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Effects of aronia berry (poly)phenols on vascular function and gut microbiota: a double-blind randomized controlled trial in adult men

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Short running head: Aronia Berry Consumption on Vascular Function.

Abbreviations: Randomized Controlled Trial, RCT; Augmentation Index, AI; cardiovascular disease, CVD; coronary artery disease, CAD; coronary heart disease,

CHD; flow-mediated dilation, FMD; pulse wave analysis, PWA; pulse wave velocity, PWV; randomized controlled trial, RCT; total (poly)phenols, TP; anthocyanins, ACN.

Clinical trial registry: The National Institutes of Health (NIH)-randomized trial records held on the NIH ClinicalTrials.gov website (NCT03041961). *Aronia Berry Consumption on Vascular Function.*

Abstract

Background: Aronia melanocarpa is a rich source of (poly)phenols. Previous research has demonstrated that berries may provide cardiovascular health benefits in high-risk populations. However, very few studies have investigated the effects of daily consumption of dietary achievable amounts of berries in healthy subjects.

Objective: This study aims to investigate the effects of aronia berries on vascular function and gut microbiota composition in a healthy population.

Methods: A double-blind, placebo-controlled, parallel designed study was conducted in 66 healthy men randomly allocated to a (poly)phenol rich extract (116 mg, 75 g berries), a whole fruit powder (12 mg, 10 g berries) or placebo (maltodextrin) for 12 weeks. Flow-mediated dilation, arterial stiffness, blood pressure, heart rate and serum biochemistry were assessed. Plasma (poly)phenol metabolites were analyzed using LC-MS. Gut microbiota composition was determined via 16S rRNA Sequencing in stool samples.

Results: Consumption of aronia whole fruit and extract powder for 12 weeks led to a significant increase in FMD over control of 0.9 ± 0.4% (95% CI: 0.13, 1.72) and 1.2 ± 0.4% (95% CI: 0.36, 1.97), respectively. Acute improvements in FMD were also observed 2h after consumption of aronia extract on day 1 (1.1 ± 0.3, p=0.003) and 12 weeks later (1.5 ± 0.4%, p=0.0001). Circulating plasma phenolic metabolites increased upon consumption of the aronia treatments. Although no changes were found in gut microbiota diversity, consumption of aronia extract increased the growth of Anaerostipes (+10.6%, p=0.01), while aronia whole fruit showed significant increases in Bacteroides (+193 %, p=0.01). Correlation analysis identified significant

associations between changes in FMD, aronia-derived phenolic metabolites, and specific gut microbial genera.

Conclusions: In healthy men, consumption of aronia berry (poly)phenols improved endothelial function and modulated gut microbiota composition indicating that regular aronia consumption has the potential to maintain cardiovascular health in individuals at low risk of cardiovascular disease.

. Introduction

Diet is one of the most important lifestyle factors greatly influencing the incidence and progression of cardiovascular diseases (CVD) (1). Evidence from epidemiological studies suggests that high intake of fruit and vegetables reduces the risk of developing coronary heart disease and stroke (2). This may be due, in part, to bioactive compounds present in fruits and vegetables, such as (poly)phenols (3). A growing body of evidence from human intervention studies indicates that chronic consumption of foods rich in (poly)phenols, such as tea, cocoa and berries, may improve long term vascular health (4). A limited number of randomized controlled trials (RCTs) have demonstrated significant improvements in blood lipids, blood pressure and endothelial function following consumption of berries or other anthocyanin rich foods (5-7). However, due to the low number of RCTs and large heterogeneity between study designs, study populations and interventions used, there are some inconsistencies in the findings (8).

The gut microbiome has an important influence on human health and recent evidence indicates that it can be modulated by the consumption of (poly)phenols. For example, consumption of red wine by healthy volunteers over 4 weeks was paralelled with a significant increase of several microbial communities including *Bifidobacterium,*

Bacteroides and *Enterococcus* (9). Anthocyanin microbial metabolites from red wine were also previously associated with higher levels of *Bifidobacterium,* which has been linked with beneficial health effects (10, 11). Similarly, significant increases in *Bifidobacterium* was observed 6-weeks after consumption of anthocyanin-rich wild blueberry (12). To what extent the modulation of the gut microbiome is important in the context of the cardiovascular health benefits of polyphenols, is currently not known, but recent studies suggest that gut microbial metabolism may be a key factor in explaining health benefits and mechanisms of action of polyphenols (13, 14).

One berry of growing interest is *Aronia melanocarpa*, as it has one of the highest (poly)phenol content compared with other fruits (15, 16). Only a few RCT's have been conducted with aronia berries and they have shown that chronic aronia berry consumption decreased blood pressure in individuals with CVD (17), metabolic syndrome (18) or at high risk of CVD (17, 19). Whether or not regular aronia consumption has the potential to maintain and/or improve cardiovascular health in healthy individuals at low risk of CVD, has not been evaluated. In addition, the effects of aronia berry consumption on gut microbial ecology have not been previously investigated. The primary aim of the present study was to investigate the effects of acute and chronic consumption of dietary achievable amounts of aronia berries in the form of an encapsulated powder on endothelial function, in a population of young individuals at low risk of CVD. We also investigated the effects of aronia berry (poly)phenol consumption on the gut microbiome and which correlations the gut microbiome have with vascular effects.

2. Methods

2.1. Intervention Study Subjects

Sixty-six healthy male volunteers aged 18-45 years were recruited from King's College London and the surrounding area. Health was ascertained by a routine clinical physical examination and specific medical history questionnaire. Volunteers with manifest cardiovascular disease including coronary artery disease, cerebrovascular disease and peripheral artery disease were excluded (Figure 1). Additional exclusions were: hypertension (≥140 mmHg SBP and ≥90 mmHg DBP), body mass index ≥30kg/m^2, diabetes mellitus and metabolic syndrome, acute inflammation, terminal renal failure, malignancies and abnormal heart rhythm (<60 or >100 bpm). Subjects were also excluded if they had allergies to berries or other significant foods, were using medication or other dietary supplements, smoked an irregular number of cigarettes per day or were planning to quit in the next 6 months.

2.2. Study Design

A three-arm, double-blind, parallel, randomized controlled trial was conducted (Figure 1B). Informed consent was obtained and subjects were randomized to the treatments. We investigated the effects of two formulations of aronia berry capsules on vascular function compared with a placebo control capsule. Measurements were taken at baseline and 2 h post-acute consumption, and this was repeated 12 weeks after chronic capsule consumption.

Once participants were screened and included, they attended two visits, at baseline and 12 weeks later. Participants were instructed not to alter their usual dietary habits nor their physical activity throughout the study. Participants were asked to refrain from eating high (poly)phenol products 24 h prior to the first visit, such as red wine and

beer, fruits and vegetables, berries, nuts, olive oil, coffee, chocolate or cocoa products and tea. They were specifically advised to eat dairy products (yoghurt, eggs, milk), white rice, products containing white flour, meat and fish. Dietary recalls (24 h) were completed at the start of each visit to monitor the low (poly)phenol diet. Food diaries and physical activity questionnaires were completed before and during the trial to ensure their habitual dietary and fluid intake remained consistent. Additionally, participants were fasted 12 h prior to each visit. Measurements of FMD, peripheral BP, PWV, AIx as well as blood samples were all taken at baseline (0 h) then, again 2 h post-acute consumption of one capsule (Figure 2). Stool samples were collected by the participants, using Omnigene-gut stool kits (DNA Genotek), and stored at -20°C at the beginning of each visit. The volunteers were instructed to take one capsule every morning with a glass of water. Participants were followed up three times throughout the 12-week period via phone to ensure no adverse events occurred.

The study investigated acute (0-2 h), chronic (0-12 weeks) and acute on chronic (12 weeks, 2h) effects of aronia berry (poly)phenols. For the purpose of clarity, the next definitions will be used throughout the text: acute effects (2 h vs. 0 h on day 1), chronic effects (0 h at week 12 vs. 0 h on day 1) and acute on chronic effects (2 h at week 12 vs. 0 h at week 12). The primary endpoint was endothelial function measured by flow-mediated dilation (FMD) using high-resolution ultrasound upon chronic consumption of capsules. Secondary endpoints included pulse-wave velocity (PWV), augmentation index (AIx) and blood pressure (peripheral and central) as determined automatically by a blood pressure monitoring system and applanation tonometry (Sphygmocor) after acute and chronic consumption of the treatments. Tertiary outcomes were plasma glucose, blood lipids, plasma phenolic metabolites and gut microbiota.

A team of qualified researchers enrolled participants on the study and assigned the interventions. Participants and researchers administering interventions and assessing outcomes were blinded to the intervention groups. An independent researcher generated the random allocation to treatment sequence using a random number generator with the purpose to allocate a specific number to every volunteer. This way, information about group allocation remained concealed. When study visits and analysis of primary outcome were completed, the independent researcher provided the codes for unblinding and treatment grouping. The study was conducted in accordance to the guidelines stated in the current revision of the Declaration of Helsinki. All procedures were approved by King's College London Ethics Committee (HR-15/16-3739; clinicaltrials.gov Registration number: NCT03041961). Volunteers were assessed and data were collected in detailed case report forms between February and July 2017 in the Metabolic Research Unit at the Department of Nutritional Sciences of King's College London.

2.3. Aronia berry and control capsules

The aronia powders were provided in capsules and manufactured by Naturex-DBS, LLC (Sagamore, Massachusetts, USA). The "aronia extract" capsules were concentrated extracts rich in (poly)phenols and processed by the removal of fiber and organic acids from 75 g aronia berries, containing 116 mg total (poly)phenols quantified using HPLC (200 mg via Folin Ciocalteu) (**Table 1**). The "aronia whole fruit" capsules contained the equivalent to 10 g of the whole aronia berry fruit, and 12 mg of total (poly)phenols quantified using HPLC (20 mg via Folin Ciocalteu) (Table 1). The control capsules, matched in appearance to both treatment capsules, contained maltodextrin and no (poly)phenols. The capsules were all matched in weight,

carbohydrates and calories (**Supplemental Table 1**). Capsules were stored in plastic bottles with patient randomization number and unique treatment allocation number.

2.4. Biochemical analyses

Blood samples collected in EDTA/heparin tubes (Greiner Bio-One Ltd., Gloucestershire, UK) were centrifuged (1700 *g*; 15 min; 4˚C) 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, LDL and HDL-cholesterol, TAG (enzymatic photometric assay; RocheDiagnostics), glucose, Hb_{A1c} and whole blood count were analyzed according to standard procedures (Biochemistry department, King's College Hospital, Denmark Hill, London).

2.5. Dietary assessment of background diet

To assess dietary intake, 7-day food diaries by EPIC (European Prospective Investigation of Cancer; University of Cambridge) were completed by participants before the first visit (baseline) and at 11 weeks, before attending the second visit, to ensure habitual diets did not change during the study period. Participants were instructed to provide as much detail as possible about all food and drinks consumed. Average daily macro- and micronutrient composition of participant's diet were analyzed with the use of Nutritics (Nutritics Professional Diet Analysis, version 3.74; Nutritics Ltd). Polyphenol intakes were assessed using the online free database Phenol-Explorer (http://phenol-explorer.eu).

2.6. Vascular measurements

FMD of the brachial artery was measured as previously described (20). Both operator and participant were blinded during analysis. Briefly, the diameter and flow velocity of the brachial artery was measured using a 12 MHz transducer (Vivid I, GE healthcare, Buckinghamshire, UK) and automatic edge-detection software (Brachial Analyser, Medical imaging applications, Iowa City, USA) yielding standard deviations of mean differences between repeated measurements of less than 1%. Brachial artery diameter was measured 2 cm proximal to the elbow. Reactive hyperaemia was induced by 5 min of distal lower arm occlusion with sphygmomanometric cuff inflated to 180 mm Hg. Blood flow was recorded at baseline using the Doppler mode. A forearm blood-pressure cuff was placed distal to the antecubital fossa and inflated to 180 mmHg for 5 min. Diameter was measured at baseline and immediately after cuff deflation at 20, 40, 60 and 80 sec, the diameter was assessed and FMD calculated as maximal relative diameter gain relative to baseline. The FMD was expressed as $(diameter_{max}\text{-}diameter_{baseline})/diameter_{baseline}$ x100.

Central BP parameters including AIx were measured by applanation tonometry using the SphygmoCor® (AtCor medical, Gloucestershire, UK). Via a transfer function, the pressure waveform of the ascending aorta was synthesized. PWV was determined using measurements taken at the carotid and femoral artery as previously described (21). All data analyses was conducted blinded.

2.7. *UHPLC Orbitrap MS analysis of plasma (poly)phenols*

Sample preparation and solid phase extraction for plasma (poly)phenol analysis was performed as described previously (22). The detection of plasma (poly)phenol metabolites was performed on a Exactive^{TM} Orbitrap Mass Spectrometer (Thermo Scientific, CA, USA) after separation on a Accela 1250 pump UHPLC system (Thermo

Scientific, CA, USA). The autosampler injected of 5 µL of each sample in a Zorbax Eclipse Plus RRHD column 2.1 × 50 mm, 1.8 m with a compatible Eclipse Plus guardcolumn 2.1 × 5 mm, 1.8 m (Agilent, Waldbronn, Germany). The mobile phase consisted of 0.1% HCOOH (solvent A) and acetonitrile with 0.1% HCOOH (solvent B) in a 10 min gradient program. Quantification analysis of the plasma (poly)phenols was done using Xcalibur 2.2 (Thermo Scientific, CA, USA).

2.8 Faecal sample collection and microbiome analysis

Fecal samples were collected in the week before each study visit using OMNIgene®•GUT self-collection tubes (DNA Genotek, Ottawa, Canada) and were stored in -20°C until further analysis. Microbiome analysis was performed by Clinical-Microbiomics A/S (Copenhagen, Denmark) as described elsewhere (23). Briefly, Total microbial DNA was extracted from faeces using the 96-well NucleoSpin Soil DNA Isolation Kit (Macherey-Nagel, Düren, Germany). PCR was performed with 16S rDNA primers S-D-Bact-0341-b-S-17 and reverse primer S-D-Bact-0785-a-A-21 with Illumina adapters attached (24) in order to target the V3-V4 regions. The following PCR program was used: 98 °C for 30 sec, 25x (98° C for 10 s, 55 °C for 20 s, 72 °C for 20 s), 72 °C for 5 min. Sequencing was performed on an Illumina MiSeq desktop sequencer using the MiSeq Reagent Kit V2 (Illumina, San Diego) for 2x250 bp paired-end sequencing.

The 64-bit version of USEARCH (25) and mothur (26) were used in combination with several in-house programs for bioinformatics analysis of the sequence data. Following tag identification and trimming, all sequences from all samples were pooled. Sequences were clustered at 97 % sequence similarity. Additional suspected chimeric OTUs were discarded based on comparison with the Ribosomal Database Project

classifier training set v9 (27) using UCHIME (28). Taxonomic assignment of OTUs was done using the method by Wang et al. (29) using the database from the Ribosomal Database Project. To find modifications in the microbiota structure associated with the aronia treatments, the samples were also longitudinally analyzed by RandomForest. Briefly, Random Forest is a powerful classifier that identifies the best subset of features (here, relative genus abundance) that can discriminate between categories (time points). In particular, we applied the algorithm to the three groups separetely (aronia extract, aronia whole fruit and control). The significance in the abundance of the relevant taxa were validated by Wilcoxon signed-rank tests.

2.9. Power calculation and statistical analysis

Power calculations were performed for the primary end point: change in FMD response after chronic consumption. The power was based on the inter-individual variability for FMD measurement of the operator (SD = 1%). Assuming an 80% power, and a 0.05 significance level, the total number of subjects required to provide sufficient power to detect a 1% difference change in FMD in a three-arm parallel study is 60 (n=20 per arm). Assuming a 10% drop out, 22 participants per arm were recruited. Changes in FMD (%) between control and treatment groups were tested using a one-way ANOVA with Tukey's post-hoc test. A two-way ANOVA with Tukey's post-hoc test was performed on dietary assessment data to test for any significant differences in time and treatment. One-way ANCOVA with BMI and chronic (poly)phenol intake as covariates was performed on baseline dietary assessment data to ensure there were no differences in polyphenol, micro- or macronutrient intakes. Correlations are presented as Pearson's r for non-normal distribution and as Spearman for normal distribution. Statistical analysis were performed with the use of IBM SPSS Statistics

22.0 (Statistical Product and Service Solutions; IBM Corp) and GraphPad Prism version 7 for Windows, (GraphPad Software, La Jolla California USA). Statistical significance is accepted at $p<0.05$.

3. Results

3.1. Participant flowchart and baseline characteristics

A total of 84 volunteers were considered for participation in the study, of which 18 were excluded and 66 were included and randomised into the three intervention groups (**Figure 1**). The first study visit started in February and the last study visit ended in July 2017. A total of 3 follow-up calls were performed per participant between study visits. Two participants discontinued the study after the first visit, and 64 completed both visits (drop-out rate of 3%). The baseline characteristics of the different groups of healthy young men were all within the normal range and no differences were found between treatment groups (**Table 2**).

3.2. Safety and tolerance of the interventions

All the treatments were well tolerated and only 4 potential adverse events were reported over the course of the study, and were considered unrelated to the treatments: 2 of the participants in the aronia extract group reported unusual tiredness, 1 volunteer in the aronia whole fruit group reported a persistent cough for a few weeks and in the control group, 1 volunteer reported food poisoning. **Supplemental Table 2** shows the 10 safety parameters assessed, which remained in the normal healthy range after 12 weeks of treatment.

3.4. Dietary assessment of food diaries and evaluation of background diet

Analysis of the 7-day food diaries of study participants revealed no significant differences in micro- and macronutrient intakes as well as polyphenol intake between any treatment group prior to the start of the study (**Supplemental Table 3**). At baseline participants had an average daily (poly)phenol intake of 531 ± 357 mg, of which 23 ± 8 mg of that being anthocyanins (Supplemental Table 3).

3.3. Efficacy of aronia interventions on vascular function

Our primary outcome was changes in FMD after 12 weeks chronic supplementation. Repetitive intake of aronia extract and aronia whole fruit significantly improved FMD by 1.0 ± 0.2 % and 0.8 ± 0.3 % at baseline of week 12 in comparison to baseline of day 1, respectively. When compared to control treatment, changes in FMD after aronia extract and aronia whole fruit consumption were significantly higher by 1.2 % (95 % CI: 0.36, 1.97) and 0.9 % (95 % CI: 0.13, 1.72), respectively (Figure 2A**,** **Supplemental Table 4**).

Acute improvements in FMD were also investigated with a significant increase in FMD of 1.4 ± 0.2 % and 1.5 ± 0.2 % observed after 2 h consumption of aronia extract on day 1 and week 12 in comparison with baseline, respectively (Figure 2B, Supplemental Table 4). In comparison with control, aronia extract increased significantly by 1.1 % (95 % CI: 0.37, 1.78) and 1.7 % (95 % CI: 0.62, 2.34) after 2 h on day 1 and week 12, respectively (Figure 2C). No significant acute FMD changes with respect to baseline or control were observed for the aronia whole fruit group (Figure 2, Supplemental Table 4).

No significant differences in the secondary outcomes including peripheral and central blood pressure, arterial stiffness and blood lipids were observed in any of the treatment groups (Supplemental Tables 4 and **5**).

3.5 Phenolic metabolites increase in plasma after aronia consumption

Detailed targeted metabolomics analysis of plasma samples was performed and 63 phenolic metabolites were quantified at baseline and after consumption of all capsules, including derivatives of hippuric acids, benzoic acids, hydroxycinnamic acids, phenylacetic acids, propionic acids, benzaldehydes, catechols, pyrogallols, flavonols

and valerolactones (**Supplemental Figure 1**). Most metabolites were present in nanomolar concentrations, except for hippuric acid, benzoic acid, phenylacetic acid and 3-(4-hydroxyphenyl)propionic acid, which were present at micromolar levels even at baseline. The aronia extract group showed increases in total plasma (poly)phenol concentrations of 166 ± 171 µM and 30 ± 156 µM after 2 h and 12 weeks, respectively. The whole aronia fruit group also showed total plasma (poly)phenol increases of 43 ± 125 µM and 14 ± 106 µM after 2 h and 12 weeks of consumption, respectively. No significant differences in total plasma (poly)phenols were found between the three intervention groups at baseline (One-way ANCOVA with BMI and chronic (poly)phenol intakes as covariates (Cohen's f <0.1)), with the exception of phenylacetic acid ($p=0.01$), 2-hydroxybenzoic acid ($p<0.001$), homovanillic acid ($p=0.048$) and homovanillic acid sulfate ($p=0.04$), which were significantly higher in the aronia whole fruit group (data not shown).

At 2 h postconsumption, 48 compounds increased significantly with respect to baseline in the aronia extract group (19 hydroxycinnamic acid derivatives, 13 benzoic acids, 5 flavonols, 4 phenylacetic acids, 2 propionic acids, 2 benzaldehydes, 1 hippuric, 1 pyrogallol and 1 valerolactone), while 22 compounds increased significantly after consumption of the aronia whole fruit (9 benzoic acids, 4 hydroxycinnamic acids, 3 phenylacetic acids, 2 flavonols, 2 benzaldehydes, 1 hippuric and 1 pyrogallol derivative). Only one compound, 1-Methylpyrogallol-*O*-sulfate, increased significantly after consumption of the control capsule.

Chronic intake of the capsules for 12 weeks led to significantly increased fasting plasma levels of 18 compounds in the aronia extract group, 10 compounds in the whole berry group and 4 compounds in the control group. The increases observed in

the aronia berry groups were predominantly driven by phenylacetic acids, benzoic acids, hydroxycinnamic acids, flavonols and benzaldehydes (**Figure 3**).

On week 12, plasma phenolic metabolites also increased significantly 2 h post-consumption of aronia extract (2 hippuric acids, 5 benzoic acids, 7 hydroxycinnamic acid derivatives, 2 phenylacetic acids, 2 benzaldehydes, 2 flavonols, 1 propionic acid and 1 valerolactone) and aronia whole fruit (2 hippuric acids, 5 benzoic acids, 6 hydroxycinnamic acid derivatives, 2 benzaldehydes, 1 phenylacetic acid, 1 propionic acid, 1 catechol derivative and 1 flavonol), while only 1 compound (phenylacetic acid) increased in the control group.

Correlation analysis between changes in plasma metabolites and changes in FMD with respect to baseline revealed significant correlations in 20 metabolites after aronia extract consumption and in 5 metabolites after consumption of the whole fruit treatment (**Table 3**).

3.6 Effects of aronia berry consumption on gut microbial abundancy

Faecal samples were taken on the first and last day of the study to conduct genomic analysis of microbial communities. To test the hypothesis that aronia supplements can lead the human gut microbiome to different configurations, we first analyzed the overall microbiome diversity - described in terms of the diversity within a sample, (i.e. alpha diversity) and between samples (beta diversity). Microbial diversity was very high and not significant in any of the treatment groups after aronia intake (data not shown). Next we visualized the data on a broad scale by principal component analysis and observed that *Anaerostipes* and *Bifidobacterium* clustered towards opposite directions as compared to *Faecalibacterium and Clostridium* genera (**Supplemental Figure 2**).

Random Forests was applied to predict the bacterial genera that would discriminate between the different treatment groups. Treatment-discriminatory bacterial genera were identified with distinctive modifications in their relative abundances, which were validated by Wilcoxon signed-rank tests with the following results: the aronia extract group had a significant higher abundance of *Anaerostipes* (+10.6%, p=0.01), and the aronia whole fruit group showed significant increases in *Bacteroides* (+193 %, p=0.01), whereas *Clostridium XiVb* was significantly higher (+2.5 %, p=0.01) after placebo treatment (**Figure 4**). The difference of the changes in % abundancy were also calculated between treatment groups and revealed significant higher increases in *Anaerostipes* (21 %, p=0.04) when comparing aronia extract group to placebo group (Figure 4).

3.7 Correlations between gut microbiome, plasma (poly)phenol metabolites and FMD

To explore the relationship between the gut microbiome and (poly)phenol metabolism, correlation heatmaps were created (**Figure 5**). The change in a subset of metabolites measured in circulation after 12-week intake of extract capsules (corrected from control) were correlated with the corresponding changes in gut microbial genera abundances (adjusted from control) (Figure 5A). In a similar way, a correlation heatmap was performed using data from whole fruit groups (adjusted from control) (Figure 5B). Figure 4 shows that significant correlations were found in both matrices, with more significant associations after intake of the extract compared to whole fruit. The highest number of associations between gut microbial genera and plasma (poly)phenol metabolites were found for *Prevotella* (correlated with 9 plasma metabolites), *Dialister* (correlation with 8 plasma metabolites), *Desulfovibrio* (correlated with 7 metabolites) and *Bifidobacteria* (correlated with 6 plasma metabolites),upon intake of aronia extract (Figure 5A). Following aronia whole fruit

consumption, *Lactobacillus* and *Dialister* were correlated with 3 and 4 aronia metabolites respectively (Figure 5B).

To investigate the link between the microbiome and vascular health we performed a correlation analysis between FMD results and the select gut microbial genera. More specifically, independent correlations (for extract (n=23) and whole fruit (n=23)) were performed between changes from baseline in FMD (corrected from control) and changes in microbial abundancies (adjusted from control). The results show that for the extract group FMD was significantly correlated with *Dialister* (spearman ρ: 0.42), *Phascolarctobacterium* (spearman ρ: -0.45) and *Roseburia* (spearman ρ: -0.45). No significant correlations were found in the aronia whole fruit group.

4. Discussion

In the present study, daily consumption of aronia whole fruit powder or a polyphenol rich aronia extract for 12 weeks significantly improved endothelial function in a group of healthy young men. The amounts used in the present study are equivalent to consuming 10 and 75 g of aronia fruit per day, which is an amount that can be easily achieved within a normal diet. The magnitude of the effects (changes in FMD of 0.9-1.2% in comparison with control) are similar to the changes obtained after chronic consumption of other polyphenol rich foods such as cocoa (3, 30-33). Such improvements in vascular function are clinically relevant, as an improvement in FMD of 1% was associated with a decrease in 8 to 10% overall CVD risk over 4 years in a recent meta-analysis of 23 randomized controlled trials (34).

Consumption of both aronia treatments for 12 weeks led to improvements in FMD of the same magnitude, despite the low (poly)phenol content of the aronia whole fruit in comparison with the aronia extract. One possible explanation for this is the presence of non-extractable (poly)phenols bound to fiber in the aronia whole fruit capsules. It has been reported that non-extractable (poly)phenols constitute approximately 50% of the total (poly)phenol content in fruits (34), and 95% of those non-extractable compounds are released from the food matrix by microbial fermentation and the action of intestinal digestive enzymes (35). Thus, non-extractable (poly)phenols could be responsible for the significant increases in phenolic metabolites seen in the plasma of volunteers after the aronia whole fruit consumption, and potentially responsible for the improvements in vascular function after chronic consumption of the capsules. We cannot discard though that there may be other bioactive components in the whole fruit powder, such as fibers, that could contribute to the effect on FMD and to the prebiotic activity of this treatment. However, the amounts of fiber present in the whole fruit

403 capsules are low in comparison with habitual dietary intake, and unlikely to exert
404 effects on their own, but it is possible that they may act synergistically with the
405 polyphenols leading to beneficial effects. Given that the aronia extract had no fiber
406 and exerted similar effects on FMD than the whole fruit, this suggests that the
407 polyphenols from aronia may be the most likely compounds responsible for the
408 beneficial effects observed. Further work is warranted in this area.

409 Acute improvements in FMD were also observed 2 h after consumption of aronia
410 extract, but not after aronia whole fruit capsules. A comprehensive targeted
411 metabolomic analysis revealed a 4 times higher significant increase in plasma
412 phenolic metabolites after acute consumption of the aronia extract in comparison with
413 the aronia whole fruit, which may explain why the effects on FMD were only significant
414 after consumption of the aronia extract. A total of 24 metabolites correlated
415 significantly upon intake of aronia extract with the most abundant ones being
416 conjugated hydroxycinnamic acids and benzoic acids, such as isoferulic,
417 dihydroferulic acids or hydroxybenzoic acids. This data agrees with our previous work
418 showing significant associations between circulating plasma phenolic acid metabolites
419 and improvements in endothelial function (20, 35, 36). Although the exact mechanisms
420 of action are not known, dietary (poly)phenols may mediate improvements in vascular
421 function by increasing the steady-state level of NO in endothelial cells, for example by
422 inhibiting NADPH oxidase (37, 38). We have previously demonstrated that NADPH
423 oxidase activity significantly decreased after acute consumption of blueberry
424 (poly)phenols, and such inhibition correlated with improvements in FMD and plasma
425 levels of phenolic metabolites (18). Aronia (poly)phenols may act in a similar way as
426 the phenolic metabolites correlating with the FMD reported here have similar
427 structures and may also be able to act as NADPH inhibitors (35, 18). The chronic

effects observed here could also be mediated via gene expression alterations, which was shown by few studies addressing the molecular mechanisms-of-action of (poly)phenols in vitro and in vivo using nutrigenomic approaches (39, 40).

Our results also showed that aronia berry consumption can modulate the gut microbiome. A significant increase in *Anaerostipes* abundance was found after the aronia extract consumption. It is suggested that the *Anaerostipes* genus plays an important functional role in the gut ecosystem due to the ability to produce butyrate from lactate (41). Butyrate was associated with beneficial effects in various diseases such as genetic metabolic diseases, hypercholesterolemia, insulin resistance, and ischemic stroke and colon cancer (42). *Bacteroides* had a significant increase of abundance after intake of the aronia whole fruit capsules. Similarly, a study showed significant increases in *Bacteroides* when healthy volunteers consumed red wine (poly)phenols for one month (9). A few studies have associated *Bacteroides* with improved health. For example, it was found that *Bacteroides* abundance increased in obese individuals that lost weight (43) and decreased in patients with inflammatory bowel disease (44), but also that polysaccharide A, which is produced by *Bacteroides,* could prevent inflammatory bowel disease in animals (45). In agreement with the findings above, other (poly)phenol-rich food sources have shown increased numbers of *Enterococcus, Prevotella, Bacteroides, Bifidobacterium, Bacteroides uniformis, Eggerthella lenta, and Blautia* (46, 47).

Over half of the quantified plasma phenolic acids in the current study were previously identified as gut microbial breakdown products (48, 49). Although the importance of the gut microbiome on the metabolism of polyphenols has been long recognized, to date little is known regarding which bacteria is responsible for the production of individual metabolites. To explore such relationships, we correlated bacterial genera

453 with plasma (poly)phenol metabolites of volunteers after consumption of the aronia
454 treatments. A significant number of correlations were found, in particular in volunteers
455 who consumed the aronia extract. For example, *Prevotella, Bifidobacteria, Dialister*
456 *and Desulfovibrio* showed the highest number of associations with plasma 3-
457 hydroxyhippuric acid, benzoic acids, cinnamic acids and phenylacetic acids.
458 *Prevotella* and *Lactobacillus* were also significantly associated with ferulic acid,
459 dihydrocaffeic acid, or isoferulic acid-3-*O*-sulfate, suggesting that several families of
460 gut microbes are involved in the metabolism of aronia berry (poly)phenols. This is
461 supported by evidence showing that gut microbes catabolize (poly)phenols into
462 smaller phenolic acids including hydroxycinnamic acids, phenylacetic acids, and
463 phenylpropionic acids (50).

464 The correlation analysis between gut microbial genera and FMD revealed significant
465 associations for *Dialister, Phascolarctobacterium* and *Roseburia*, which also showed
466 correlations with plasma phenolic acids such as isoferulic acid-3-*O*-*β*-*D*-glucuronide,
467 gallic acid, dihydrocaffeic acid, and isoferulic acid-3-*O*-sulfate (Figure 5A). These
468 bacteria are also capable to produce short chain fatty acids such as propionic and
469 butyric acid (51, 52). Evidence suggests that these short chain fatty acids might have
470 a beneficial impact on gut barrier, glucose homeostasis, obesity and vascular health
471 (53, 54). Indeed, it was recently shown that propionic acid could protect against
472 hypertensive cardiovascular damage (55). The link between aronia (poly)phenols, gut
473 microbiome and improved vascular function could therefore be linked to the ability of
474 gut microbes such as *Dialister* to produce potentially bioactive phenolic metabolites
475 and short chain fatty acids (propionic and butyric acids) that could be beneficial for
476 vascular health.

477

478 Our work is limited by the small number of participants and further studies with a larger
479 number of participants are needed to confirm our findings. Another notable limitation
480 of this work is that the study population consisted of a group of healthy young men.
481 Therefore, our findings cannot be directly extrapolated to all segments of the general
482 population. The study also has notable strengths. This is reflected by the use of gold
483 standard techniques for measuring vascular function, a double blind RCT design, and
484 an extensive metabolomics analysis of plasma (poly)phenols in tandem with the
485 primary outcome made it possible to correlate plasma levels with FMD as well as with
486 gut microbial genera.

487 In conclusion, our present data indicate that consumption of dietary achievable
488 amounts of Aronia berries can lead to clinically relevant improvements in endothelial
489 function. Furthermore, we linked plasma phenolic metabolites to vascular benefits and
490 changes in gut microbial populations. Our results indicate that consumption of aronia
491 berry (poly)phenols as part of a balanced and healthy diet may help to maintain
492 cardiovascular health in young male individuals at low risk of CVD.

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performed by Clinical Microbiomics, SR, AC and GI. DC and GI analyzed food diaries. GI, ARM, MLS, and EW contributed to writing the manuscript. EF is employed by Naturex Inc. Naturex Inc. is an international group specializing in plant extraction and natural ingredients for food, health and beauty sectors.There are no other conflict of interest to declare.

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Tables

Table 1. (Poly)phenol content of intervention capsule

Per capsule (500 mg)	Extract	Whole fruit	Control
		Mean ± SD	
Flavonols (mg)	35 ± 0.3	2.6 ± 0.1	0 ± 0
Quercetin (µg)	1625 ± 395	575 ± 430	0 ± 0
Quercetin-3-glucoside (µg)	8879 ± 970	534 ± 33	0 ± 0
Quercetin-3-galactoside (µg)	7859 ± 68	816 ± 42	0 ± 0
Quercetin-3-rhamnoside (µg)	9 ± 1.5	0 ± 0	0 ± 0
Quercetin-3-rutinoside (µg)	15797 ± 613	610 ± 20	0 ± 0
Kaempferol (µg)	18 ± 19	27 ± 30	0 ± 0
Kaempferol-3-glucoside (µg)	229 ± 21	0 ± 0	0 ± 0
Myricetin (µg)	6.5 ± 7	2.5 ± 2.5	0 ± 0
Myricetin-3-glucoside (µg)	76 ± 8.5	0 ± 0	0 ± 0
Isorhamnetin (µg)	71 ± 24	32 ± 18	0 ± 0
Hesperetin (µg)	1.5 ± 1.5	1 ± 1.5	0 ± 0
Naringenin (µg)	5.5 ± 1	2 ± 0.5	0 ± 0
Hydroxycinnamic acids (mg)	33 ± 0.4	1.7 ± 0	0 ± 0
Vanillic acid (µg)	0.5 ± 0.5	0 ± 0	0 ± 0
p-coumaric (µg)	119 ± 17	3.5 ± 4	0 ± 0
m-coumaric (µg)	28 ± 12	1.5 ± 1.5	0 ± 0
o-coumaric (µg)	11 ± 4	0.5 ± 0.5	0 ± 0
Caffeic acid (µg)	819 ± 22	125 ± 1.5	0 ± 0
Dihydrocaffeic acid (µg)	25 ± 11	2.5 ± 6.5	0 ± 0
Ferulic acid (µg)	84 ± 35	6.5 ± 4	0 ± 0
Isoferulic acid (µg)	225 ± 150	17 ± 19	0 ± 0
Neochlorogenic acid (µg)	8360 ± 374	425 ± 49	0 ± 0
Chlorogenic acid (µg)	21341 ± 1180	1116 ± 50	0 ± 0
Cryptochlorogenic (µg)	1746 ± 17	30 ± 2	0 ± 0
Benzoic acids (mg)	2.8 ± 0	0.5 ± 0	0 ± 0
Benzoic acid (µg)	295 ± 23	16 ± 17	0 ± 0
Gallic acid (µg)	61 ± 15	1 ± 0.5	0 ± 0
4-hydroxybenzoic acid (µg)	59 ± 1.5	23 ± 3	0 ± 0
3-hydroxybenzoic acid (µg)	6.5 ± 7	0.5 ± 0.5	0 ± 0
2-Hydroxybenzoic acid (µg)	25 ± 28	5 ± 5.5	0 ± 0
Protocatechuic acid (µg)	2396 ± 105	450 ± 18	0 ± 0
Phloretin (µg)	0.5 ± 0.5	0 ± 0	0 ± 0
Epicatechin (µg)	101 ± 5.5	0 ± 0	0 ± 0
Total phenolics (mg)	71 ± 1.5	4.8 ± 0.6	0 ± 0
Anthocyanins (mg)	29 ± -	3.6 ± -	0 ± 0
Proanthocyanidins (mg)	16 ± 3.2	3.3 ± 0.9	0 ± 0
Total (poly)phenols (mg)	116 ± 4.7	12 ± 1.4	0 ± 0

Table 2. Baseline characteristics of the population included in the study.

Population characteristics	All (n=66)	Extract (n=23)	Whole fruit (n=23)	Control (n=20)
		Mean ± SD		
Age (years)	24 ± 5.3	24 ± 6.3	24 ± 5.2	23 ± 4.4
Height (cm)	177 ± 7.2	178 ± 7.3	176 ± 5.9	176 ± 8.5
Weight (Kg)	71 ± 8.3	74 ± 7.5	70 ± 9.8	69 ± 6.8
BMI (kg/m^2)	23 ± 2.1	23 ± 1.9	23 ± 2.6	22 ± 1.6
PSBP (mmHg)	119 ± 10.6	119 ± 12.6	119 ± 8.4	118 ± 11
PDBP (mmHg)	68 ± 7.9	67 ± 8.2	69 ± 9.1	68 ± 6.2
CSBP (mmHg)	101 ± 7.9	101 ± 8.6	102 ± 8.3	100 ± 7
CDBP (mmHg)	70 ± 9.2	69 ± 9.8	71 ± 10.1	70 ± 7.4
HR (bpm)	62 ± 9.8	62 ± 11.4	63 ± 9.4	62 ± 8.9
PWV (m/s)	5.5 ± 1.1	5.6 ± 1.2	5.7 ± 1.2	5.1 ± 1
AIx (%)	-3.6 ± 10	-4.4 ± 10.3	-2.6 ± 9.4	-3.8 ± 10.7
Body fat (%)	15 ± 4.0	15 ± 3.9	14 ± 4.4	15 ± 3.4
BMR (Kcal)	1787 ± 188	1848 ± 171	1770 ± 192	1736 ± 191
PGLU (mmol/L)	5 ± 0.3	5 ± 0.3	5.0 ± 0.4	4.9 ± 0.3
PLT ($10^9/L$)	225 ± 38.8	225 ± 46.1	221 ± 38	228 ± 31.9
Urea (mmol/L)	5.8 ± 1.3	6.0 ± 1.5	5.5 ± 0.8	5.9 ± 1.5
Creatin (mmol/L)	80 ± 10.2	84 ± 12.2	77 ± 7.7	77 ± 9
ALP (IU/L)	66 ± 16.3	65 ± 14.3	63 ± 16.4	70 ± 18.4
AST (IU/L)	26 ± 12.6	30 ± 18.5	24 ± 7.7	23 ± 6.7
GGT (IU/L)	17 ± 14.4	19 ± 20.2	16 ± 11.3	17 ± 9.5
CHOL (mmol/L)	4.1 ± 0.7	4.3 ± 0.7	4.0 ± 0.6	4.1 ± 0.8
TRIG (mmol/L)	0.8 ± 0.4	1.0 ± 0.5	0.7 ± 0.3	0.8 ± 0.3
HDL (mmol/L)	0.4 ± 0.3	1.3 ± 0.2	1.5 ± 0.3	1.3 ± 0.2
LDL (mmol/L)	2.4 ± 0.6	2.5 ± 0.6	2.2 ± 0.5	2.4 ± 0.8
LDH (IU/L)	150 ± 22.9	156 ± 17	147 ± 27.6	147 ± 22.6
Smoking (%)	14 ± 5.6	9	13	20

Table 3. Plasma (poly)phenols correlation with FMD.

Δ FMD vs Δ plasma (poly)phenols	Aronia extract (2 h) (n=43)	Aronia extract (12 wks) (n=43)	Aronia extract (12 wks, 2 h) (n=43)	Aronia whole fruit (12 wks, 2 h) (n=43)
	Spearman ρ			
Total (poly)phenols	0.34			
2-Hydroxyhippuric acid	0.36			
Protocatechuic acid			0.41	
2-Hydroxybenzoic acid			0.30	
3-Hydroxybenzoic acid		0.30	0.41	0.38
4-Hydroxybenzoic acid	0.35			
Vanillic acid-4'-*O*-sulfate	0.33			
Isovanillic acid		0.31		
Gallic acid			0.45	
Dihydroferulic acid	0.58			
Dihydro isoferulic acid	0.41			
p-Coumaric acid			0.30	
o-Coumaric acid				0.36
Ferulic acid-4'-*O*-sulfate				0.32
Isoferulic acid-3'-*O*-β-*D*-glucuronide	0.39			
Dihydroferulic acid-4'-*O*-β-*D*-glucuronide	0.32			
Dihydro isoferulic acid-3'-*O*-β-*D*-glucuronide	0.37			
Dihydro isoferulic acid-3'-*O*-sulfate		0.35		
Phenylacetic acid	0.48		0.44	0.33
3,4-Dihydroxyphenylacetic acid				0.31
4-Hydroxybenzaldehyde			0.39	
Catechol-*O*-sulfate	0.44			
Kaempferol			0.42	
Quercetin-3'-*O*-β-*D*-glucuronide	0.40			
(4R)-5-(3'-Hydroxyphenyl)-γ-valerolactone-4'-*O*-sulfate	0.39			

Correlations between changes in plasma metabolite concentrations (with respect to baseline) and FMD changes (with respect to baseline). Correlations were performed by correlating control and aronia extract outcomes in one analysis as a second indepent analysis was done using control and aronia whole fruit outcomes. This was repeated for data obtained from acute (2 h), chronic (12 wks) and acute on chronic (12 wks, 2 h) measurements. No correlations were found between aronia whole fruit and FMD on the acute and chronic level. Spearman was used for correlations of non-parametric data. All data represented had $p < 0.05$.

Figure Legends

Figure 1. CONSORT study flow diagram and study design**. A.** Participant flowchart. **B.** Study design. FMD, flow-mediated dilation; PWV, pulse wave velocity; AIX, augmentation index.

Figure 2. Improvements in endothelial function measured as FMD after aronia consumption**. A** FMD change from baseline (CFB) three months after chronic consumption of the control (n=19), aronia whole fruit (n=20) or aronia extract (n=22) capsules. **B.** FMD change from baseline (CFB) 2h post-consumption of the control (n=19), aronia whole fruit (n=20) or aronia extract (n=22) capsules. **C.** FMD change from baseline (CFB) at 2h post-consumption and after three months chronic consumption of the control (n=19), aronia whole fruit (n=21) or aronia extract (n=22) capsules.

Figure 3. Plasma (poly)phenol metabolites after consumption of aronia berries. Control (n=20), aronia whole fruit (n=23), aronia extract (n=23).
Figure 3A. Total hippuric acids
Figure 3B. Total benzoic acids
Figure 3C. Total cinnamic acids
Figure 3D. Total phenylacetic acids
Figure 3E. Total benzaldehydes
Figure 3F. Total catechols
Figure 3G. Total pyrogallols
Figure 3H. Total flavanols
Figure 3I. Total propionic acids

Figure 4. Changes in the gut microbiome associated to aronia treatments. Top 10 features from aronia extract (A), aronia whole fruit (B) and control (C) data sets, as revealed by Random Forests. Red dots denote bacterial genera significantly discriminant of the final microbiome structure respect to the initial configuration for

each treatment. Differences in the relative abundances of each relevant genera were investigated using Wilcoxon signed-rank tests and visualized by both dot plots and box plots. Paired samples from the same individual were connected by a black line.

Figure 5. Correlation heatmap of plasma metabolites and gut microbiome. **A.** Changes in metabolite concentrations (adjusted from control) versus changes in abundance of microbial genera (adjusted from control) upon 12-week consumption of aronia extract (n = 23). **B.** Changes in metabolite concentrations (adjusted from control) versus changes in abundance of microbial genera (adjusted from control) upon 12-week consumption of aronia whole fruit (n = 23). Correlations were performed in two independent analyses: **A.** aronia extract (adjusted from control) and **B.** aronia whole fruit (adjusted from control). Values are represented as spearman rho, *$p < 0.05$.