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Circulating anthocyanin metabolites mediate vascular benefits of blueberries: insights from randomized controlled trials, metabolomics, and nutrigenomics

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ABSTRACT

Potential health benefits of blueberries may be due to vascular effects of anthocyanins which predominantly circulate in blood as phenolic acid metabolites. We investigated which role blueberry anthocyanins and circulating metabolites play in mediating improvements in vascular function and explore potential mechanisms using metabolomics and nutrigenomics. Purified anthocyanins exerted a dose-dependent improvement of endothelial function in healthy humans, as measured by flow-mediated dilation (FMD). The effects were similar to those of blueberries containing similar amounts of anthocyanins while control drinks containing fiber, minerals, or vitamins had no significant effect. Daily 1-month blueberry consumption increased FMD and lowered 24h-ambulatory-systolic-blood-pressure. Of the 63 anthocyanin plasma metabolites quantified, 14 and 17 correlated with acute and chronic FMD improvements, respectively. Injection of these metabolites improved FMD in mice. Daily blueberry consumption led to differential expression (>1.2-fold) of 608 genes and 3 microRNAs, with Mir-181c showing a 13-fold increase in peripheral blood mononuclear cells. Patterns of 13 metabolites were independent predictors of gene expression changes and pathway enrichment analysis revealed significantly modulated biological processes involved in cell adhesion, migration, immune response, and cell differentiation. Our results identify anthocyanin metabolites as major mediators of vascular bioactivities of blueberries and changes of cellular gene programs.

Key words: polyphenols; blueberries; endothelial function; omics; nutrition; metabolomics

INTRODUCTION

Cardiovascular aging is a dynamic process that goes along with endothelial dysfunction, intimal hyperplasia, and arterial stiffness and may lead to arteriosclerosis and atherosclerosis.(1) In fact, the prevalence of coronary, peripheral, and cerebrovascular artery disease increase with age, and is in the order of 25% in individuals older than 75 years, and accounts for the majority of invalidity and mortality in older people.(2) Nutritional interventions are promising approaches to slow cardiovascular aging.(3, 4) The protective effects of these approaches including the Mediterranean diet may be mediated by a high fruit and vegetable intake and novel food bioactives consumed along with them.(3, 5) A growing body of nutritional science highlights the complex mechanisms and pleiotropic pathways of cardiometabolic effects of different foods to support healthy cardiovascular aging.(3) Therefore, there is a growing need to generate robust scientific evidence on the mechanistic and clinical effects of specific foods and in particular the role of bioactive compounds in them that may mediate the effects.(7) To be able to understand the mechanisms-of-action it is paramount to identify which bioactive compounds in fruits and vegetables are responsible for such beneficial effects and demonstrate causality using accredited endpoints and understand how such compounds are absorbed, distributed, metabolized, and excreted in healthy humans. To date, the most promising classes of food bioactives present in fruits and vegetables are polyphenols.(8, 9)

Blueberries are rich in polyphenols, in particular anthocyanins, but also contain other phenolic compounds in smaller amounts such as procyanidins, flavonols and phenolic acids, as well as being a rich source of fiber, vitamins, and minerals.(10) Blueberries have initially been investigated due to their potential beneficial effects on age-dependent decline in cognitive function(11) but were recently shown to improve cardiovascular function.(12) Data from the Nurses' Health Study II demonstrated that a high intake of blueberries and

strawberries and the high intake of anthocyanins associated with it (as calculated based on food frequency questionnaires) was inversely associated with the risk of myocardial infarction.(13) While these data underscore potential real world relevance that anthocyanin intake with blueberries could lower cardiovascular risk, epidemiological data inherently only provide associative evidence and are further limited by the lack of biomarkers of intake. Causality between anthocyanin intake in berries and cardiovascular benefits in healthy humans has not been established so far. Furthermore, the biological mechanisms-of-action are not fully understood and this may be due to the fact that anthocyanins are not present in circulation in relevant amounts but rather as low molecular weight phenolic acid compounds which are the result of chemical and microbial degradation (14, 15). However, the role of the circulating metabolites that reach the target organs (cardiovascular system) and can, therefore, only feasibly be regarded as the molecules causing the biological effects is not defined so far.(9)

As depicted in the graphical abstract, the aims of this work were to investigate which role anthocyanins and their circulating metabolites play in blueberry related vascular benefits, evaluate potential chronic effects, and explore their mechanisms-of-action.

METHODS

Human Studies

Four studies were conducted in healthy male volunteers (see **Supplementary FIGUREs 1-4 for** CONSORT study flow diagrams and study protocols).

Human Study 1: In a double-blind, 5-arm randomized crossover controlled study, healthy volunteers (n=5) received the 5 treatments in random order on 5 different days separated by one week of washout: control drink, control drink with fiber, control drink plus a mix of minerals and vitamins, pure ACN (total ACN content of 160 mg, (Medox, Norway)), or a wild blueberry drink, made of 11 g of freeze-dried wild blueberry powder (Wild Blueberry Association of North America) dissolved in 500 ml low nitrate water (**TABLE 1**). The amounts of fibre, minerals, vitamins, and ACN were similar to the amounts present in the wild blueberry drink. The control was matched for flavor and color. Flow-mediated vasodilation (FMD) was measured before (0 h) and at 1, 2, and 6 h post consumption.

Human Study 2: To investigate the dose-response of ACN, a randomized, controlled double-blind crossover trial was conducted (n=10)**.** FMD was measured at baseline before (0 h), at 2, and 6 h post consumption of ACN capsules (0 [control], 80, 160, 240, 320, or 480 mg ACN) on 6 different days with one week wash-out between study days.

Human Study 3: An uncontrolled, single arm, single blind pilot study was performed to investigate the timecourse of the chronic effects of wild blueberry on FMD. Volunteers (n=5) had 11g of wild blueberry powder, equivalent to 100g fresh wild blueberries, and containing 362mg total (poly)phenols dissolved in 500 ml water bi-daily over 28 days, and FMD was measured at baseline, day 7, 14, 21, and 28.

Human Study 4**:** An acute-on-chronic 2-armed, double-blind, parallel randomized controlled trial was conducted (n=40, 20 in each arm) comparing effects of wild blueberry drink (11 g wild blueberry powder, bi-daily) with matched control drinks (11 g powder, bi-daily) over 28

days. The primary outcome was improvement in FMD. Secondary endpoints were pulse-wave-velocity (PWV) and aortic augmentation index (AIX). and 24h-ambulatory-blood-pressure (BP) was taken in a sub-population n=22). Measurements were taken on day 1 (baseline) and after 28 bi-daily consumption at 0 and 2 h. Blood samples were drawn at all timepoints to measure plasma blueberry (poly)phenols and their metabolites. Blood lipids (triglycerides, total cholesterol, HDL, LDL), glucose and routine clinical lab parameters were also determined. To explore potential mechanism-of-action, mRNA and miRNA analyses were performed on peripheral blood mononuclear cells (PBMC) isolated from blood samples that were collected after an overnight fasting period from 10 volunteers at the beginning and the end of the 28-day period of blueberry consumption.

Animal study

To prove the bioactivity of circulating phenolic acid metabolites, a 3-armed randomized double-blinded crossover study was carried out in ten 6-week old male C57BL/6 mice with a 7-day wash-out period. The interventions were plasma (poly)phenol metabolite mixes comprising of metabolites that correlated with human FMD acutely, chronically (adjusted for human equivalent dose according to the FDA guidelines (16); **TABLE 2B**), and vehicle control (0.9% saline solution matched for methanol content at 5.8%). Mice were anesthetized with isofluran, and FMD measured as previously published(17) before and after blinded administration of 100 µl through intracardiac injection. The analyses of ultrasound images were performed by an operator blinded to allocation of treatments. The metabolites were purchased or synthesized(18), dissolved in methanol and diluted with saline. Animal procedures were approved by the local authorities (84-02.04.2014.A312) at Duesseldorf University.

Vascular measurements

All vascular measures were performed by a trained researcher in a temperature-controlled room after a period of 15 minutes rest as detailed below. FMD of the brachial artery was measured as previously described(19) using a 12 MHz transducer (Vivid I, GE Healthcare, Berlin, Germany) with automatic edge-detection software (Brachial Analyzer, Medical Imaging Applications, Iowa City, IA, USA). A single trained operator performed the analysis of all images within a single study. Office BP (mean of 2nd and 3rd measurements) was taken using an automated clinical digital sphygmomanometer (Dynamap, Tampa, FL, USA). 24 h ambulatory BP measurements were performed on day 1 and day 28, using a Tonoport V monitor (GE Healthcare, Berlin, Germany). PWV and AIX were measured by applanation tonometry using the SphygmoCor® (SMART medical, Gloucestershire, UK) system determined from measurements taken at the carotid and femoral artery as previously described.(4)

Biochemical analysis

The blood samples collected in EDTA/heparin tubes were spun (1,700*xg*; 15 min; 4ºC) immediately after collection. Samples for (poly)phenol analysis were spiked with 2% formic acid. All samples were aliquoted and frozen at -80°C until analysis. Screening clinical parameters including total, LDL and HDL-cholesterol, triglycerides (enzymatic photometric assay; RocheDiagnostics), Hb_{A1c}, glucose (hexokinase assay) and whole blood count (flow cytometry; Sysmex) were measured using standard techniques by the Institute for Clinical Chemistry, University Hospital Düsseldorf, Germany.

Nutrient and (poly)phenol analysis of wild blueberry interventions

Nutrient analysis was performed by Medallion Labs (Minneapolis, US) using standard procedures. (Poly)phenol analysis of blueberry interventions was performed as previously described.(10)

UPLC-Q-TOF MS analysis of plasma (poly)phenols

Plasma and urinary analysis of (poly)phenol metabolites was performed using microelution solid phase extraction coupled with UPLC-Q-TOF MS and authentic standards for quantification as previously described.(20)

Gene expression analyses

Peripheral blood mononuclear cells (PBMC) isolation: PBMCs were isolated from whole blood using BD Vacutainer tubes (Becton Dickinson, Franklin Lakes, USA). Blood samples were collected after an overnight fasting period from 10 volunteers at the beginning and the end of the 28-day period of consumption of 11 g of freeze-dried wild blueberry powder dissolved in 500 mL low nitrate water twice a day. Eight mL of blood collected into BD Vacutainer tubes were immediately centrifuged at room temperature in a horizontal rotor for 20 min at 1,500*xg*. The cell layer was collected and wished twice with sterile PBS with centrifugation at 300*xg* for 10 min between each washing step. The obtained pellet of PBMCs was immediately frozen at -80°C and kept at this temperature until use.

Total RNA extraction: The PBMCs were lysed using lysing buffer solution from the RNeasy Micro Kit (Qiagen, Hilden, Germany). Total RNA extraction has been performed using RNeasy Micro Kit as recommended by the manufacturer. RNA quality and quantity were checked by 1% agarose gel electrophoresis and by the determination of the absorbencies at 260 and 280 nm on NanoDrop ND-1,000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The total RNA were stored at -80°C until used.

Microarray analysis: Total RNA (50 ng per sample) for 20 RNA samples (10 from the volunteers at the beginning and 10 at the end of the 28-day study period) was amplified and fluorescently labelled to produce Cy5 or Cy3 cRNA using the Low Input Quick Amp Labeling two color Kit (Agilent, Santa Clara, USA) in the presence of spike-in two colors control as recommended by the manufacturer. After purification, 825 ng of labeled cRNA were hybridized onto G4845A Human GE 4x44K v2 microarray (Agilent, USA) according to the manufacturer's instructions. The G4845A Human GE 4x44K v2 microarray contains 27958 Entrez Gene RNAs sequences. After hybridization, microarrays were scanned with Agilent G2505 scanner (Agilent, USA) and data were extracted with Feature Extraction software (Agilent, USA) using linear and Lowess normalization. Genespring GX10 software (Agilent, Santa Clara, CA, USA) was used to quantify the signal and background intensity for each feature and to substantially normalize the data by the 75^{th} percentile method. Statistical analyses were performed using Genespring GX10 software to identify differentially expressed genes using Student's t test and the probability values were adjusted false discovery rate to eliminate false positives. Genes with FDR corrected $p<0.05$ and with a fold change >1.2 were referred to as differentially expressed genes (608). A gene list was chosen based on strict criteria to perform a multivariate analysis on plasma (poly)phenol concentrations. At first, the coefficient of variation of fold change expression was calculated between individuals where genes with less than 20% variation were designated resulting in a list of 152 genes. A fold change cut-off of at least 1.3 was chosen resulting in 20 remaining genes (**Figure 3A**). Next, a Pearson correlation followed by stepwise multivariate linear regression analysis of metabolite concentrations against the top 20 selected genes was performed. The analysis was executed 20 times, inserting every-time one gene against the 63 metabolites.

miRNA expression analysis: The impact of blueberry consumption on the expression of miRNAs was analyzed using Human miRNA (V3) 8x15K microarrays (Agilent, Santa Clara, USA). MiRNAs were labeled using miRNA labeling and hybridization kit from Agilent technologies (Agilent, Santa Clara, USA) as recommended by the manufacturer. Briefly, 100 ng of each total RNA sample were treated with calf intestinal phosphatase for 30 min at 37°C before denaturing the samples using pure DMSO at 100°C for 5 min and rapid transfer in an ice water bath to prevent RNA reannealing. RNA samples were labeled with pCp-Cy3 using T4 RNA ligase by incubation at 16°C for 2 h. After purification with microBioSpin columns, labeled samples were hybridized to Agilent human miRNA microarrays. Hybridizations were performed for 24 h at 55°C after which the microarrays were washed in GE Wash Buffer 1 (Agilent, Santa Clara, CA, USA) and GE Wash Buffer 2 (Agilent, Santa Clara, CA, USA) for 5 min. Following washing step, the microarrays were scanned with Aligent Microarray Scanner (Agilent, Santa Clara, CA, USA). The scanned images were analyzed using Feature Extraction Software (Agilent, Santa Clara, CA, USA). Genespring GX10 software (Agilent, Santa Clara, CA, USA) was used to quantify the signal and background intensity for each feature and to substantially normalize the data by the 75^{th} percentile method. The statistical significance was the corrected ratios of hybridization signal intensity between blueberry-exposed samples and control samples. miRNAs selected by these criteria are referred to as the "differentially expressed miRNAs".

Biological interpretation: to extract maximum biological information of differentially expressed genes, together with gene ontology (biological processes) and gene networks, genes were also classified according to their role(s) in cellular or metabolic pathways using Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/pathway.html) and Metacore (https://portal.genego.com) databases. Gene network interactions were based on data mining tools where a score was

given to every genes occurring in the same research abstracts. Potential transcription factors involved in the regulation of differentially expressed genes were searched using network algorithms for transcription factors development in Metacore software. Potential target genes of miRNA were identified using miRWalk database (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/).

Power calculation and statistical analyses

FMD was defined as the primary outcome. Based on previous intervention studies with blueberries, we expected a change in FMD by 1-2%.(12) The intra- and inter-individual variability for FMD measurements established in our laboratory are 0.9% (standard deviation of difference between repeated FMD measurements in n=20 middle aged healthy subjects, unpublished) and 1% (standard deviation within a group of healthy old subjects).(21) Study 1-3: Assuming a SD of difference between repeated FMD measurements of 0.9%, intra-individual measurements in 5 and 10 experimental subjects would provide sufficient power to detect an absolute change in FMD of 1.5% and 0.9% (two sided a of 5%, power = 0.80). Study 4: Assuming a SD of change in FMD of 1%, 20 experimental and 20 control subjects would provide sufficient power to detect an absolute change in FMD of 0.9% (two sided a of 5%, power = 0.80). Changes in FMD values were compared to control by one-way repeated measurements ANOVA (study 1-2) and are presented as mean values and 95% confidence intervals. In study 3, FMD values were compared to baseline by one-way repeated measurements ANOVA. In study 4, changes in FMD values at 2h, 1 month, and 1 month/2h relative to baseline values at day 1/0h were compared to control by one-way repeated measurements ANOVA (study 1-2) and are presented as mean values and 95% confidence intervals. Sample size calculations for the mouse study were based on our previously described method (17) to detect significant FMD changes with a power of 0.8 and error probability of 0.05. Changes in % FMD in between control and acute/chronic mixes were

performed using one-way repeated measurements ANOVA with Dunnett's post-hoc test. Correlations are presented as Pearson's r for normal distribution and as Spearman for non-normal distribution. Analyses were performed in Prism 6 and 7 and SPSS 20 (IBM). See above sections for microarray analyses.

RESULTS

The characteristics of the healthy volunteers (mean age 33±6 years [study 4]) are shown in **Supplementary TABLE 1**.

Anthocyanins are major contributors of endothelial function improvement after blueberry consumption

We first evaluated the potential role of anthocyanins as major bioactives in blueberries to increase endothelial function. In a randomized crossover study **(Study 1; Supplementary FIGURE 1),** we compared the effects of fiber, minerals and vitamins, and anthocyanins with the effects of blueberries containing the compounds in similar amounts on flow-mediated vasodilation (FMD). Our present results demonstrated that neither the vehicle control drink, the control with fiber, nor the control with minerals and vitamins in similar amounts as present in 100g of fresh blueberries (**TABLE 1**) had a significant effect on FMD at 2 and 6h post-consumption (**FIGURE 1A**). More importantly, 160 mg of pure anthocyanins was sufficient to increase FMD in a similar magnitude as blueberries containing 150 mg anthocyanins did.

Pure anthocyanins dose-dependently increase endothelial function

To further evaluate the causal role of anthocyanins in the mediation of vascular effects of blueberries, we performed dose-response experiments with pure anthocyanins **(Study 2; Supplementary FIGURE 2)**. Our data demonstrate a clear dose-dependent increase in FMD

at 2 and 6 h after consumption of pure anthocyanins (0-480 mg), respectively (**FIGURE 1B**). The amount to achieve half-maximal FMD improvements (ED_{50}) was 131 mg (95%CI:59,290 mg) and 150 mg (95%CI:78,290 mg) ACN at 2h and 6 h, respectively. The top of the sigmoid curve fit indicated maximal FMD increases of 1.3% (95%CI:0.8,1.9%) and 1.1% (95%CI: 0.6,1.5%), respectively. This was similar to our previously published dose-response study with blueberries.(12) The current analysis of dose-response data from the previously published study(12) is shown in **FIGURE 1C**. It showed that the amount of anthocyanins in blueberries to achieve half-maximal FMD improvements was 120 mg and did not significantly differ from pure anthocyanins. The top of the sigmoid curve fit indicated a maximal FMD increases after blueberries of 2.1% (95%CI:1.8,2.5%) which is significantly larger than achieved with anthocyanins when consumed alone.

Daily blueberry consumption leads to sustained effects on endothelial function and blood pressure

We then evaluated (a) which anthocyanin metabolites circulate in blood and may, therefore, qualify as bioactives causing the vascular functional effects, and (b) whether acute effects translate into sustained effects with a potential to impact vascular health.

In a pilot open label study **(Study 3; Supplementary FIGURE 3)** to evaluate the timecourse of chronic effects, we administered wild blueberries containing 150 mg ACN bi-daily (300 mg/day) over one month and measured FMD every week in the morning after over night fasting. FMD significantly increased already after one week, increased further after 2 weeks, and plateaued thereafter (**FIGURE 2A**). This suggests that at least 2 weeks of daily blueberry consumption are necessary to achieve a sustained improvement in endothelial function that persists after overnight fasting.

We then performed a one month randomized controlled intervention study (**Study 4; Supplementary FIGURE 4**) with additional secondary end-points including 24-h-blood-pressure-measurements, AIX, PWV, and blood lipids, and detailed metabolomics analyses (**Supplementary TABLE 2**). The very first (acute) consumption of wild blueberry containing 150 mg of ACN significantly increased FMD by 1.5% (95%CI:0.6,2.3%) at 2 h post-ingestion as compared to control (**FIGURE 2B**). After 28 days of bi-daily consumption, FMD was significantly increased after overnight fasting in the wild blueberry group as compared to control by 2.3% (95%CI:1.4,3.2%). Interestingly, no further improvement in FMD was observed when blueberries were acutely consumed on day 28 (acute-on-chronic; 0.3% [95%CI:-1.3,0.6%]) suggesting a saturation of effect indicating that acute and chronic effects may be mediated via similar pathways.

The improvement in FMD was accompanied by a lowering of 24h-SBP (-5.6 mmHg [95%CI:-0.2,-11.1mmHg)]. Changes in 24h-DBP were not significantly different from control (-5.5 mmHg [95%CI:-13.0,1.9 mmHg)]. No changes were seen with respect to PWV, AIX, or blood lipids in our present study.

Acute and chronic blueberry effects are linked to circulating anthocyanin metabolome

To identify potential mediators of vascular effects, we performed a metabolomics analysis of circulating anthocyanin metabolites. A total of 63 phenolic metabolites were quantified in plasma taken from subjects after blueberry at 2h after first dose and after 1 month of bi-daily consumption after an overnight fast. Most of the metabolites were conjugated and non-conjugated phenolic acid derivatives, with only 3 of them being flavonoid derivatives (see **Supplementalry TABLE 2**; data published elsewhere (22)). To link the circulating metabolites with vascular effects, we performed univariate correlation analyses with the increases in FMD at 2 h and after 28-day consumption of blueberry and all individual plasma

metabolites (**TABLE 2A**). Fourteen phenolic metabolites significantly correlated with the acute effects and 21 with the chronic responses, with 9 of them correlating with both acute and chronic responses.

Anthocyanin metabolites lead to endothelial function improvements when injected into a translational experimental model

To demonstrate the biological activity of the circulating ACN metabolites that correlated with the FMD responses in the human study (Study 4), we facilitated a translational animal model that allows the measurement of FMD in living mice.(17) We assessed FMD in crossover study in 10 anesthetized mice before and at 15 min after injection of mixtures of the anthocyanin metabolites that were significantly correlated with acute and chronic blueberries effects in study 4 or vehicle (**TABLE 2B**). Both metabolite mixes led to significant increases in FMD over vehicle ('acute'-metabolites: 8.7% [95%CI:3,15%]; 'chronic'-metabolites: 8.3% [95%CI:2,14%]; **FIGURE 2C**).

Gene and miRNA expression changes linked with chronic consumption of blueberries

To gain insight into the nutrigenomic effect of chronic wild blueberry consumption, we performed exploratory gene expression analyses on PBMCs from a subgroup (n=10) of the subjects participating in Study 4 comparing samples taken at baseline before and after having received blueberries over 28 days. The analyses showed that 608 genes were significantly differentially expressed (**Supplementary FIGURE 5**), with 357 genes up-regulated and 251 identified as down-regulated with fold-changes varying from -1.58 to 1.61 (see **FIGURE 3A** for top 20). A homogenous fold change expression across all ten individuals was observed with a minor variation in volunteer 1 for a subset of the genes (**Supplementary FIGURE 5**). To evaluate the biological significance of the nutrigenomic data, a functional annotation of the 608 genes according to biological processes (gene ontology) was performed. Gene

network analysis was conducted to investigate gene-gene interactions. Among the 35 significant gene networks identified, 11 are known to be involved in the regulation of chemotaxis and inflammation/immune response, 9 in cell adhesion and cytoskeleton organization, and few networks regulating signal transduction, apoptosis, or development. Together with gene network analyses, pathway enrichment analyses using KEGG and Metacore databases showed that among the most overrepresented pathways identified are those involved in the regulation of cell adhesion, cell migration, inflammation, and cell differentiation processes (**FIGURE 3A, Supplementary FIGURE 6**).

Together with the impact of blueberry polyphenol compounds on the expression of genes, we also analyzed their impact on expression of small non-coding RNA, microRNA, involved in the post-transcriptional regulation of gene expression. Our nutrigenomic study identified 3 differentially expressed miRNA in PBMCs of the volunteers consuming blueberries for 1 month: miR-181c-3p*, miR-126-5p*, miR-30c-5p (**Supplementary TABLE 3**). The most striking finding was the 13-fold increase in expression of miR-181c. With the aim to retrieve potential biological effects of modulation of the 3 miRNAs, we identified target genes of these miRNAs using the MirWalk database and performed network and pathway analyses. Our comparison of differentially expressed genes with potential target genes of differentially expressed miRNAs revealed 69 genes in common. This observation suggests that 11% of differentially expressed genes could be regulated at post-transcriptional level by the miRNAs. Bioinformatic analyses to identify pathways and networks in which target genes are involved in have shown that among the most over-represented ones are those involved in the regulation of focal adhesion, chemotaxis, cytoskeletal reorganization, cytokine-cytokine interactions, cellular development but also lipid absorption, accumulation and excretion (**FIGURE 3B, Supplementary FIGURE 6**).

Metabolites predict expression changes in the top 20 selected genes

One third (n=21) of the quantified 63 metabolites showed significant correlation with improvements in FMD. To explore whether circulating blueberry derived phenolic metabolites might be responsible for the changes in gene activity in PBMCs, a correlation followed by a stepwise multivariate analysis was performed on a selection of top 20 genes with inter-individual variability less than 20%. From these top 20 genes, 15 are involved in processes of inflammation or have a functional link to cardiovascular disease development. Twelve metabolites were identified as significant independent predictors for changes in expression of the 20 genes (**FIGURE 3A**). Notably, the R^2 of the individual multivariate linear regression models including sets of metabolites being significant independent predictors of gene changes were 0.73-0.99 suggesting that 73-99% of the variability in gene expression changes were explained by the metabolites. Two of the 12 metabolite independent predictors of gene expression changes (namely quercetin 3-*O*-ß-*D*-glucuronide and homovanillic acid) positively correlated with improved human vascular function and were also part of the polyphenol mix that significantly increased FMD in mice.

DISCUSSION

While blueberries contain many potentially 'healthy' bioactive molecules including vitamins, fibre, and minerals, our present results demonstrate that anthocyanins are major bioactive compounds in blueberry that can account for the increases in endothelial function after blueberry consumption. Our data demonstrate for the first time that purified anthocyanins cause dose-dependent improvements in endothelial function. The comparison of pure anthocyanins dose-responses experiments with the previously obtained results with anthocyanin-rich blueberries (12) supports that the majority of blueberry effects can be explained by anthocyanins but also indicate that the beneficial effects of blueberries are still larger than achieved with the consumption of anthocyanins alone. This may be due to other blueberry (poly)phenols likely chlorogenic acids, which have been previously shown to be capable of inducing favorable effects on endothelial function.(23) The amounts of vitamins, fibres, and minerals but also the amount of flavanols (3 mg of flavanol monomers)(24) present in blueberries were too low to exert significant effects. However, we cannot discard possible synergistic and/or antagonistic effects of components when consumed as a whole food, rather than individual compounds, as well as matrix effects affecting the liberation or absorption of anthocyanins from blueberries, which have been previously described to play a role in the context of flavanols.(25)

We also report for the first time in healthy adults that chronic blueberry consumption leads to a significant sustained improvement in endothelial function and lowering of 24-h-SBP. A few studies in at-risk populations, however, have demonstrated a reduction in blood pressure after blueberry consumption.(26) The potential clinical relevance of the findings is underscored by the fact that the lowering of blood pressure in the magnitude observed in our study of 5 mmHg is similar to what is commonly observed in clinical studies with blood pressure

lowering medication (e.g. ACE inhibitors) in patients.(27) Taken together, our data demonstrate that blueberries not only acutely and transiently improve endothelial function (12) but also induce sustained improvement in endothelial function and SBP after repetitive consumption over 1 month.

The molecular mechanisms-of-action of blueberries have still not been fully characterized and this may be due to the fact that only recently significant advances in the understanding of the absorption and metabolism of anthocyanins and blueberry polyphenols were made indicating that, for instance, anthocyanins primarily circulate as phenolic metabolites.(12, 15) However, most clinical studies with cardiovascular outcomes have not reported plasma or urine levels of circulating polyphenol metabolites in the participants. In a targeted metabolomics approach, we identified here in healthy humans a panel of circulating metabolites that correlated with vascular FMD responses and demonstrated in a translational model that these metabolites are indeed bioactive and can improve FMD after injection. Importantly, these metabolites do not represent metabolites specific for anthocyanins and are common metabolites of other common dietary (poly)phenols.(9) It may be argued that it is a limitation of the present work that we did not quantify parent anthocyanins and their phase II metabolites. However, we believe that such results would likely not impact the outcomes of our present work as the majority of anthocyanins are transformed into low molecular weight phenolic metabolites, which we quantified in this work. Intact anthocyanins are present in very low concentrations in plasma, and typically represent less than 1% of the anthocyanin metabolome, therefore, do not significantly contribute to the pool of circulating anthocyanin metabolites (12,15).

Future work is needed to dissect if it is one or several of the metabolites or combinations of metabolites that mediate the effects and if the individual metabolites act via the same or

different potentially synergistic mechanism(s). The investigation of structure-function relationships with these candidate metabolites may help to identify the molecular target structure and investigate potential class effects. Many (poly)phenols share similar phase II and gut microbiome derived metabolites, and similar metabolites have been observed to correlate with FMD after consumption of coffee (23) and cranberries (28). Furthermore, whether these effects, slow or even reverses components of cardiovascular aging itself and can increase healthspan or longevity remains to be determined. Future randomized controlled trials in larger populations including older subjects with relevant clinical endpoints will answer this question.

Identifying molecular targets of polyphenol has proven to be a real challenge due to the complex mechanisms and pleiotropic pathways of cardiometabolic effects of different foods. We here used an exploratory nutrigenomic approach aiming at identifying gene networks in circulating mononuclear cells that were modulated by blueberry consumption in healthy humans and explored which gene and expression changes correlate with circulating phenolic metabolites and vascular responses. It is a limitation of this approach that gene expression changes were investigated in blood cells and not endothelial cells. However, immune responses, mediated by both circulating and resident leukocytes,(29) play pathophysiological roles in the development and progression of CVD, including neutrophil recruitment, coronary atherosclerotic plaque development and stability, heart failure, and endothelial dysfunction.(30, 31) A few studies have proposed molecular mechanisms-of-action of other (poly)phenols *in vitro* and *in vivo* using nutrigenomic approaches.(32) These studies suggest that dietary (poly)phenols exert anti-inflammatory properties by binding to molecular targets in human cells making them attractive candidates for dietary CVD prevention strategies. Our present data corroborate these overall observations and add to the current body of knowledge

by supplying *in vivo* data on nutrigenomic effects of blueberries and link these with anthocyanin-metabolites in healthy humans. Among the significant gene networks identified from genes whose expression was affected by blueberry, one third is involved in the regulation of immune response and inflammation. This observation suggests that blueberry consumption can modulate inflammatory cellular processes of PBMCs that represent cellular targets of vascular function maintenance. The fact that we also identified circulating anthocyanin metabolites in plasma as strong predictors of expression changes in the most overexpressed genes with the majority being involved in inflammation suggest that these metabolites may play a mechanistic role in the mediation of effects. Only 2 metabolites correlated both with vascular function improvements and gene expression changes indicating that the mechanism by which anthocyanin metabolites modulate vascular function and PBMC expression changes may differ and the interaction is likely complex. We also observed significant changes in the expression of 3 micro RNAs. The little data available for miR-30c-5p and miR-181c-3p suggests that they could play a role in cancer development but a possible link with cardiovascular disease remains to be determined. miR-126-5p was described as being involved in enhancing the inflammatory responses of monocytes(33) or showing increased expression in patients with carotid artery disease(34) and acute pancreatitis.(35) Taken together, our nutrigenomic data showed that blueberry consumption can modulate the expression of genes and miRNA towards an anti-inflammatory and CVD protective profile, revealing new molecular targets that may be underlying the health properties of berries.

In conclusion, our results demonstrate a key role of anthocyanin metabolites in the mediation of biological activities of blueberries that could contribute to healthy cardiovascular aging. We provide further scientific evidence that in healthy humans chronic blueberry consumption

leads to sustained cardiovascular benefits which are linked with circulating anthocyanin metabolites and the modulation of cellular gene programs towards an anti-inflammatory and CVD protective profile. Future studies will help to further characterize the mechanisms-of-action of individual metabolites, establish general structure-function relationships, and identify relevant interactions. As the identified metabolites are common for a range of food bioactive classes, this knowledge represents an important building block necessary for the development of evidence based dietary recommendations for food bioactives in primary prevention.

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REFERENCES

1. Heiss C, Rodriguez-Mateos A, Kelm M. Central role of eNOS in the maintenance of endothelial homeostasis. Antioxid Redox Signal. 2015;**22**:1230-1242. https://doi.org/10.1089/ars.2014.6158

2. Diehm C, Schuster A, Allenberg JR, Darius H, Haberl R, Lange S, *et al.* High prevalence of peripheral arterial disease and co-morbidity in 6880 primary care patients: cross-sectional study. Atherosclerosis. 2004;**172**:95-105. https://doi.org/10.1016/S0021-9150(03)00204-1

3. Heiss C, Spyridopoulos I, Haendeler J. Interventions to slow cardiovascular aging: Dietary restriction, drugs and novel molecules. Exp Gerontol. 2018;**109**:108-118. https://doi.org/10.1016/j.exger.2017.06.015

4. Heiss C, Sansone R, Karimi H, Krabbe M, Schuler D, Rodriguez-Mateos A, *et al.* Impact of cocoa flavanol intake on age-dependent vascular stiffness in healthy men: a randomized, controlled, double-masked trial. Age (Dordr). 2015;**37**:9794. https://doi.org/10.1007/s11357-015-9794-9

5. Mathers JC. Impact of nutrition on the ageing process. Br J Nutr. 2015;**113 Suppl**:S18-22. https://doi.org/10.1017/S0007114514003237

6. Zhan J, Liu YJ, Cai LB, Xu FR, Xie T, He QQ. Fruit and vegetable consumption and risk of cardiovascular disease: A meta-analysis of prospective cohort studies. Critical reviews in food science and nutrition. 2017;**57**:1650-1663. https://doi.org/10.1080/10408398.2015.1008980

7. Mozaffarian D, Wu JHY. Flavonoids, Dairy Foods, and Cardiovascular and Metabolic Health: A Review of Emerging Biologic Pathways. Circ Res. 2018;**122**:369-384. https://doi.org/10.1161/CIRCRESAHA.117.309008

8. Rodriguez-Mateos A, Vauzour D, Krueger CG, Shanmuganayagam D, Reed J, Calani L, *et al.* Bioavailability, bioactivity and impact on health of dietary flavonoids and related compounds: an update. Archives of toxicology. 2014;**88**:1803-1853. https://doi.org/10.1007/s00204-014-1330-7

9. Del Rio D, Rodriguez-Mateos A, Spencer JP, Tognolini M, Borges G, Crozier A. Dietary (poly)phenolics in human health: structures, bioavailability, and evidence of protective effects against chronic diseases. Antioxid Redox Signal. 2013;**18**:1818-1892. https://doi.org/10.1089/ars.2012.4581

10. Rodriguez-Mateos A, Cifuentes-Gomez T, Tabatabaee S, Lecras C, Spencer JP. Procyanidin, anthocyanin, and chlorogenic acid contents of highbush and lowbush blueberries. J Agric Food Chem. 2012;**60**:5772-5778. https://doi.org/110.1021/jf203812w

11. Joseph JA, Denisova NA, Bielinski D, Fisher DR, Shukitt-Hale B. Oxidative stress protection and vulnerability in aging: putative nutritional implications for intervention. Mech Ageing Dev. 2000;**116**:141-153. https://doi.org/10.1016/S0047-6374(00)00128-7

12. Rodriguez-Mateos A, Rendeiro C, Bergillos-Meca T, Tabatabaee S, George TW, Heiss C, *et al.* Intake and time dependence of blueberry flavonoid-induced improvements in vascular function: a randomized, controlled, double-blind, crossover intervention study with mechanistic insights into biological activity. Am J Clin Nutr. 2013;**98**:1179-1191. https://doi.org/10.3945/ajcn.113.066639

13. Cassidy A, Mukamal KJ, Liu L, Franz M, Eliassen AH, Rimm EB. High anthocyanin intake is associated with a reduced risk of myocardial infarction in young and middle-aged women. Circulation. 2013;**127**:188-196. https://doi.org/10.1161/CIRCULATIONAHA.112.122408.

14. Feliciano RP, Istas G, Heiss C, Rodriguez-Mateos A. Plasma and Urinary Phenolic Profiles after Acute and Repetitive Intake of Wild Blueberry. Molecules 2016;**21**. https://doi.org/10.3390/molecules21091120.

15. Czank C, Cassidy A, Zhang Q, Morrison DJ, Preston T, Kroon PA, *et al.* Human metabolism and elimination of the anthocyanin, cyanidin-3-glucoside: a (13)C-tracer study. Am J Clin Nutr. 2013;**97**:995-1003. https://doi.org/10.3945/ajcn.112.049247

16. Guidance for industry : estimating the maximum safe starting dose in initial clinical trials for therapeutics in adult healthy volunteers. In: US Department of Health and Human Services FaDA, Centre for Drug Evaluation and Research, ed. Rockville, MD; 2005:1-27.

17. Schuler D, Sansone R, Freudenberger T, Rodriguez-Mateos A, Weber G, Momma TY, *et al.* Measurement of endothelium-dependent vasodilation in mice--brief report. Arterioscler Thromb Vasc Biol. 2014;**34**:2651-2657. https://doi.org/10.1161/ATVBAHA.114.304699

18. Feliciano RP, Mecha E, Bronze MR, Rodriguez-Mateos A. Development and validation of a high-throughput micro solid-phase extraction method coupled with ultra-high-performance liquid chromatography-quadrupole time-of-flight mass spectrometry for rapid identification and

quantification of phenolic metabolites in human plasma and urine. J Chromatograph A. 2016;**1464**:21-31. https://doi.org/10.1016/j.chroma.2016.08.027

19. Rodriguez-Mateos A, Hezel M, Aydin H, Kelm M, Lundberg JO, Weitzberg E, *et al.* Interactions between cocoa flavanols and inorganic nitrate: additive effects on endothelial function at achievable dietary amounts. Free Radic Biol Med. 2015;**80**:121-128. https://doi.org/10.1016/j.freeradbiomed.2014.12.009.

20. Feliciano RP, Boeres A, Massacessi L, Istas G, Ventura MR, Nunes Dos Santos C, *et al.* Identification and quantification of novel cranberry-derived plasma and urinary (poly)phenols. Arch Biochem Biophys. 2016;**599**:31-41. https://doi.org/10.1016/j.abb.2016.01.014.

21. Horn P, Amabile N, Angeli FS, Sansone R, Stegemann B, Kelm M, *et al.* Dietary flavanol intervention lowers the levels of endothelial microparticles in coronary artery disease patients. Br J Nutr. 2014;**111**:1-8. https://doi.org/10.1017/S0007114513003693.

22. Feliciano R, Istas G, Heiss C, Rodriguez-Mateos A. Plasma and Urinary Phenolic Profiles after Acute and Repetitive Intake of Wild Blueberry. Molecules. 2016;**21**:1120. https://doi.org/10.3390/molecules21091120.

23. Mills CE, Flury A, Marmet C, Poquet L, Rimoldi SF, Sartori C, *et al.* Mediation of coffee-induced improvements in human vascular function by chlorogenic acids and its metabolites: Two randomized, controlled, crossover intervention trials. Clin Nutr. 2017;**36**:1520-1529. https://doi.org/10.1016/j.clnu.2016.11.013

24. Sansone R, Ottaviani JI, Rodriguez-Mateos A, Heinen Y, Noske D, Spencer JP, *et al.* Methylxanthines enhance the effects of cocoa flavanols on cardiovascular function: randomized, double-masked controlled studies. Am J Clin Nutr. 2017;**105**:352-360. https://doi.org/10.3945/ajcn.116.140046

25. Cifuentes-Gomez T, Rodriguez-Mateos A, Gonzalez-Salvador I, Alanon ME, Spencer JP. Factors Affecting the Absorption, Metabolism, and Excretion of Cocoa Flavanols in Humans. J Agric Food Chem. 2015;**63**:7615-7623. https://doi.org/110.1021/acs.jafc.5b00443

26. Basu A, Du M, Leyva MJ, Sanchez K, Betts NM, Wu M, *et al.* Blueberries decrease cardiovascular risk factors in obese men and women with metabolic syndrome. J Nutr. 2010;**140**:1582-1587. https://doi.org/ 10.3945/jn.110.124701

27. Heart Outcomes Prevention Evaluation Study I, Yusuf S, Sleight P, Pogue J, Bosch J, Davies R, *et al.* Effects of an angiotensin-converting-enzyme inhibitor, ramipril, on cardiovascular events in high-risk patients. N Engl J Med. 2000;**342**:145-153. https://doi.org/10.1056/NEJM200001203420301

28. Rodriguez-Mateos A, Feliciano RP, Boeres A, Weber T, Dos Santos CN, Ventura MR, *et al.* Cranberry (poly)phenol metabolites correlate with improvements in vascular function: A double-blind, randomized, controlled, dose-response, crossover study. Mol Nutr Food Res. 2016;**60**:2130-2140. https://doi.org/10.1002/mnfr.201600250

29. Sullivan GW, Sarembock IJ, Linden J. The role of inflammation in vascular diseases. J Leukoc Biol. 2000;**67**:591-602. https://doi.org/10.1002/jlb.67.5.591

30. Montecucco F, Liberale L, Bonaventura A, Vecchie A, Dallegri F, Carbone F. The Role of Inflammation in Cardiovascular Outcome. Curr Atheroscler Rep. 2017;**19**:11. https://doi.org/10.1007/s11883-017-0646-1

31. Fernandez-Ruiz I. Immune system and cardiovascular disease. Nat Rev Cardiol. 2016;**13**:503. https://doi.org/10.1038/nrcardio.2016.127

32. Monfoulet LE, Mercier S, Bayle D, Tamaian R, Barber-Chamoux N, Morand C, *et al.* Curcumin modulates endothelial permeability and monocyte transendothelial migration by affecting endothelial cell dynamics. Free Rad Biol Med. 2017;**112**:109-120. https://doi.org/10.1016/j.freeradbiomed.2017.07.019.

33. Huang J, Zhu L, Qiu C, Xu X, Zhang L, Ding X, *et al.* MicroRNA miR-126-5p Enhances the Inflammatory Responses of Monocytes to Lipopolysaccharide Stimulation by Suppressing Cylindromatosis in Chronic HIV-1 Infection. J Virol. 2017;**91**. https://doi.org/10.1128/JVI.02048-16

34. Sezer Zhmurov C, Timirci-Kahraman O, Amadou FZ, Fazliogullari O, Basaran C, Catal T, *et al.* Expression of Egfl7 and miRNA-126-5p in Symptomatic Carotid Artery Disease. Genet Test Mol Biomarkers. 2016;**20**:125-129. https://doi.org/10.1089/gtmb.2015.0252

35. Kusnierz-Cabala B, Nowak E, Sporek M, Kowalik A, Kuzniewski M, Enguita FJ, *et al.* Serum levels of unique miR-551-5p and endothelial-specific miR-126a-5p allow discrimination of patients in the early phase of acute pancreatitis. Pancreatology. 2015;**15**:344-351. https://doi.org/10.1016/j.pan.2015.05.475

TABLE 1: Composition of interventions used in human studies (ACN, anthocyanin).

	Control	**Control** (+fiber)	**Control** (fiber+minerals+vitamins)	**ACN**	**Blueberry**
Dietary fiber (g)		5	5		4.4
Potassium (mg)			75		68
Fructose (g)	3.5	3.5	3.5	0.05	3.6
Total beta carotene (IU)			50		58
Vitamin C (mg)			12.5		1.7
Calcium (mg)			20		17
Iron (mg)			0.63		0.58
Vitamin E (IU)			1.88		0.39
Vitamin B1 (mg)			0.18		0.03
Vitamin B2 (mg)			0.19		0.01
Vitamin B6 (mg)			0.25		0.02
Phosphorus (mg)			15.6		12.9
Magnesium (mg)			12.5		6.5
Zinc (mg)			0.63		0.67
Manganese (mg)			2.25		2.87
Niacin (mg)			2.5		0.61
Anthocyanins (mg)				160	150
Flavanol monomers (mg)					3
Flavanol oligomers (mg)					49
Flavonols (mg)					31
Chlorogenic acid (mg)					64

TABLE 2: Details of plasma metabolism from human (A) and animal (B) studies

A		Correlation analysis human study (values are Pearson's r, all p<0.05)		B	Estimated concentration mouse plasma after IC injection (mM)	
		2h FMD	28 d FMD		2 h Metabolites	28 d Metabolites
Cinnamic acids	Ferulic acid		0.51			0.1
	Ferulic acid-4-*O* -sulfate	0.36	0.43		1.6	1.2
	Ferulic acid 4-*O* -ß-D-glucuronide		0.44			2.8
	Isoferulic acid 3-*O* -ß-D-glucuronide	0.43	0.48		0.5	1.1
	Dihydroferulic acid	0.54	0.43		1.0	1.5
	Dihydroferulic acid 4-*O* -ß-D-glucuronide	0.42			1.1	
	Dihydroisoferulic acid 3-*O* -ß-D-glucuronide		0.41			0.1
	Dihydroisoferulic acid 3-*O* -sulfate		0.43			0.6
	Dihydrocaffeic acid 3-*O* -sulfate		0.63			1.6
	p-Coumaric		0.40			0.1
	Cinnamic acid		0.42			0.3
	Chlorogenic acid	0.44		➔	1.0	
Benzoic acids	Vanillic acid	0.60	0.61		6.0	10.0
	Homovanillic acid	0.40	0.52		0.9	1.4
	Protocatechuic acid	0.37			0.3	
	Syringic acid	0.41	0.42		0.1	0.2
	4-Hydroxybenzoic acid	0.37			0.4	
	2,4-Dihydroxybenzoic acd		0.64			0.4
	4-Methylgallic acid-3-*O* -sulfate	0.40	0.41		0.8	0.5
Phenols	4-Methylcatechol-2-*O* -sulfate		0.42			12.7
	1-Methylpyrogallol-*O* -sulfate	0.62	0.50		0.9	1.8
Hippuric acids	Hippuric acid		0.57			368.9
	3-Hydroxyhippuric acid		0.56			10.5
Phenylacetic acids	3-Hydroxyphenyl acetic acid		0.46			3.0
	4-Hydroxyphenyl acetic acid	0.41	0.40		3.0	5.0
Flavonols	Quercetin 3-O-ß-D-glucuronide	0.38			104.5	

A Univariate correlation between human plasma metabolites (study 4) and change in FMD at 2 h (2h FMD), 'acute effect') and after 28 days (28 d FMD, 'chronic effect') of blueberry consumption in humans (values are Pearson's r and all $p<0.05$) and **B** composition of metabolite mixes that were injected intracardially (IC) in 100 μLvehicle into mice to demonstrate bioactivity of 2 h and 28 d metabolite profiles. Values represent estimated instantaneous concentrations (μM) in mouse plasma.

FIGURE LEGENDS

Graphical abstract

FIGURE 1: Importance of anthocyanin (ACN) in blueberry-mediated acute improvements in vascular function. Comparison of ACN effect on flow-mediated dilation (FMD) with (A) other components of blueberry at 2 and 6 h after ingestion (see **TABLE 1** for composition of interventions), (B) dose-response of ACN at 2 and 6 h (0 mg control arbitrarily set to 1), and (C) dose-response of blueberry at 2 h (adapted from (12)). * $p < 0.05$ vs 0 mg ACN control.

FIGURE 2: Circulating anthocyanin metabolites improve vascular function. (A) Pilot study to demonstrate time course of FMD during daily consumption of blueberries. (B) FMD values at baseline after acute, chronic, and acute on chronic ingestion of control (white bars) and blueberry (blue bars). In theses subjects a targeted metabolomics analysis of plasma metabolites was performed. We identified metabolites that correlated with the 2h and day 28 changes (*see **TABLE 2A** for correlation analysis) and composed a chemically pure mix of the identified metabolites that we injected intracardially into mice. (C) FMD in mice at before and after 15 min injection of mixtures of anthocyanin metabolite profiles that correlated with of acute (2h) and chronic (28 days) FMD improvements in human study (*see **TABLE 2B** for composition).

FIGURE 3: (A) Summary of individual stepwise regression analyses identifying circulating metabolites as significant independent predictors of gene expression changes of the top 20 differentially expressed genes after 1 month of blueberry consumption (n=10). * designate that also significant correlations existed with changes in flow-mediated dilation. **(B)** Schematic illustration of biological processes and molecular functions occuring in PBMC upon blueberry intake. A subset of genes from the 608 significant differentially expressed list were chosen for

this figure as they are involved in previously established cell processes which are highlighted in blue.

Figures

Figure 1

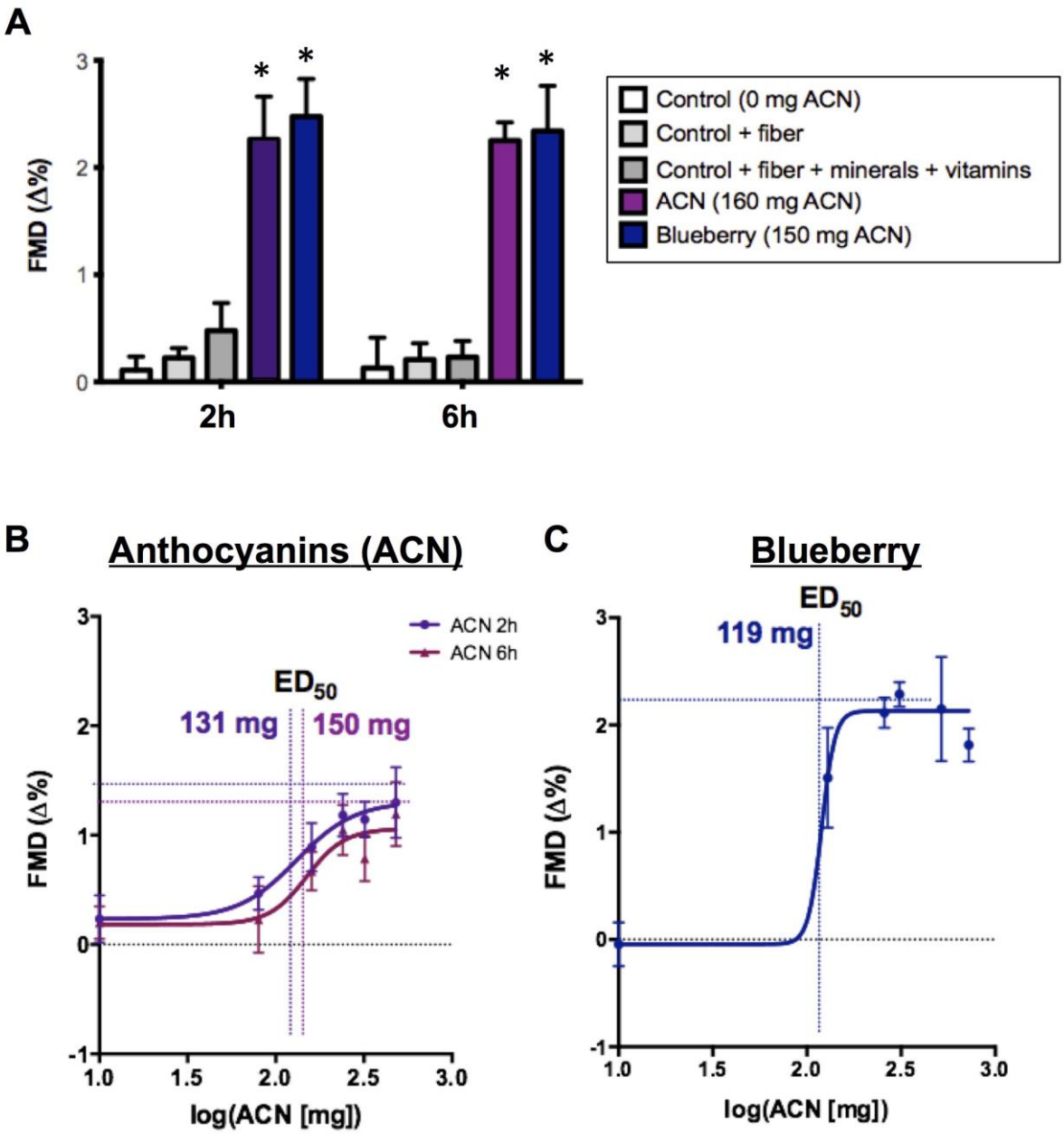

Figure 2

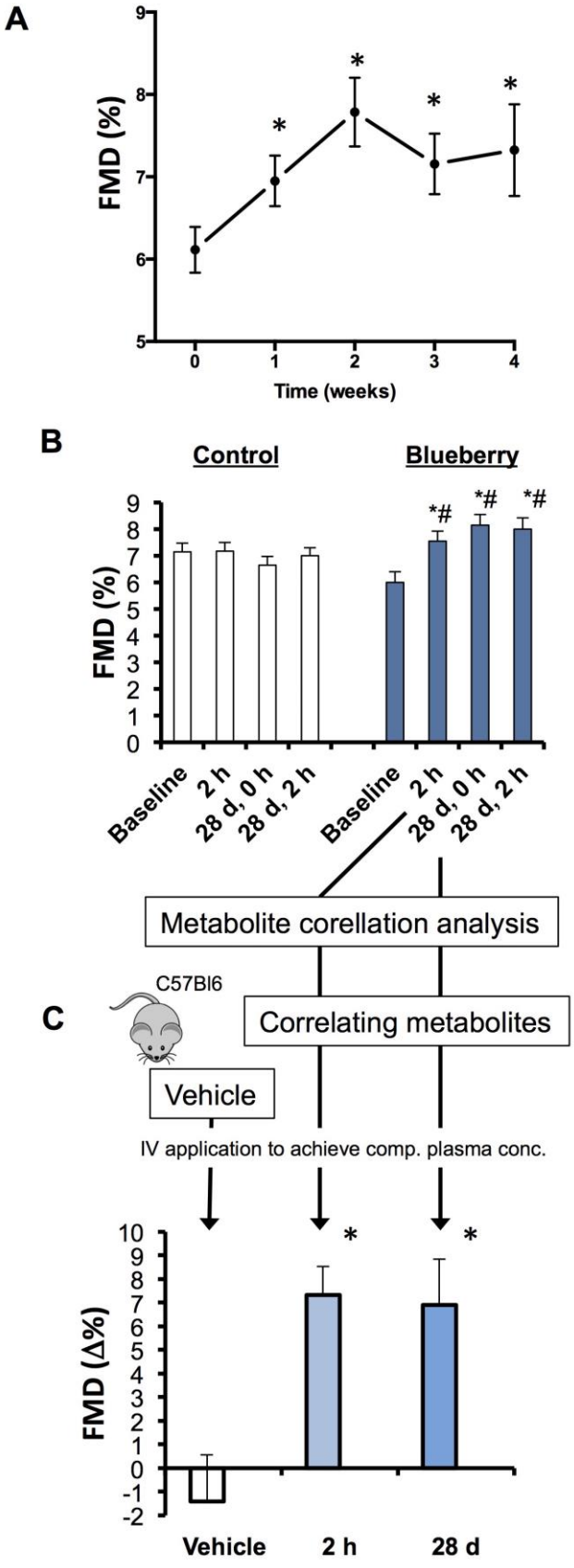
A
FMD (%)
Time (weeks)
B
Control
Blueberry
FMD (%)
Baseline
2 h
28 d, 0 h
28 d, 2 h
Metabolite corellation analysis
C
C57Bl6
Correlating metabolites
Vehicle
IV application to achieve comp. plasma conc.
FMD (Δ%)
Vehicle
2 h
28 d

Figure 3

A

Genes	Fold change	Isovanillic acid	Homovanillic acid*	Caffeic acid-4-*D*-glucuronide	Dihydro isoferulic acid 3-*O*-ß-glucuronide	2,3-Dihydroxybenzoic acid	Gallic acid	3,4-Dihydroxybenzaldehyde	4-Hydroxybenzaldehyde	Pyrogallol-*O*-2-sulfate	2-Methylpyrogallol-*O*-sulfate	(+)-R-(2,4-Hydroxyphenoxy) propionic acid	Quercetin 3-*O*-ß-*D*-glucuronide*	Adjusted R^2 of model
LANCL3	1.62		+				+	+		+				0.79
*PTPRC**	1.55			+				+					+	0.77
*ARHGEF15**	1.50		+		+	+						+		0.98
*MAP3K21**	1.48		+	+	+		+		+					0.99
*FOXI2**	1.44		+	+						+				0.92
RNA28S5	1.43	+						+				+	+	0.99
*NAT8L**	1.41				+		+				+		+	0.83
*RABEPK**	1.41		+					+		+				0.87
*SCARNA16**	1.40		+					+	+		+	+		0.97
LOC100130920	1.35	+		+								+	+	0.97
*FOXO3**	1.35			+	+		+		+	+			+	0.73
ZNF770	1.34				+	+					+	+		0.92
*ZASP**	1.33	+	+		+			+		+				0.99
*CNOT7**	1.33	+						+		+	+			0.97
*TNS1**	1.32	+		+			+				+			0.92
*NABP1**	1.32	+						+				+	+	0.83
*GSTK1**	-1.33				+	+			+			+	+	0.96
*THEMIS2**	-1.36			+					+				+	0.90
*RNASET2**	-1.37			+		+			+	+	+			0.85
SNRPB	-1.38					+		+			+	+		0.97

B