysis.

Office central BP parameters, including central heart rate adjusted AIx (AIx@HR75), SBP, DBP and heart rate were measured by applanation tonometry (SphygmoCor®; AtCor Medical), which uses a transfer function to synthesize the pressure waveform of the aorta. Carotid-femoral PWV was determined using tonometry at the carotid and femoral artery as previously described [21].

Office BP was measured using an automated clinical digital sphygmomanometer Omron M3 on the right upper arm, seated, after 10 min rest in a quiet room with the arm supported at heart level, legs uncrossed, back supported and with an empty bladder. Three measurements were taken 1 min apart with the last 2 averaged for the final value.

Brachial FMD was measured as previously described [8,22]. Briefly, diameter and flow velocity of the brachial artery were measured 2 cm proximal to the elbow with a 12 MHz transducer (Vivid I; GE Healthcare) and automatic edge-detection software (Brachial Analyzer; Medical Imaging Applications). Reactive hyperemia was induced by 5 min of forearm occlusion (distal to the antecubital fossa) with a sphygmomanometric cuff inflated to 180 mmHg. Blood flow was recorded at baseline in Doppler mode. Diameter was measured at baseline and immediately after cuff

deflation at 20, 40, 60, and 80 s. FMD was calculated as maximal relative diameter gain relative to baseline and was expressed as $\frac{(diameter\ 60\ sec - diameter\ baseline)}{diameter\ baseline} * 100$.

2.5. Dietary assessment of background diet

Dietary habits, using the validated EPIC Study 7-day food diary [20], were completed by participants before the first visit (baseline) and at the 11th week, before visit 2, to assess habitual diets or their change during the study. Participants were instructed to provide as much detail as possible about all food and drinks consumed, including seasoning added, information on cooking methods and brand names. Photographs were provided to help participants estimate portion sizes. The average daily macro- and micronutrient composition of each participant's diet was analyzed with the use of Nutritics (Nutritics 5.6 Research Edition, Nutritics Ltd, Dublin, Ireland) (Poly)phenol intakes were assessed with content data from both Phenol-Explorer (http://phenol-explorer.eu/) and the US Department of Agriculture (USDA) database [23–25]. Data from food diaries were coded by trained operators using a standardized operating protocol developed internally to reduce coding errors.

2.6. Biochemical analysis

Blood samples collected in EDTA/heparin tubes (Bunzl Healthcare) were centrifuged at 3000 rpm at 4 °C for 15 min immediately after collection. Plasma samples for (poly)phenol analysis were spiked with 2% formic acid and frozen at −80 °C. All clinical chemistry parameters, including total cholesterol, triglycerides, LDL and HDL cholesterol, high-sensitivity C-reactive protein (hs-CRP), cortisol, glucose, liver enzymes and whole blood count, were analyzed according to standard procedures. Samples were kept at 4° and processed on the same day (Affinity Biomarker Laboratories, London).

2.7. Urine collection

At both pre-visits 1 and 2, participants were given a 3 L urine bottle containing 3 g of ascorbic acid powder and instructed to collect all their urine from the time they left the research unit on the pre-visit until they came back 24 h after for the actual study day. They were provided a cooling bag with ice packs to keep the sample at a low temperature to avoid degradation of (poly)phenol metabolites. After returning the urine (visits 1 and 2), a representative sample was saved and centrifuged (3000 rpm, 15 min, 4 °C) immediately after return. Samples were then aliquoted with and without 2% formic acid and stored at −80 °C.

Table 1
(Poly)phenol content of intervention capsule.

	Average	CoV (%)
Procyanidins (>DP10) (%)	5.50	7.56
Total anthocyanins (%)	4.65	0.44
Cyanidin-3-galactoside (%)	*3.06*	*0.50*
Cyanidin-3-glucoside (%)	*0.14*	*3.67*
Cyanidin-3-arabinoside (%)	*1.05*	*0.59*
Cyanidin-3-xyloside (%)	*0.15*	*1.09*
Cyanidin (%)	*0.25*	*1.34*
Neochlorogenic acid (%)	3.52	0.92
Chlorogenic acid (%)	3.43	0.45
Caffeic acid (%)	1.67	1.13
Quercetin-O-glycosides (%)	1.16	2.47
Procyanidins (DP2-DP10) (%)	0.86	1.19
Rutin (%)	0.28	1.20
Quercetin (%)	0.12	1.06
Total PP quantified individually (%)	21.2	2.08
Total PP quantified individually (mg/capsule of 500 mg)	**105.9**	

CoV, coefficient of variation; DP, degree of polymerisation; PP, (poly)phenols.

2.8. Liquid Chromatography-Mass Spectrometry analysis of plasma and urine (poly)phenols

(Poly)phenol metabolites were extracted from urine and plasma samples using micro-elution solid phase extraction (μ-SPE) and measured by UPLC-Q-q-Q MS by a validated method [26]. Briefly, diluted urine samples (dilution 1:5) or undiluted plasma samples were acidified with 4% phosphoric acid (v:v 1:1). The mixture (600 μL) was loaded onto Oasis 96-well reversed-phase HLB (hydrophilic-lipophilic balanced) sorbent μ-SPE plates (Waters, Eschborn, Germany) and eluded with 90 μL of methanol after washing. Identification and quantification of (poly)phenol metabolites were performed on a SHIMADZU Triple Quadrupole Mass Spectrometer (LCMS8060, SHIMADZU, Kyoto, Japan). Eluded samples (5 μL) were injected through a Raptor Biphenyl column 2.1 × 50 mm, 1.8 μm (Restek, Bellefonte, USA) with a compatible Raptor Biphenyl Guard Cartridges 5 × 2.1 mm (Restek, Bellefonte, USA) in the UPLC system. A 14-min gradient joined by a 2-min equilibration was applied to the run under a flow rate of 0.5 mL/min at 30 °C. The metabolites in samples were identified by comparing retention times with standards in corresponding multiple reaction monitoring (MRM) transitions and quantified by calibration curves made from standard mixes using SHIMADZU LabSolutions™ LCMS Software.

2.9. Faecal sample collection and DNA extraction

Faecal samples were self-collected as close as possible to each study visit in OMNIgene GUT self-collection tubes (DNA Genotek) and stored at −80 °C on the day of the collection until further analysis. DNeasy PowerSoil Pro kits (QIAGEN, Germany) were used for the DNA extraction, and involved a bead-beating based mechanical lysis and chemical lysis of the samples. Extracted DNA samples were quantified using Qubit 4 fluorometer and Qubit™ dsDNA HS Assay Kit (Thermofisher Scientific, USA). All microbiome analysis were performed on the Per Protocol population, i.e., the Intention-To-Treat (ITT) population removed from any major protocol deviations (n = 9) and subjects failing to take at least 80% of the study treatment (n = 1). Moreover, several fecal samples were not analyzed due to the consumption of antibiotics within 3 months of visit (n = 4), missing faecal samples (n = 2), and failure to pass the quality check based on hierarchical clustering (n = 1). The remaining population considered for all microbiome analyses consisted of 85 subjects (Aronia, n = 42, and Control, n = 43).

2.10. Libraries preparation and shotgun metagenomic sequencing

Shotgun metagenomics sequencing was performed by CosmosID, Inc (Rockville, MD, USA). Briefly, DNA libraries were then prepared using the Nextera XT DNA Library Preparation Kit (Illumina Inc., USA) and Nextera Index Kit (Illumina Inc., USA). The standard protocol was used for total DNA input of 1 ng and libraries were constructed following 12 PC R cycles. A minimum of 2.2* 2 M high-quality paired end reads of 150 bp were generated per sample.

2.11. Sequencing data pre-processing

First, quality control was performed with fastp [27]. In brief, Illumina sequencing adapters were removed, low quality reads were trimmed or discarded and read too short (<60 bp) were discarded. Then, reads mapped to the human genome (CHM13 GCA_000983455.2) with bowtie 2 were removed [28]. Finally,

2.2 M* 2 high-quality paired-end reads were randomly selected in each sample with fastq-sample (github.com/fplaza/fastq-sample).

2.12. Gene abundance table generation

The gene abundance table was generated with the METEOR software suite [29]. First, selected high quality reads were mapped with bowtie 2 to the updated Integrated Gene Catalog of the human gut microbiota, comprising 10.4 million of genes [30]. Alignments with nucleotide identity <95% were discarded and gene counts were computed with a two-step procedure previously described that handles multi-mapped reads [31]. Finally, raw gene counts were normalized according to gene length.

2.13. MetaGenomic species (MGS) abundance table generation

The gene catalogue has been previously clustered into 1990 MetaGenomic Species (MGS, clusters co-abundant genes belonging to the same microbial species) with MSPminer [32,33]. The abundance of an MGS in a sample was defined as the mean abundance of its 100 marker genes (i.e., species-specific core genes that correlate the most altogether). If less than 10% of the marker genes were seen in a sample, the abundance of the MGS was considered as null. Abundances at higher taxonomic ranks were computed as the sum of the MGS that belong to a given taxa. MGS richness was assessed as the number of MGS detected in a sample (that is, whose abundance is strictly positive).

2.14. Microbiome functional potential

Three databases were used to estimate gene functional potential: Kyoto Encyclopedia of Genes and Genomes (KEGG), the eggNOG database, and the TIGRFAM [34–36]. Genes from the IGC2 catalogue were mapped with DIAMOND onto KEGG orthologs (KO) from the KEGG database (version 8.9) [37]. Each gene was assigned to the best-ranked KO among hits with e-value < 10−5 and a bit score >60. The same procedure was used with eggNOG (version 3.0). The gene catalogue was searched against TIGRFAM profiles (version 15.0) using HMMER 3.2.1 [38]. The presence of KEGG modules, Gut-Metabolic Modules (GMMs) and Gut-Brain Modules (GBMs) in MGS [39,40] was then assessed. A functional module consists in an ensemble of KOs (or NOGs, or TIGRFAMs).

2.15. Power calculation and statistical analysis

Power calculations were performed for the primary endpoint, change in 24 h BP response after chronic consumption. Based on literature and internal data, the minimum relevant differences expected in the change of SBP and DBP versus baseline between Aronia (500 mg) and Control were 6 and 4.5 mmHg respectively with expected SDs of 9 and 6.5 mmHg respectively [5,8,41–43]. Therefore, a total of 90 and 82 recruits was necessary to ensure 80% power to detect a significant difference in SBP and DBP change between treatment groups respectively for two-sided tests at 2.5% (alpha correction to Control the type I error rate due to multiple primary endpoints). Assuming a 10% drop out, 100 subjects were required into this two-treatment parallel-design study (n = 50 per arm).

Outcome analysis was conducted using ANCOVA with baseline as a covariate to compare responses due to either aronia berry extract or placebo capsule (fixed factors). Sensitivity analysis with age and baseline BMI as covariates was also performed for the primary outcome to test the influence of these factors. Response to treatment was calculated as change from baseline (CFB).

Distributions of dependent variables were verified by Shapiro Wilk testing. If not normally distributed, log-transformation was applied. If normality of the log-transformed variable could not be validated, non-parametric analysis using Mann Whitney U test without adjustment variables was performed. Adjusting Type I error rates for the primary endpoint family used the Bonferroni method. Correlations are presented as Pearson's *r* for normal, and Spearman's rho for non-normal distributions and adjustments for multiple comparisons were performed using the Benjamini–Hochberg procedure. Statistical analysis was performed with IBM SPSS Statistics 26.0 (Statistical Product and Service Solutions; IBM Corp.), R version 3.6.0 (https://www.r-project.org) and GraphPad Prism version 8 for Windows (GraphPad Software). All the statistical tests were applied over ITT population unless otherwise stated. A p-value of 0.05 was used as statistical significance threshold along with Cliff's delta as effect size measure [44]. The multi-omics data integration analysis was carried out through a parallel and vertical integration scheme [45] considering the association of features found to be significantly different between Aronia and Control groups from different datasets (that is plasma and urine metabolites, clinical variables and gut microbiome) and displayed as heatmaps of correlation using the *Hmisc* R package.

3. Results

3.1. Baseline characteristics of the study population

A total of 323 volunteers visited the unit and were screened, 221 were excluded and 102 (47 men, 55 women) were assigned randomly to Aronia or Control groups as the intention-to-treat (ITT) sample (Fig. 2).

Ninety-seven participants completed all visits (5% dropout rate) and were thus included in the acute, chronic, and acute on chronic analysis. Baseline characteristics of both intervention groups were balanced (Table 2 and Supplemental Table S2), with no significant differences between Control and Aronia group found, except for a higher asleep heart rate in the Aronia group ($p = 0.031$).

As expected from randomization, the average age was similar for both Aronia and Control groups (56.2 years), with mean BMI in the normal range (24.7 kg/m^2) and an average CVD risk in the next 10 years (QRISK®3 score) at 5.3% (Table 2 and Supplemental Table S2). Only 5 smokers were included, 4 allocated to the Control group. Participants were very active, with an average IPAQ score of 5574 ME T-min/week, well above the cutoff of 3000 ME T-min/week defining high physical activity. Participants were in the low prehypertension range with an overall average of 121.7/80.4 mmHg, and metabolic variables were within the normal range (Supplemental Table S2).

3.2. Background diet

The analysis of 7-day food diaries also showed no significant differences between the Aronia and Control groups for micro- and macro-nutrients and (poly)phenols at baseline, except a borderline higher vitamin B3 ($p = 0.047$) in Control group. Daily average baseline (poly)phenol intake was 1364 ± 705 mg in the Aronia group and 1738 ± 1287 mg in Control group. Main contributors to the intake of total (poly)phenols at baseline were coffee (29%), tea (27%) and fruits (11%) (Supplemental Table S3), with no difference after 12-weeks between groups in nutrients or (poly)phenol intake except for flavonols ($p = 0.042$), marginally higher in the Control group (Supplemental Table S4).

3.3. Effects of aronia berry (poly)phenol consumption on cardiometabolic outcomes

No significant differences were found in ITT or PP populations for our primary outcome mean brachial 24 h SBP and DBP (SBP_{br} and DBP_{br}) at 12 weeks, nor when age and BMI were included as covariates (Table 2 and Supplemental Table S5). Secondary outcomes of office peripheral and central BP were not different between groups (Table 2), although SBP decreased significantly at 12 weeks in the Aronia group compared with baseline (95% CI = −4.85, −0.38 mmHg), but not for the placebo (−2.26 to 1.71 mmHg).

The Aronia group had a significant decrease in 24 h and awake peripheral AIx_{br} and central AIx_{ao} (Δ24h AIx_{br} = −6.5%, p = 0.004; Δ24h AIx_{ao} = −3.1%, p = 0.007, Δawake AIx_{br} = −6.3%, p = 0.012; Δawake AIx_{ao} = −2.9%, p = 0.023) (Table 2). Awake PWV also significantly declined in the Aronia group compared with Controls (Δawake PWV = −0.24 m/s, p < 0.05). However, approximately 24 h after the last capsule was taken, no significant differences were found in office PWV (ΔPWV = 0.01 m/s, p = 0.8) nor AIx (ΔAIx = −0.5%, p = 0.8) measured by SphygmoCor®.

No significant differences in other secondary outcomes (FMD, blood flow velocity, heart rate, cortisol and blood lipids) were found. Changes from baseline were significant for HDL cholesterol and cortisol levels in the Aronia group only (CFB_{HDL} = +0.07, 95% CI = 0.004, 0.141 mmol/L and $CFB_{cortisol}$ = −22.5, −42.4, −2.6, mmol/L).

3.4. Tolerance, safety and compliance to the intervention

Compliance to the intervention was high, averaging 99.1 ± 5.6%. Only one participant was below the 80% compliance limit. Overall, the aronia berry (poly)phenol extract was well tolerated as consumed daily for 12 weeks, with no serious adverse event reported, and only 2 adverse events in 2 participants (both from the Aronia group) out of 102 found to be "possibly" related to the intervention. One participant experienced various symptoms including bad taste in the mouth, nausea, and change in skin texture, while another reported "feeling unwell" since the first capsule and decided to drop out after 7 weeks. Other adverse events of ad-hoc nausea, migraines, cold, or joint pain were found to be "unlikely" related or "unrelated" to the intervention. Regarding safety, of 38 blood parameters measured at baseline, all remained in the normal range after 12-weeks' intervention with no significant changes (data not shown).

3.5. Increase in plasma and urine metabolites after aronia berry extract consumption

A total of 92 phenolic metabolites were quantified in plasma and 24-h urine samples. Metabolites included derivatives of flavonoids (flavonols, n = 7; flavan-3-ols, n = 5), cinnamic acids (n = 21), benzoic acids (n = 22), hippuric acids (n = 5), benzene diols and triols (n = 7), benzaldehydes (n = 3), phenylacetic acids (n = 4), phenylpropanoic acids (n = 16) and valerolactones (n = 1). Most

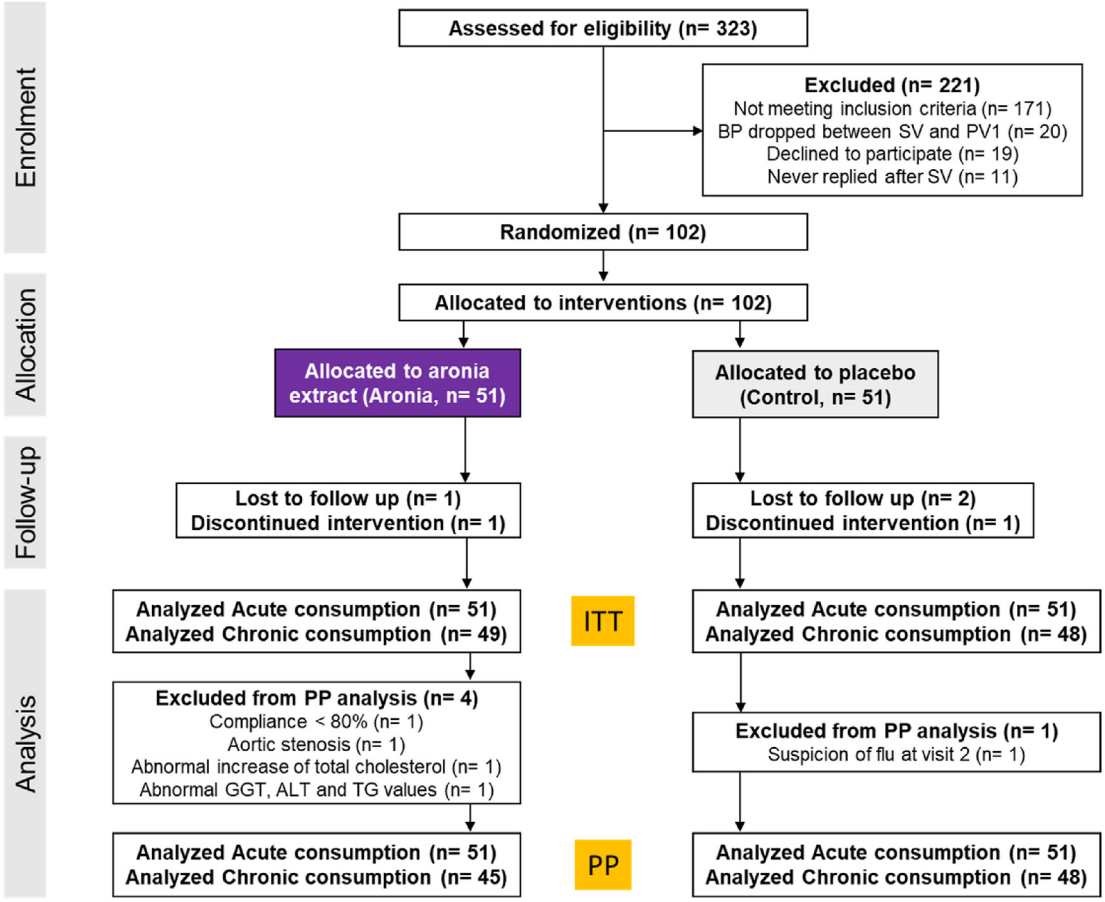


Fig. 2. CONSORT participant flowchart. ALT, Alanine aminotransferase; BP, Blood pressure; GGT, γ-glutamyl transferase; ITT, Intention to treat; PP, Per protocol; PV1, Previsit 1; SV, Screening visit; TG, Triglycerides.

Table 2
Effects of aronia berry after 12 weeks of daily consumption on vascular outcomes.

	Aronia		Control		Changes from baseline		Differences after 12 weeks
	Baseline (V1)	12 weeks (V2)	Baseline (V1)	12 weeks (V2)	Aronia	Control	Aronia versus Control
Sex (male/female, n)	23/28		24/27		–		–
Age (years)	56.2 ± 8.7		56.2 ± 9		–		–
Height (cm)	168.4 ± 8.1		170.8 ± 9.2		–		–
Weight (kg)	71 ± 9.9	71.4 ± 10.4	70.8 ± 11.6	71.1 ± 11.5	0.42 (−0.9; 0.9)	0.02 (−0.5; 0.5)	0.41 (−0.3, 1.1)
BMI (kg/m2)	25.1 ± 3.2	25.1 ± 3.1	24.3 ± 3.1	24.2 ± 3.2	0.12 (−0.06; 0.3)	−0.006 (−0.2; 0.2)	0.12 (−0.1, 0.4)
Body fat (%)	27.1 ± 8.2	27.4 ± 7.8	25.9 ± 8.9	25.6 ± 8.9	0.44 (−0.2; 1.8)	−0.12 (−0.8; 0.5)	0.56 (−0.4, 1.5)
Basal metabolic rate (Kcal)	1518 ± 257	1521 ± 271	1538 ± 303	1551 ± 314	2.6 (−9.6, 15)	3.8 (−8.8, 16)	−1.2 (−19, 16)
IPAQ (MET-min/week)	5360 ± 3663		5789 ± 3996		–		–
Smokers (n)	1		4		–		–
24 h SBP_{br} (mmHg)	121 ± 9.3	118 ± 7.4	120 ± 10.1	118 ± 8.3	−2.0 (−3.4; −0.8)*	−1.8 (−3.1; −0.5)*	−0.24 (−2.1, 1.6)
24 h DBP_{br} (mmHg)	69.7 ± 5.5	68 ± 4.7	69.5 ± 8.4	68 ± 7.1	−1.7 (−2.7; −0.7)*	−1.2 (−2.3; −0.2)*	−0.48 (−1.9, 0.9)
24 h heart rate (bpm)	70 ± 7.2	69.8 ± 7.6	69.5 ± 9.7	67.7 ± 10.4	−0.21 (−2.0; 1.6)	−1.6 (−3.3; 0.2)	1.4 (−1.2, 3.9)
24 h SBP_{ao} (mmHg)	117 ± 13.1	113 ± 9.7	116 ± 11.4	116 ± 9.9	−1.4 (−3.3; 0.4)	−0.29 (−2.2; 1.4)	−1.1 (−3.6, 1.3)
24 h AIx_{ao} (%)	30.1 ± 12	29.1 ± 10.6	29.7 ± 10.4	32.2 ± 11.4	−0.89 (−2.5; 0.7)	2.2 (0.6; 3.7)*	−3.1 (−5.3, −0.9)**
24 h AIx_{br} (%)	−14.5 ± 23.2	−16.8 ± 20.8	−15.7 ± 20.5	−10.6 ± 22.4	−2.2 (−5.3; 0.9)	4.3 (1.3; 7.3)*	−6.5 (−11, −2.1)**
24 h PWV (m/s)	9.4 ± 1.1	9.4 ± 1.2	9.2 ± 1.1	9.3 ± 1.1	−0.05 (−0.3; 0.2)	0.11 (−1.0; 0.3)	−0.16 (−0.5, 0.1)
Asleep SBP_{br} (mmHg)	108 ± 11.3	107 ± 9.5	109 ± 12.7	107 ± 10.4	−1.2 (−3.3; 0.9)	−1.4 (−3.6; 0.7)	0.24 (−2.8, 3.2)
Asleep DBP_{br} (mmHg)	59.9 ± 6.6	59.3 ± 6.6	60.6 ± 9.1	59.4 ± 7	−0.95 (−2.5; 0.6)	−0.57 (−2.2; 1.0)	−0.38 (−2.6, 1.8)
Asleep heart rate (bpm)	63.6 ± 7.2	63.3 ± 8	60 ± 8.7	59.6 ± 10.8	0.21 (−1.6; 2.6)	−0.33 (−2.2; 1.5)	0.55 (−2.1, 3.2)
Asleep SBP_{ao} (mmHg)	106 ± 15.3	104 ± 12.6	108 ± 14.6	107 ± 12.7	−1.3 (−4.1; 1.6)	−0.26 (−3.7; 2.6)	−1.0 (−5.0, 3.0)
Asleep AIx_{ao} (%)	31.9 ± 13.1	31 ± 12	33.7 ± 12.6	35.7 ± 13.9	−0.70 (−3.3; 1.9)	1.5 (−0.9; 4.1)	−2.2 (−5.8, 1.3)
Asleep AIx_{br} (%)	−11.3 ± 26	−13.2 ± 23.7	−7.7 ± 25	−3.9 ± 27.5	−1.4 (−6.3; 3.6)	3.0 (−1.9; 7.9)	−4.4 (−11, 2.6)
Asleep PWV (m/s)	8.9 ± 1.3	8.9 ± 1.3	8.7 ± 1.3	8.8 ± 1.3	−0.08 (−0.3; 0.2)	0.14 (−0.1; 0.4)	−0.22 (−0.6, 0.1)
Awake SBP_{br} (mmHg)	127 ± 9.7	125 ± 7.9	125 ± 9.4	124 ± 8.7	−1.9 (−3.5; −0.4)*	−1.8 (−3.4; −0.2)*	−0.11 (−2.4, 2.1)
Awake DBP_{br} (mmHg)	74.4 ± 5.7	72.7 ± 5	73.5 ± 8.4	72.2 ± 7.7	−1.8 (−3.0; −0.6)*	−1.3 (−2.5; −0.2)*	−0.47 (−2.1, 1.2)
Awake heart rate (bpm)	73.5 ± 8.4	73.5 ± 8.3	73.9 ± 11	71.8 ± 11	−0.12 (−2.1; 1.9)	−1.8 (−3.9; 0.2)	1.7 (−1.2, 4.6)
Awake SBP_{ao} (mmHg)	122 ± 12.8	119 ± 10.1	121 ± 10.7	121 ± 9.6	−1.8 (−3.8; 0.2)	−0.94 (−2.9; 1.3)	−0.8 (−3.6, 2.0)
Awake AIx_{ao} (%)	28.3 ± 12.8	27.3 ± 10	27.4 ± 10.6	29.7 ± 10.9	−1.1 (−3.2; 0.9)	1.8 (−0.7; 3.8)	−2.9 (−5.7, −0.2)**
Awake AIx_{br} (%)	−17.9 ± 24.7	−20.4 ± 19.7	−20.1 ± 20.7	−15.6 ± 21.4	−2.7 (−6.6; 1.3)	3.6 (−0.3; 7.5)	−6.3 (−12, −0.7)**
Awake PWV (m/s)	9.6 ± 1.1	9.6 ± 1.2	9.4 ± 1.1	9.5 ± 1	−0.15 (−0.4; 0.2)	0.13 (−0.1; 0.4)	−0.28 (−0.8, −0.01)*
Office SBP (mmHg)	123 ± 10.3	120 ± 8.3	121 ± 8.2	121 ± 8.1	−2.2 (−4.9; −0.4)*	−0.16 (−2.3; 1.7)	−2.1 (−4.7, 0.6)
Office DBP (mmHg)	81 ± 5.6	79.8 ± 5.1	79.9 ± 6.1	78.7 ± 6.8	−0.79 (−2.2; 0.6)	−1.1 (−2.6; 0.3)	0.36 (−1.6, 2.4)
Office peripheral heart rate (bpm)	67.1 ± 9.4	66.7 ± 8.5	66 ± 9.3	63.6 ± 11.1	−0.49 (−2.3; 1.3)	−2.2 (−4.4; −0.4)*	1.7 (−0.9, 4.3)
Office PWV (m/s)	7.4 ± 1.3	7.7 ± 1.7	7.5 ± 1.6	7.7 ± 1.4	0.32 (−0.7; 0.7)	0.31 (−1.0; 0.7)	0.01 (−0.6, 0.6)
Office AIx (%)	22.5 ± 11.7	21.3 ± 9.3	20.7 ± 9.8	20 ± 11.3	−0.96 (−3.5; 1.1)	−0.50 (−2.7; 1.7)	−0.46 (−3.5, 2.6)
Office central SBP (mmHg)	115 ± 10.5	113 ± 8.6	114 ± 7.9	113 ± 8.9	−1.7 (−3.6; 0.2)	−0.31 (−2.3; 1.6)	−1.4 (−4.1, 1.4)
Office central DBP (mmHg)	81.4 ± 7.1	81 ± 5.1	80.9 ± 6.1	79.6 ± 7.2	−0.10 (−1.7; 1.4)	−1.1 (−2.7; 0.4)	1.0 (−1.2, 3.3)
Office central heart rate (bpm)	62.5 ± 8.2	61.8 ± 7.6	60.5 ± 9.8	59.3 ± 10.8	−0.76 (−2.5; 1.0)	−1.0 (−2.8; 0.7)	0.26 (−2.2, 2.7)
Flow mediated dilation (%)	5.1 ± 1.4	5.2 ± 1.4	5.1 ± 1.4	5.6 ± 1.6	0.15 (−0.3; 0.6)	0.56 (0.1; 1.0)*	−0.42 (−1.0, 1.2)
Blood flow velocity (cm/sec)	92.3 ± 24.4	89.1 ± 26	86.8 ± 23	91 ± 26.1	−0.98 (−7.8; 5.8)	3.4 (−3.5; 1.3)	−4.4 (−14, 5.3)
10-year QRISK®3 score (%)	5.6 ± 3.8	5.3 ± 3.7	5.3 ± 4.2	5 ± 4.1	−0.16 (−1.4; 0.7)	−0.36 (−1.2; 0.5)	0.11 (−0.4, 0.6)
Cortisol (mmol/L)	245 ± 77.1	222 ± 68.7	239 ± 88.2	234 ± 79.6	−22 (−41; −2.2)*	−6.3 (−25; 13)	−16 (−43, 12)
Fasted plasma glucose (mmol/L)	5.2 ± 0.4	5.1 ± 0.5	5.1 ± 0.6	5 ± 0.5	−0.08 (−0.2, 0.03)	−0.15 (−0.3, −0.03)*	0.07 (−0.1, 0.2)
Cholesterol (mmol/L)	5.4 ± 0.9	5.5 ± 1.2	5.4 ± 1	5.3 ± 1	0.04 (−0.2; 0.2)	−0.12 (−0.4; 0.7)	0.15 (−0.1, 0.4)
Triglycerides (mmol/L)	0.9 ± 0.4	0.9 ± 0.5	0.9 ± 0.4	0.8 ± 0.3	0.004 (−0.78; 0.9)	−0.02 (−0.1; 0.6)	0.03 (−0.09, 0.14)
HDL (mmol/L)	1.7 ± 0.5	1.7 ± 0.6	1.7 ± 0.4	1.7 ± 0.4	0.07 (0.2; 0.1)*	0.05 (−0.1; 0.1)	0.02 (−0.08, 0.11)
LDL (mmol/L)	3.5 ± 1.1	3.7 ± 1.3	3.5 ± 0.8	3.5 ± 0.9	0.18 (−0.8; 0.4)	0.02 (−0.2; 0.2)	0.16 (−0.1, 0.4)

Values as Mean ± SD or Mean (95% confidence interval). Differences were calculated from ANCOVA (Bonferroni post-hoc test) comparing Aronia with Control changes from baseline. Changes from placebo following the 12-week intervention: *p < 0.05, **p < 0.01, ***p < 0.001. ao, aortic; br, brachial; DBP, diastolic blood pressure; PWV, pulse wave velocity; SBP, systolic blood pressure; V1, visit 1; V2, visit 2.

urinary metabolites were present at nmol and μmol concentration, while hippuric acid was at mmol concentration, even at baseline. No significant differences in plasma or urine (poly)phenols metabolites were found between the 2 groups at baseline, except for 1 urinary metabolite, 2-hydroxybenzene-1-glucuronide, which was significantly higher in the Control group (data not shown).

Following 12-week daily consumption, 5 urinary (poly)phenol metabolites (1 benzoic acid derivative: 2,3-dihydroxybenzoic acid, and 4 cinnamic acid derivatives: 4-O-caffeoylquinic acid, 5-O-caffeoylquinic acid, 3-O-feruloylquinic acid and 4-O-feruloylquinic acid) significantly increased in the Aronia compared to Control group (Supplemental Table S6). For plasma, no significant changes were found in any metabolite after 12-weeks, when measured in fasting morning blood collected 24 h after consumption of the last capsule (Supplemental Table S7). However, significantly increased plasma phenolic metabolites were found 2 h post-consumption of the aronia capsules, on day 1 (12 cinnamic acids, 5 benzoic acids and 1 flavonol derivative) and 12 weeks later (6 cinnamic acids, 5 benzoic acids and 1 phenylpropanoic acid) (Table 3).

3.6. Effects of aronia berry (poly)phenol consumption on gut microbiome diversity, composition, and function

The effects exerted by aronia berry (poly)phenols consumption on gut microbiome richness and composition were evaluated in 85 subjects of the PP population (Aronia, n = 42; Control, n = 43). We strictly focused the analysis on individuals for whom the microbiome data were available and paired for both timepoints. After 12 weeks of intervention, a significant increase in microbiome gene richness was found when considering the variation at the end of trial compared with baseline in the Aronia versus Control group (Aronia: 12,335 ± 112,117, Control: −30,053 ± 108,906, p = 0.021) (Fig. 3).

No changes in alpha diversity measured as Shannon–Wiener and Simpson indices nor beta diversity assessed via Bray–Curtis dissimilarity were found, indicating that there were no differences between groups and timepoints considering the overall gut microbiome structures (data not shown). However, we found differences in the microbial community compositions between the two intervention groups. A significant increase of several species (n = 12) in the Aronia compared to Control group was depicted such as *Intestinimonas butyriciproducens* (msp 0364, p = 0.014, effect-size = 0.30), *Lawsonibacter asaccharolyticus* (msp 0478, p = 0.04, effect-size = 0.26), *Butyricimonas faecihominis* (msp 0151, p = 0.05, effect-size = 0.23), *Bacteroides xylanisolvens* (msp 0021, p = 0.05, effect-size = 0.23) among others, along with a significant decrease of *Senegalimassilia anaerobia* (msp 1009, p = 0.03, effect-size = −0.26) and *Haemophilus parainfluenzae* (msp 0881, p = 0.03, effect-size = −0.20) taxa (Fig. 4).

Moreover, we also reported an enrichment of several species (although not statistically significant) in aronia compared to control group considering an absolute Cliff's 'delta >0.2 between aronia and control group. We notably observed an increase of *Faecalibacterium prausnitzii* 2 (msp 0893, p = 0.11, effect-size = 0.20) (Fig. 4).

The impact of the treatment was also observed in the gut functional potential. An estimation of ~2% variation in the metabolic potential after 12 weeks was captured, identified by 17 functional modules significantly different between the groups, mainly the Gut-Brain Module for the propionate production and the Gut Microbial Module leading to gamma-Aminobutyric acid (GABA) biosynthesis among others, both enriched in the Aronia compared to Control group (Supplemental Table S8).

3.7. Integrated multi-omics analysis to investigate the associations between gut microbiome, cardiometabolic outcomes and circulating (poly)phenol metabolites

Our objective was to look for potential interactions between gut microbiome species, clinical outcomes and circulating (poly)phenol metabolites. More precisely, we strictly focused on the 18 microbial taxa significantly different between the Aronia and the Control group following the 12-week intervention and we correlated changes in the abundances of bacteria with changes from baseline of urinary, plasma, and clinical variables from the different available datasets (Fig. 5 A and B).

In addition, a total of 43 plasma metabolites correlated positively with 8 different microbial species after 12-weeks' intake of aronia extract (Fig. 5 A). Among the most significant, plasma 3-hydroxy-4-methoxybenzoic acid-5-sulfate and 2-hydroxy-3/6-methoxybenzene-1-sulfate correlated with *B. xylanisolvens* ($p < 0.01$), while 2-hydroxybenzoic acid and 3-(4'-methoxyphenyl) propanoic acid-3'-sulfate correlated significantly with *Flavonifractor* and *Clostridium* species (msp 0977, $p < 0.01$ and $p < 0.001$, respectively).

In the Aronia group, 35 urinary compounds were positively related to the abundance of specific bacterial species (Fig. 5 B) - notably, 3'-hydroxyphenylacetic acid and 3-hydroxybenzoic acid-4-sulfate with *I. butyriciproducens* and *Roseburia* sp. CAG:471 (msp 0733), respectively ($p < 0.001$). Moreover, quercetin and 3-(4'-methoxyphenyl)propanoic acid-3'-sulfate were correlated with a *Christensenellales* CAG:74 (msp 0622) and *L. asaccharolyticus*, respectively ($p < 0.01$).

After aronia consumption, 27 of 36 clinical outcomes significantly correlated negatively with 11 bacterial species (Supplemental Fig. S9). The strongest negative correlations were between changes from baseline in 24 h and awake SBP_{ao} and SBP_{br} and changes in *Roseburia* sp. CAG:471 ($p < 0.001$), as well as between Asleep AIx_{ao} and AIx_{br} and changes in *Oscillospiraceae* CAG:129 (msp 0747, $p < 0.01$), indicating that higher abundance correlated with lower BP and arterial stiffness.

We observed that the increased levels of *L. asaccharolyticus* and *F. prausnitzii* species were positively associated to the increase of aronia-related urinary compound 3,4-dihydroxybenzoic acid (Spearman's rho = 0.26 and 0.29, FDR≤ 0.05, respectively). Moreover, variations in levels of such compounds were negatively correlated with the increase in arterial stiffness measured as awake PWV (Spearman's rho = −0.32, FDR≤ 0.05).

Independent Spearman's correlation analysis was run to determine the relationship between changes in urinary and plasma (poly)phenols and changes in the primary outcome (Δ24h SBP_{br}/DBP_{br}), Δoffice SBP_{br}, and significant outcomes (Δawake PWV, Δ24h AIx_{ao}, Δawake AIx_{ao}, Δ24h AIx_{br} and Δawake AIx_{br}) in the Aronia group with respect to baseline (Fig. 5 A and B). The plasma metabolite analysis revealed a significant negative correlation between Δ24h, Δawake AIx_{ao} and AIx_{br} and (4 R)-5-(3′-hydroxyphenyl)-γ-valerolactone-4′-sulfate, while Δoffice SBP_{br} was negatively correlated with 4'-methoxycinnamic acid-3'-sulfate, 4-hydroxy-3-methoxybenzoic acid, and 3-methoxybenzoic acid-4-sulfate. We also found 9 significant positive correlations between Δawake AIx_{ao} and AIx_{br} and Δ24h BP and 6 metabolites including quercetin-7-glucuronide and 3,4-dihydroxybenzaldehyde.

Regarding the urinary metabolites, 29 significant negative correlations were found for Δawake PWV (total urinary (poly)phenols, 10 hydroxycinnamic acids, 8 hydroxybenzoic acids, 3 benzene diols and triols, 3 hippuric acids, 2 phenylpropanoic acids, 1 flavonol and 1 valerolactone), 4 for Δ24h AIx_{ao} (3 hydroxybenzoic acids, 1 flavan-3-ol), 11 for Δawake AIx_{ao} (3 hydroxybenzoic acids, 3 hydroxycinnamic acids, 3 phenylpropanoic acids, 1 flavan-3-ol and 1 benzene diol), 1 for Δ24h AIx_{br} (benzoic acid) and 4 for Δawake AIx_{ao} (2 hydroxycinnamic acids, 1 benzoic acid and 1 flavan-3-ol), highlighting the improvement of these parameters along with the increase in (poly)phenols metabolites. Only 14 out of 65 correlations among the urinary metabolites were positive, including 9 related to BP (4 for Δ24h SBP_{br}, 3 for ΔDBP_{br} and 2 for Δoffice SBP_{br}).

4. Discussion

Our study investigated the effects of 12-week aronia berry (poly)phenol consumption on cardiometabolic health and gut microbiome composition in prehypertensive middle-aged adults. We found no significant effects in blood pressure (primary outcome), endothelial function or blood lipids. However, a significant improvement in 24 h ambulatory arterial indices and significant changes in gut microbiome richness, functions and composition were found between Aronia and Control groups.

4.1. Influence of participants' dietary background and overall health

The lack of BP effects contrasts with the other 2 existing studies that tested effects of aronia berry consumption on 24 h ABPM [4,46]. Kardum et al. reported decreased 24 h and awake SBP and DBP following a 4-week daily intake of aronia juice. A plausible reason for this discrepancy is that their participants were hypertensive with average baseline BPs of 141/87 mmHg. In contrast, our volunteers were prehypertensive, but borderline normotensive, with average baseline BPs of 120/70 mmHg. In comparison, the study by Loo et al. had a prehypertensive population with baseline BPs of 133/83 mmHg, and the aronia berry (poly)phenol amounts given to volunteers was 20 times higher than our intervention, suggesting that both higher BP baseline levels and higher amounts of aronia berry (poly)phenols may be needed to exert an effect on BP.

Table 3
Aronia berry-related metabolites found increased in plasma 2 h following intake of the first active capsule on day 1 and 12 weeks later.

Name	Usual name	Acute CFP (Aronia - Control)	
		95% CI (nM)	P
Flavonols			
Quercetin-3-glucuronide	–	0.2, 16.8	**0.000**
Benzoic acids			
3-Hydroxybenzoic acid-4-sulfate	Protocatechuic acid-4-sulfate	787, 2412	**0.000**
4-Hydroxybenzoic acid-3-sulfate	Protocatechuic acid-3-sulfate	516, 1239	**0.000**
4-Hydroxybenzoic acid-3-glucuronide	Protocatechuic acid-3-glucuronide	−2.1, 9.4	**0.037**
4-Hydroxy-3-methoxybenzoic acid	Vanillic acid	−82.4, 418	**0.025**
3-Methoxybenzoic acid-4-sulfate	Vanillic acid-4-sulfate	72.5, 165	**0.000**
Cinnamic acids			
4'-Hydroxycinnamic acid-3'-sulfate	Caffeic acid-3'-sulfate	99.6, 742	**0.000**
4'-Hydroxy-3'-methoxycinnamic acid	trans-Ferulic acid	24.7, 446	**0.010**
3'-Methoxycinnamic acid-4'-sulfate	Ferulic acid-4'-sulfate	74.8, 1016	**0.000**
3'-Methoxycinnamic acid-4'-glucuronide	Ferulic acid-4'-glucuronide	−111, 638	**0.003**
3'-Hydroxy-4'-methoxycinnamic acid	Isoferulic acid	25.4, 297	**0.000**
4'-Methoxycinnamic acid-3'-sulfate	Isoferulic acid-3'-sulfate	−3.9, 54	**0.042**
4'-Methoxycinnamic acid-3'-glucuronide	Isoferulic acid-3'-glucuronide	4.7, 227	**0.002**
4-O-Caffeoylquinic acid	Cryptochlorogenic acid	4.8, 19.3	**0.000**
5-O-Caffeoylquinic acid	Chlorogenic acid	32.8, 118	**0.000**
3-O-Feruloylquinic acid	–	23.9, 57.2	**0.000**
4-O-Feruloylquinic acid	–	−1.4, 16	**0.004**
4'-Hydroxycinnamic acid	p-Coumaric acid	−2.8, 118	**0.020**
Name	Usual name	Acute on chronic CFP (Aronia - Control)	
		95% CI (nM)	P
Benzoic acids			
3-Hydroxybenzoic acid-4-sulfate	Protocatechuic acid-4-sulfate	−714, 11,079	**0.000**
4-Hydroxybenzoic acid-3-sulfate	Protocatechuic acid-3-sulfate	162, 3496	**0.000**
3-Hydroxy-4-methoxybenzoic acid-5-sulfate	4-Methylgallic acid-3- sulfate	−119, 287	**0.010**
4-Hydroxy-3-methoxybenzoic acid	Vanillic acid	−157, 1669	**0.006**
3-Methoxybenzoic acid-4-sulfate	Vanillic acid-4-sulfate	−0.5, 669	**0.000**
Cinnamic acids			
4'-Hydroxycinnamic acid-3'-sulfate	Caffeic acid-3'-sulfate	−543, 1968	**0.000**
4'-Hydroxy-3'-methoxycinnamic acid	trans-Ferulic acid	−488, 2113	**0.002**
3'-Methoxycinnamic acid-4'-sulfate	Ferulic acid-4'-sulfate	−637, 2536	**0.001**
4-O-Caffeoylquinic acid	Cryptochlorogenic acid	0, 45	**0.000**
5-O-Caffeoylquinic acid	Chlorogenic acid	1.4, 268	**0.000**
3-O-Feruloylquinic acid	–	−2.1, 165	**0.000**
Phenylpropanoic acids			
3-(3'-Hydroxyphenyl)propanoic acid	–	−1429, 6496	**0.023**

Differences were calculated from ANCOVA (Bonferroni) including baseline values as covariate, comparing Aronia with Control changes from baseline. 95% CI, 95% confidence interval; CFP, changes from placebo. P-values (P) obtained following Mann–Whitney non-parametric test.

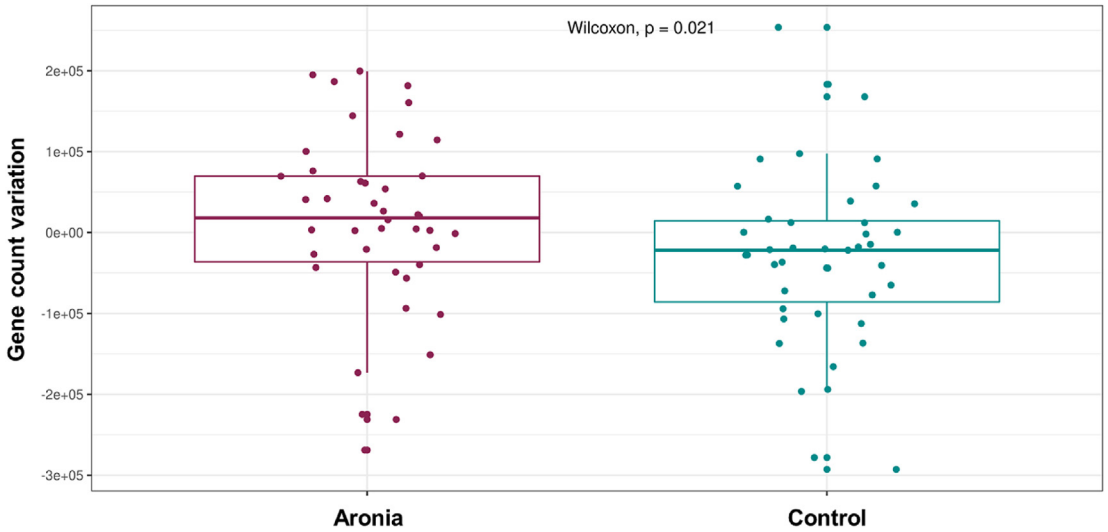


Fig. 3. Boxplot showing variation of gene count in individuals after 12-week of intervention compared to baseline. Aronia and Control groups were compared with a Wilcoxon rank-sum test.

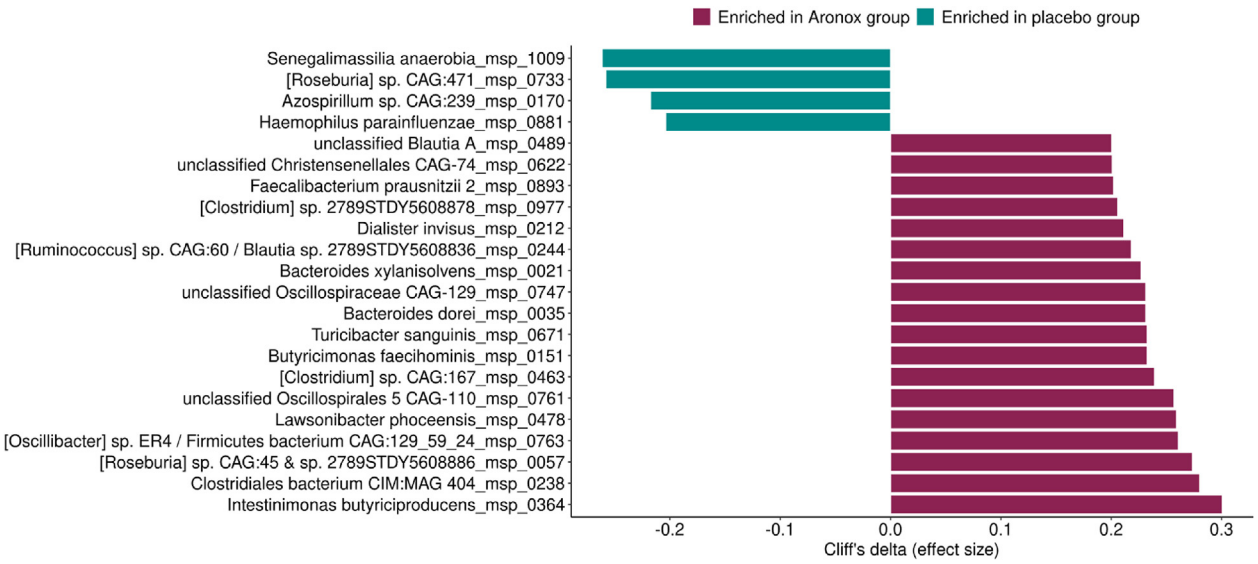


Fig. 4. Bar plot of the species significantly contrasted between Aronia and Control groups after changes from baseline. Aronia-enriched and Control-enriched species are respectively in green and burgundy. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Another plausible reason to explain the lack of BP effects here is that our volunteers followed a healthy lifestyle, with high levels of physical activity and high consumption of fruit and vegetables, fibre and (poly)phenols. Fruit and vegetable average intake was 469 g/day, above the daily 400 g ("5-a-day") recommended by Public Health England and WHO [47,48]. Participants were also close to the UK-recommended fibre intake guideline of 30 g averaging 25 g/day. Their estimated (poly)phenol intake from 7-d food diaries was 1537 ± 989 mg/day, 50% above the average 1035 ± 545 mg/day estimated in similarly-aged individuals in the UK National Diet and Nutrition Survey Rolling Programme [49] and similar to estimates from the UK "health-conscious group" of the EPIC study (1521 mg/day) [50]. The amount of aronia berry (poly)phenols administered in this study is therefore small compared with the daily (poly)phenol intake coming from the habitual diets of our volunteers here. Future studies will have to consider baseline intakes and report habitual (poly)phenol intakes throughout the study duration.

4.2. Modulation of vascular function by aronia berry (poly)phenols

Despite no changes in BP and endothelial function, improvements in arterial indices were found, with significant decreases in awake PWV, albeit borderline, and decreased 24 h and awake AIx in the Aronia group compared with the Control group. Although the clinical significance of AIx is still being explored, it is thought to represent the reflection of aortic pressure and flow waves, and as shown in meta-analyses, a higher brachial and central AIx are related to higher risk of cardiovascular event rates in follow-up [51,52]. 'Augmentation' refers to enhanced peak systolic waves in the aorta. Effects of aronia in this study may therefore be directly on the arterial wall, not picked up by forearm endothelial tests. Few other studies with (poly)phenol rich foods have found decreases in PWV and AIx without changes in blood pressure or endothelial function, such as a 4-week study on cranberry juice in patients with coronary artery disease or a 6-month isoflavone supplementation in older people, with similar decreases in PWV as observed here [53,54].

4.3. Bidirectional relationship between aronia (poly)phenols and gut microbiome and their effects on cardiovascular outcomes

It is well established that an interplay exists between host health and gut microbiome composition, and that microbiome-mediated outcomes are strongly influenced by diet [55]. Decreased gene richness of the intestinal microbiome is reported in individuals with gut dysbiosis conditions associated with insulin resistance, dyslipidemia and a more pronounced inflammatory phenotype [56] as well as in prehypertensive and hypertensive individuals and animal models [57,58]. Our findings showed that aronia berry (poly)phenols increased gene richness in the Aronia compared to Control group, and this could be linked to the positive improvements in arterial stiffness found.

In addition, several species were found to be enriched in the Aronia compared with Control group following 12-weeks' supplementation. Noteworthy, increased levels of butyrate-producer species were reported like *I. butyriciproducens* and *B. faecihominis*, taxa reported to be positively correlated with improved insulin sensitivity, lower blood pressure and lower BMI [58–60], along with *B. xylanisolvens*, a xylan-degrading bacterium. Coherently, those observations were substantiated by the increase of potential functional modules leading to the production of propionate, a diet-related gut microbial metabolite shown to play an important role in cardiometabolic health and hypertension. Although not statically significant, we also found an increase in the next-generation probiotic species *F. prausnitzii*, one of the most abundant taxa found in the gut, with a potentially important role in promoting gut homeostasis [61]. Interestingly, increased levels of those bacteria were significantly associated to a decrease in arterial stiffness and positively correlated to a circulating phenolic metabolite, 3,4-dihydroxybenzoic acid, which conjugated forms significantly increased in plasma and urine after Aronia consumption, with some evidence suggesting that it has anti-inflammatory properties in humans [62].

We further analyzed the potential interactions between gut microbiome species, clinical outcomes and circulating (poly) phenol metabolites. Strong correlations were found between the

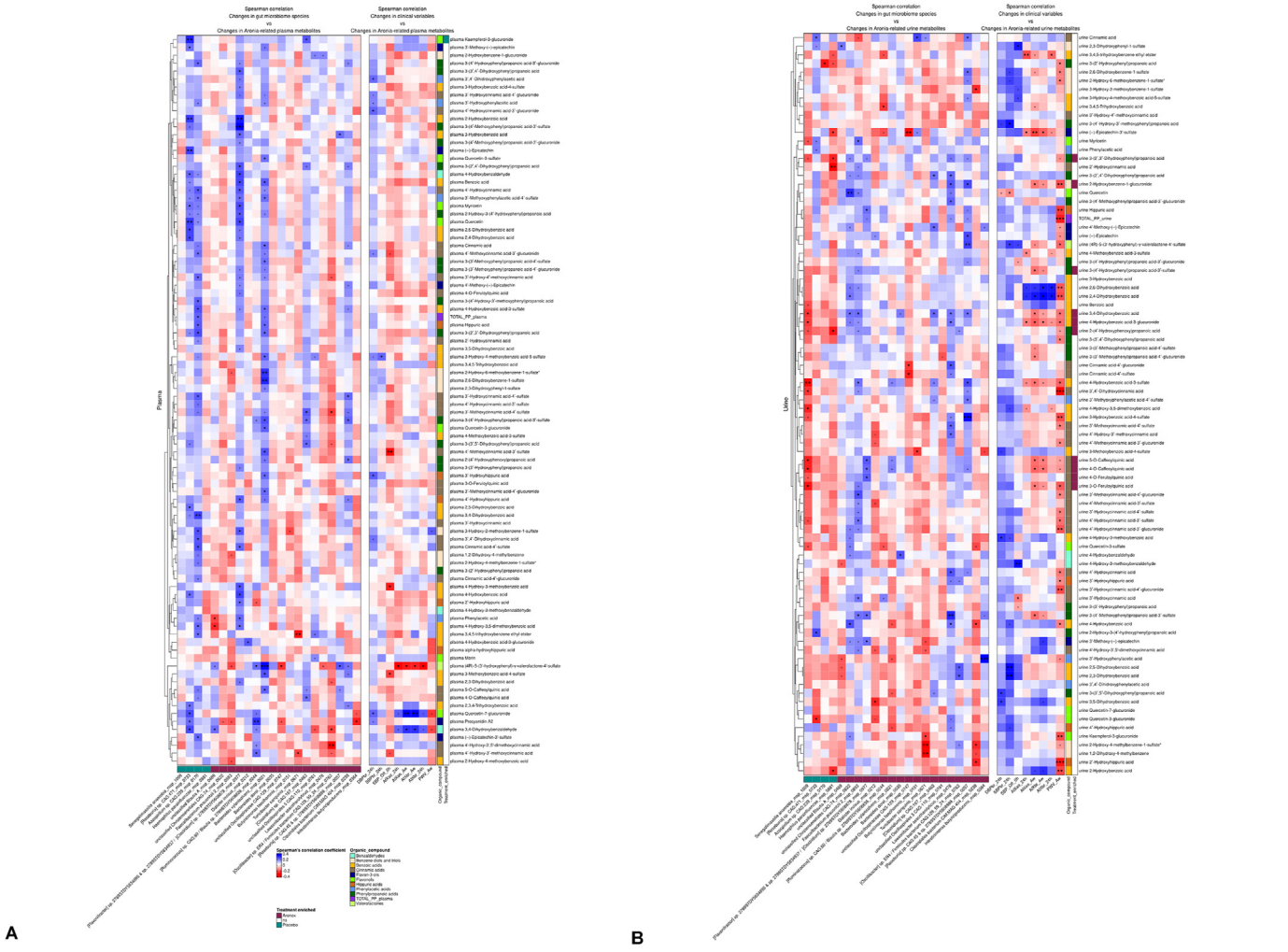


Fig. 5. Heatmap showing hierarchical Ward-linkage clustering of correlations between targeted Aronia-related compounds and significantly different features in gut microbiome, and clinical metadata (A) Plasma metabolites. (B): Urinary metabolites. The color scale represents the scaled version of Spearman's rho coefficients, with red indicating negative and blue indicating positive correlations. Adjustments were performed using the Benjamini-Hochberg procedure and rho values were filtered by keeping correlations with at least one false discovery rate (FDR) ≤ 0.05. (*FDR$\leq$ 0.05, **FDR$\leq$ 0.01, ***FDR$\leq$ 0.001). GMM, Gut Microbial Module; GBM, Gut Brain Module. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

gut microbiome and (poly)phenol metabolites, with 43 plasma metabolites correlating positively with 8 different microbial species following intake of aronia extract. A total of 23 urinary and 43 plasma phenolic metabolites, mainly cinnamic and benzoic acid derivatives, benzenes diols and triols, were significantly correlated with the decrease in PWV and AIx, in line with previous work from our team highlighting significant associations existing between plasma metabolites and enhancement of vascular function [63,64]. Finally, correlations were also found between gut microbiome and clinical outcomes, of particular importance *Oscillospiraceae* and *Oscillibacter* spp, which were significantly and negatively correlated with AIx. To our knowledge, this is the first time these species are shown to correlate with beneficial effects on arterial function.

Our data indicates that the (poly)phenols in the aronia berry extract are likely the ones responsible for the effects observed on arterial stiffness and gut microbiome in this study. The non-(poly) phenolic fraction of the aronia capsules such as macronutrients and fibers were also present in similar amounts in the control capsule, and in very small amounts as compared with the habitual ingestion from the diet, therefore unlikely to be responsible for the effects.

4.4. Strengths and limitations

The main limitation of the study was the inclusion of middle-aged individuals with a healthy lifestyle, implicating our findings cannot be extrapolated to a younger or older section of the general public, or those with different habitual diets. Moreover, despite the interesting results of the data integration analysis, this study does not allow to conclude whether there is any cause–effect relationship existing between putative health-promoting bacterial species and the vascular effects of aronia (poly)phenols. This study also has several strengths, including the use of gold standard techniques as well as a robust double-blind RCT design. The number of participants recruited (n = 102) is higher than all those from previous RCTs focused on aronia berry impacts on BP [4–8,65].

4.5. Conclusion

To conclude, the present findings suggest that daily consumption of aronia berry extract led to improvements in arterial function in healthy middle-aged people, with a concomitant and related increase in potentially health-promoting bacterial taxa. The

bidirectional relationship between (poly)phenols and gut microbiome could explain these improvements in clinical outcomes, as multiple significant associations were observed between these different parameters. Although a modest effect was found in arterial stiffness, overall aronia berry (poly)phenols supplementation did not lower blood pressure in our study population, possibly due to the healthy lifestyle of the participants. Future work should be conducted to investigated whether aronia supplementation may be effective in other at-risk populations such as hypertensives or people with CVD risk.

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Author contribution and acknowledgments

The authors' responsibilities were as follows – MLS, EF, MLecomte, PFB and ARM: Conceptualization and methodology (design and coordination of the study); MLS, FAG, DK, CD and UK: Investigation (recruitment of participants and conduction of study visits); MLS and YX: Formal analysis of the plasma and urine (poly) phenol metabolites by LC-MS; YX and SA: Formal analysis of food diaries; MLS: Formal analysis statistical analysis of clinical data, diet diaries and (poly)phenol metabolites; M Laiola and FPO: Formal bioinformatic analysis and modelling of the gut microbiome data; MLS, MLecomte, PFB, M Laiola, FPO, JKC and ARM: Writing - Original Draft, Review and Editing (wrote and revised the manuscript); and all authors: read and approved the final manuscript. EF, M Lecomte, and PFB are employed by Naturex SA, part of Givaudan. The authors would like to thank Thamarat Ahmed, Victoria Lau, Abinayah Jayanthan, Ashwa Saeed, Wenxue Pan, and Tim Wingham for their help during the study, as well as all volunteers for taking part in the trial. We also thank Anthony Sullivan, Chris Titman and Neil Loftus from Shimadzu UK Ltd. for their support on the LC–MS/MS instrument.

Conflict of interest

EF, MLecomte, PFB are employees of Naturex SA, part of Givaudan. MLS, YX, FAG, DK, CD, UK, SA, JKC and ARM were supported by a collaborative research agreement with Naturex SA, part of Givaudan. FPO and MLaiola were supported by a service agreement with Naturex SA, part of Givaudan.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clnu.2022.08.024.

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