

ORIGINAL ARTICLE

Effects of anthocyanins on cardiovascular risk factors and inflammation in pre-hypertensive men: a double-blind randomized placebo-controlled crossover study

SS Hassellund^{1,2,3}, A Flaa^{1,2,3}, SE Kjeldsen^{1,3,4}, I Seljeflot^{3,5}, A Karlsen⁶, I Erlund⁷ and M Rostrup^{1,2}

High intake of fruits and vegetables is associated with reduced cardiovascular risk. A number of fruits and vegetables are rich in anthocyanins, which constitute a subgroup of the flavonoids. Anthocyanins have demonstrated anti-inflammatory and anti-oxidative properties, and anthocyanin-rich interventions have indicated beneficial effects on blood pressure and other cardiovascular risk factors. We assessed whether a purified anthocyanin supplement improves cardiovascular metabolic risk factors and markers of inflammation and oxidative stress in prehypertensive participants, and whether plasma polyphenols are increased 1–3 h following intake. In all, 31 men between 35–51 years with screening blood pressure >140/90 mm Hg without anti-hypertensive or lipid-lowering medication, were randomized in a double-blinded crossover study to placebo versus 640 mg anthocyanins daily. Treatment durations were 4 weeks with a 4-week washout. High-density lipoprotein (HDL)-cholesterol and blood glucose were significantly higher after anthocyanin versus placebo treatment ($P=0.043$ and $P=0.024$, respectively). No effects were observed on inflammation or oxidative stress *in vivo*, except for von Willebrand factor, which was higher in the anthocyanin period ($P=0.007$). Several plasma polyphenols increased significantly 1–3 h following anthocyanin intake. The present study strengthens the evidence that anthocyanins may increase HDL-cholesterol levels, and this is demonstrated for the first time in prehypertensive and non-dyslipidemic men. However, no other beneficial effects in the short term were found on pathophysiological markers of cardiovascular disease.

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INTRODUCTION

High intake of fruits and vegetables is associated with reduced risk of cardiovascular disease (CVD) according to two systematic reviews.^{1,2} Atherosclerosis is the main underlying pathology of CVD, and food compounds that influence pathophysiological factors such as hypertension, hyperlipidemia, endothelial dysfunction, inflammation and/or oxidative stress may also beneficially affect CVD development.

A systematic review assessing effects of dietary flavonoids in 133 randomized controlled trials found that cocoa products improved systolic blood pressure (BP), diastolic BP and flow-mediated vasodilatation (measure of endothelial dysfunction), soy protein isolate improved diastolic BP and low-density lipoprotein (LDL)-cholesterol (LDL-C) and green tea improved LDL-C.³ The authors recommended more studies on other commonly consumed flavonoid subclasses such as the anthocyanins.

Anthocyanins constitute a diverse group of polyphenolic compounds present in fruits, vegetables, berries and red wine.⁴ Anti-inflammatory and anti-oxidative effects *in vitro* have been reported,^{5,6} and anthocyanin-containing compounds have been shown to reduce BP,⁷ insulin sensitivity⁸ and atherosclerosis⁹ in rodents. Some human trials have assessed BP and other CVD-related parameters after anthocyanin-containing interventions and beneficial effects have to some degree been attributed to anthocyanins.^{10–13} However, the interventions in these trials

contained low amounts of anthocyanins, or relatively higher amounts of other possibly biologically active compounds, thus making it hard to assess whether effects were caused by anthocyanins.

Medox capsules (Biolink AS, Sandnes, Norway), on the other hand, are relatively pure, that is, they contain high amounts of anthocyanins in relation to other polyphenols or other plant constituents. These anthocyanin capsules have demonstrated beneficial effects that may be relevant for CVD development in two previously published randomized placebo-controlled studies. The first study found that NF- κ B-associated markers of inflammation decreased after 3 weeks of treatment in 120 healthy individuals.¹⁴ More recently, Qin *et al.*¹⁵ found that the same anthocyanins increased high-density lipoprotein (HDL)-cholesterol (HDL-C) and reduced LDL-C significantly after 12 weeks of treatment among 120 dyslipidemic patients.

The present crossover-designed study was conducted to assess whether a supplement containing high doses of purified anthocyanins can improve cardiovascular risk factors in men with a screening BP corresponding to grade 1 hypertension or higher. We have previously published data on the lack of antihypertensive effects in this population.¹⁶ The present paper deals with effects of anthocyanins on additional established risk factors and markers of inflammation and oxidative stress, as well as polyphenol bioavailability in prehypertensive men.

¹Section of Cardiovascular and Renal Research, Oslo University Hospital, Oslo, Norway; ²Department of Acute Medicine, Oslo University Hospital, Oslo, Norway; ³Institute of Clinical Medicine, University of Oslo, Oslo, Norway; ⁴Department of Cardiology, Oslo University Hospital, Oslo, Norway; ⁵Center for Clinical Heart Research, Oslo University Hospital, Oslo, Norway; ⁶Department of Nutrition, University of Oslo, Oslo, Norway and ⁷National Institute for Health and Welfare, Helsinki, Finland. Correspondence: Dr SS Hassellund, Department of Acute Medicine, Oslo University Hospital, Ullevaal, Kirkeveien 166, N-0407 Oslo, Norway. E-mail: skjalsh@medisin.uio.no
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METHODS

The clinical trial was carried out at the Section of Cardiovascular and Renal Research, Oslo University Hospital, Ullevaal from 2007 to 2009. The study was approved by the Regional Ethics Committee. All procedures were in accordance with institutional guidelines and the Helsinki Declaration. All participants provided written informed consent. The study was registered at <http://www.clinicaltrials.gov>; NCT00634387.

Participants

The participants were recruited partly from a follow-up study performed in 2005/2006 and partly from paper advertisements and referrals by general practitioners. All participants recruited from sources other than the follow-up study, went through a thorough screening procedure including past medical history, medications, clinical examination, routine blood and urine samples and BP.

We included men between 35 and 51 years of age, with resting office systolic BP of >140 and/or diastolic BP >90 mm Hg or daytime average BP of more than 135/85 mm Hg, if they had recently undergone a 24-h ambulatory BP recording. Participants with diabetes mellitus, CVD, renal disease, or screening BP $>180/110$ mm Hg, or participants using anti-hypertensive, anti-platelet, or cholesterol-lowering medication were excluded (Figure 1).

Study design and intervention

A double-blind randomized placebo-controlled crossover intervention study was conducted. Each treatment period lasted for 4 weeks separated by a 4-week washout (Figure 2).

The anthocyanin and placebo capsules were identically packaged and had identical appearance. The Medox capsules (Biolink AS) contained 17 different purified anthocyanins (mostly cyanidin 3-O- β -glucosides and delphinidin 3-O- β -glucosides, details described by Qin *et al.*¹⁵). Each anthocyanin capsule contained 80 mg of anthocyanins that were extracted from bilberry (blueberry) (*Vaccinium myrtillus*) and black currant (*Ribes nigrum*) through high-technological patented processes. The specified anthocyanin content was guaranteed by a thorough quality control system by the manufacturer. The anthocyanin content of blueberries and/or black currants varies substantially; therefore the anthocyanin content is analyzed in every batch of anthocyanin extract that is used in Medox capsules. Based on this analysis, the weight of anthocyanin extract that equals 80 mg of anthocyanins pro capsule is estimated. When running, up to 24 000 capsules are produced every hour by a full-automatic capsule-filling machine. Every 50th capsule is weighed and if the weight is below or higher than 10% above the estimated weight that equals 80 mgs of anthocyanins, the machine stops.

Furthermore, the manufacturer also stated that the capsules contained 40 mg citric acid and 35 mg maltodextrin to maintain stability, 25 mg fruit sugars and 13 mg lipids and traces of other polyphenols. Placebo capsules consisted of maltodextrin and blue color. The capsule content of both capsules were approximately 225 mg. The detailed content of the polyphenol traces was analyzed by National Institute for Health and Welfare (Helsinki), and they reported that the chlorogenic acid content of the capsules was 0.9 mg (as caffeic acid equivalents). Other phenolic acids were present at even lower levels: gallic acid 0.49 mg, vanillic acid 0.13 mg, protocatechuic acid 0.45 mg, syringic acid 0.09 mg and ferulic acid 0.10 mg.

Four capsules each morning and evening were self administered for 4 weeks, yielding a daily intake of 640 mg anthocyanins, which corresponds

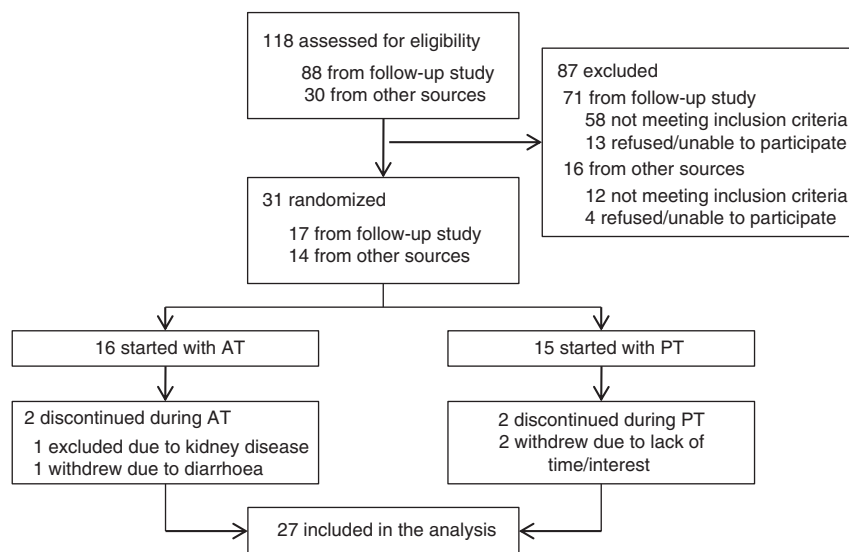


Figure 1. Flow of participants. AT, anthocyanin treatment. PT, placebo treatment.

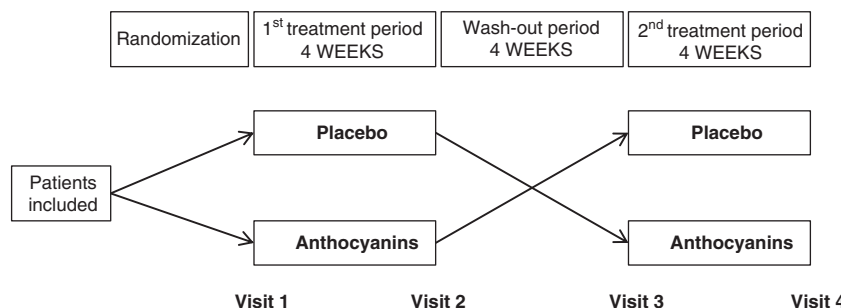


Figure 2. Study design.

to about 100 g of a mixture of fresh blueberries and black currants. Participants were told to swallow capsules with water and to avoid ingestion closely related to meals. All participants were thoroughly instructed to maintain their level of exercise, eating patterns, alcohol drinking habits and intake of other herbal products throughout the study.

The participants came to four visits at 4-week intervals. All visits were scheduled at 0800 hours. Participants were instructed not to eat, drink, or smoke for at least 8 hours before all visits. The only exception to this rule was the intake of the morning dose of placebo or anthocyanins, which was taken in the morning in order to maximize potential effects. Thus, all blood samples from visit 2 and 4 were collected 1–3 h after the intake of four study capsules. The time gap between ingestion of morning dose and assessment of outcomes was not assessed. However, on an individual level the time gap was probably very similar across the two treatment periods, as all participants were scheduled at 0800 hours and were instructed to ingest their morning dose at a regular time of the day. Two trained examiners were present at every visit. Number of unused capsules and adverse events were recorded after both treatment periods. Participant instructions were reinforced at all visits.

Randomization

Two separate computer-generated 4-block randomization lists stratified for recruitment source (follow-up study versus other sources) was made by a non-investigator. Study nurse or clinical physician enrolled participants. Scheduled participants were consecutively assigned by a medical technologist unaware of enrollment status, to treatment codes that corresponded to labels on otherwise identical concealed containers. Treatment codes were not revealed before all data was collected and analyzed.

Study outcomes

Primary endpoint was the difference in sitting systolic BP across treatment periods, published elsewhere.¹⁶ Secondary outcomes were differences in CVD-related parameters; total cholesterol, HDL-C, total cholesterol/HDL-C ratio, LDL-C, triglycerides, lipoprotein a, fasting glucose, HbA1c, albumin/creatinine ratio, insulin, HOMA-IR (homeostasis model assessment of insulin resistance), homocysteine, hematological and liver- and kidney markers, markers of inflammation and oxidative stress and plasma polyphenols.

Laboratory assessment

All blood samples were collected through an intravenous catheter in the supine position. Routine analyses were assessed using conventional laboratory methods at Oslo University Hospital, Ullevaal. Insulin was measured by competitive radioimmunoassay kit (Linco Research, Inc, St Charles, MO, USA). The HOMA-IR was calculated as serum glucose (mmol l^{-1}) multiplied by serum insulin (pmol l^{-1}) and divided by 135.¹⁷

Plasma polyphenols

Plasma polyphenols were assessed by a modification of a previously published method.¹⁸ In brief, polyphenols in plasma were hydrolyzed enzymatically by incubation with sulphatase and glucuronidase. Following hydrolysis, polyphenols were extracted with liquid-liquid extraction and analyzed by gas chromatography-mass spectrometry.

Inflammatory parameters

High-sensitivity C Reactive Protein was analyzed by immunoturbidimetric methods (Modular P, Roche, Basel, Switzerland).

TNF α , IL-6, IL-4, MCP-1, CD40L, ICAM-1, VCAM-1, P-selectin were all measured by enzyme-linked immunosorbent assay method (R&D Systems Europe, Abingdon, UK). Von Willebrand factor was determined by enzyme-linked immunosorbent assay (Asserachrom Stago Diagnostica, Asnieres, France).

L-arginine and ADMA (asymmetric dimethylarginine) were measured by high performance liquid chromatography and precolumn derivatization

with o-phthaldialdehyde (Sigma Chemicals Co, St Louis, MO, USA) as previously described.¹⁹

NOx was analyzed using Total Nitric Oxide Assay kit (R&D Systems Europe) after ultrafiltrated through a 12 kDa cut-off filter (VectaSpin Micro 12K MWCO, Whatman International Ltd, Maidstone, UK) to minimize interference with plasma proteins.

Von Willebrand factor, a marker of endothelial dysfunction, P-selectin and MCP-1 were analyzed in citrated plasma and CD40L in EDTA-plasma, otherwise serum was used.

Oxidative stress parameters

Ferric reducing/antioxidant power (FRAP) was determined in EDTA plasma, and modified FRAP was determined in extracts of EDTA plasma free of uric acid and proteins as previously described,²⁰ in order to eliminate the contribution of antioxidative power from these substances. For preparation of extracts free of uric acid and proteins, 10 μl uricase (0.1 units per 10 μl) in Tris buffer (pH 8.5, 400 mmol l^{-1}) were added to 60 μl plasma. After incubation for 5 min at room temperature, 120 μl ethanol was added to precipitate proteins. Samples were placed at 4 °C for 5 min before centrifugation at 13 000 g at 4 °C for 5 min. The uric acid- and protein-free supernatants were used for FRAP analysis.

The Diacron reactive oxygen metabolites test is considered a marker of lipid peroxidation and was performed in serum, according to the manufacturer's instructions (Diacron International, Grosseto, Italy).

Statistics

Sample size considerations are previously detailed.¹⁶ All statistical analyses were based on values at the end of the placebo period versus values at the end of the anthocyanin period rather than change scores during each treatment period. This statistical approach was according to the study protocol and was based on the fact that fewer measurements reduce total variation and thus increase statistical power. Paired-samples *t*-test was used when the differences between the treatment periods were sufficiently normally distributed, otherwise Wilcoxon signed rank test was used. Carry-over effects were analyzed in variables that were significantly different across treatment periods, by comparing whether anthocyanin effects differed between the first and the second treatment period, and equally in the placebo group.²¹ Data are presented as mean \pm s.e.m., unless otherwise specified. A two-sided significance level of 5% was used throughout. The data were analyzed using the statistical package SPSS 16.0 for Windows (SPSS Inc, Chicago, IL, USA).

RESULTS

Description of participants

The participants were 41 ± 3 (mean \pm s.d.) years of age. Their mean BP at screening was $143/96 \pm 13/6$ mm Hg (mean \pm s.d.) and it dropped to $137/85 \pm 12/7$ mm Hg (prehypertension) at the first visit. In all, 15 men had a family history of hypertension among first-degree relatives, and 1 man had a first-degree family history of CVD. None had pre-clinical organ-damage (microalbuminuria or left ventricular hypertrophy measured by electrocardiogram). A total of 11 used some kind of health-promoting product (7 used vitamins, 6 used fish oil/omega-3 supplementation and 3 used other herbal products) in addition to their habitual food consumption. Only 4 men were physically inactive.

Intervention period

In all, 27 out of 31 men completed all four visits. Average compliance was 90% in the placebo period and 88% in the anthocyanin period (estimated by capsule count). One patient withdrew due to diarrhea for several days in the middle of the active period, possibly constituting an adverse effect of the anthocyanin treatment. During the anthocyanin period, additionally 3 patients reported minor headache of short duration,

2 patients reported that their stools were darker than usual and 1 of the latter patients also reported nausea. No adverse reaction was recorded during the placebo period. Overall, no carryover effects were observed. However, carryover effects were not assessed in relation to von Willebrand factor as only blood samples from visit 2 and 4 were analyzed.²¹

Effects on cardiovascular risk factors

Table 1 shows the cardiovascular risk factors at the end of both treatment periods. Following the anthocyanin treatment, HDL-C was significantly higher ($P=0.043$), and the ratio between cholesterol and HDL-C was correspondingly lower ($P=0.049$). Furthermore, glucose levels were significantly higher ($P=0.024$) and HOMA-IR was borderline significantly higher following the anthocyanin treatment ($P=0.059$).

Effects on inflammatory and oxidative stress parameters

Von Willebrand factor was significantly higher following the anthocyanin period as compared with the placebo period ($P=0.007$). Additional investigated parameters such as cytokines, markers of endothelial cell activation, or oxidative stress markers were similar (Table 2).

Plasma polyphenols

Out of 17 phenolic acids measured, 8 were significantly higher in the end of the anthocyanin period as compared with the placebo period (sampling was 1–3 h after morning dose of study capsules) (Table 3). The potential anthocyanin metabolites gallic acid, 4'-O-methyl gallic acid, vanillic acid, protocatechuic acid and syringic acid were 185%, 725%, 131%, 190% and 667%, respectively, higher after the anthocyanin treatment. Caffeic acid and its metabolite isoferulic acid, as well as ferulic acid, were 150%, 120% and 94% higher, respectively.

Clinical chemistry analyses

Anthocyanins did not significantly affect white blood cells, platelets, fibrinogen, uric acid, urea, creatinine, aspartate transaminase or alanine transaminase.

DISCUSSION

In the present randomized double-blinded placebo-controlled study, we demonstrated for the first time that purified anthocyanins increase HDL-C levels in non-dyslipidemic prehypertensive subjects. Accordingly, the ratio of total cholesterol/HDL-C was

Table 1. Laboratory parameters related to risk of cardiovascular disease following the placebo and the anthocyanin treatment periods

Parameter	n	Placebo	Anthocyanins	P
S-Cholesterol, mmol l ⁻¹	27	4.88 ± 0.16	4.96 ± 0.17	0.432
S-HDL-C, mmol l ⁻¹	27	1.18 ± 0.09	1.24 ± 0.08	0.043
S-Cholesterol/ S-HDL-C ratio	27	4.60 ± 0.28	4.34 ± 0.26	0.049
S-LDL, mmol l ⁻¹	27	3.09 ± 0.16	3.19 ± 0.15	0.341
S-Triglycerides, mmol l ⁻¹ a	27	1.37 ± 0.18	1.18 ± 0.13	0.127
S-Lipoprotein(a), mg l ⁻¹ a	27	228 ± 63	206 ± 55	0.110
S-Glucose, mmol l ⁻¹	27	5.08 ± 0.07	5.22 ± 0.07	0.024
S-HbA1C, %	27	5.41 ± 0.05	5.40 ± 0.05	0.746
S-Insulin, pmol l ⁻¹ a	24	39 ± 4	47 ± 7	0.127
HOMA-IR ^a	24	1.50 ± 0.14	1.87 ± 0.26	0.059
S-Homocysteine, μmol l ⁻¹	27	10.70 ± 0.54	10.71 ± 0.52	0.983
U-albumine/U-creatinine, mg per mmol	26	0.27 ± 0.03	0.32 ± 0.05	0.331

Abbreviations: HDL-C, high-density lipoprotein-cholesterol; HOMA-IR, homeostasis model assessment of insulin resistance; LDL, low-density lipoprotein.

^aAnalyzed by Wilcoxon signed rank test. Parameters are presented as mean ± s.e.m.

Table 2. Markers of inflammation and oxidative stress following the placebo and the anthocyanin periods

Parameter	n	Placebo	Anthocyanins	P
hsCRP, mg l ⁻¹	27	1.14 ± 0.17	0.17	0.253
MCP-1, pg ml ⁻¹	26	179 ± 7	7	0.302
P-selectin, pg ml ⁻¹	26	21.7 ± 1.3	1.3	0.937
ICAM, ng ml ⁻¹	27	193 ± 8	8	0.632
VCAM, ng ml ⁻¹	27	574 ± 24	24	0.369
CD40L, pg ml ⁻¹	27	79 ± 5	5	0.380
IL-6, pg ml ⁻¹	27	1.35 ± 0.18	0.18	0.088
TNF-α, pg ml ⁻¹	27	2.12 ± 0.21	0.21	0.147
IL-4, pg ml ⁻¹	27	0.25 ± 0.01	0.01	0.857
von Willebrand factor, %	26	74 ± 4	4	0.007
L-arg, μmol l ⁻¹	24	80 ± 3	3	0.666
ADMA, μmol l ⁻¹	24	0.66 ± 0.03	0.03	0.970
L-arg/ADMA ratio	24	124 ± 6	6	0.337
NOx, μmol l ⁻¹	26	39 ± 3	3	0.691
FRAP, μmol l ⁻¹	26	926 ± 21	21	0.362
Modified FRAP, μmol l ⁻¹	26	165 ± 10	10	0.617
dROMs, Carr U	26	295 ± 10	10	0.699

Abbreviations: ADMA, asymmetric dimethylarginine; dROMs, diacrons reactive oxygen metabolites; FRAP, ferric reducing/antioxidant power; hsCRP, high-sensitivity C reactive protein; ICAM, intercellular adhesion molecule; IL, interleukin; MCP, monocyte chemotactic protein; TNF, tumor necrosis factor; VCAM, vascular cell adhesion molecule. Variables are presented as mean values ± s.e.m.

Table 3. Plasma phenolic acids 1–3 h after intake of anthocyanins following the placebo and the anthocyanin period, respectively (n = 27)

Parameter	Placebo	Anthocyanins	P
Gallic acid	7 ± 1	20 ± 1	<0.001
4-O-methylgallic acid	8 ± 3	66 ± 6	<0.001
Vanillic acid ^a	26 ± 5	60 ± 4	<0.001
Protocatechuic acid	20 ± 2	58 ± 4	<0.001
Syringic acid	3 ± 0	23 ± 2	<0.001
Homovanillic acid	57 ± 4	63 ± 4	0.219
3,4-dihydroxyphenylacetic acid (DOPAC)	81 ± 4	85 ± 4	0.424
Hippuric acid	611 ± 118	672 ± 80	0.509
Caffeic acid	20 ± 4	50 ± 5	<0.001
Isoferulic acid	10 ± 3	22 ± 3	<0.001
Ferulic acid	18 ± 3	35 ± 4	<0.001
Dihydroisoferulic acid	49 ± 19	59 ± 25	0.773
Dihydrocaffeic acid ^a	22 ± 4	38 ± 10	0.256
Dihydroferulic acid	46 ± 11	71 ± 18	0.202
3-hydroxybenzoic acid	16 ± 2	20 ± 3	0.230
3-hydroxyphenylacetic acid	118 ± 14	134 ± 16	0.389
3-(3-hydroxyphenyl)-propionic acid ^a	230 ± 63	342 ± 91	0.136

^aAnalyzed by Wilcoxon signed rank test. All concentrations are given in nmol l⁻¹. Parameters are presented as mean ± s.e.m.

increased. Intriguingly, glucose and levels of von Willebrand factor also increased following anthocyanin treatment. Although an increase of HDL-C may be beneficial, the increase of glucose and von Willebrand factor may indicate unfavorable effects. All other investigated risk factors, as well as markers of oxidative stress and inflammation did not differ across treatment periods.

The majority of intervention studies investigating anthocyanins have used foods containing several types of polyphenols. Medox capsules, on the other hand, contain purified anthocyanins and they have been assessed in two previous placebo-controlled studies. In the first Medox-trial from 2007,¹⁴ HDL-C was found to be non-significantly higher after 3 weeks. In a recent Medox-study,¹⁵ HDL-C increased by 13.7% and LDL-C decreased by 13.6% in dyslipidemic patients after 12 weeks. The authors suggested that this effect was due to a change in the level and activity of cholesteryl transport protein, responsible for reverse cholesterol transport. Our study found a less pronounced HDL-C increase and furthermore, LDL-C levels were unaltered, indicating a modest effect on blood lipids. However, data from these three trials may indicate that duration is more crucial than dose with respect to HDL-C and LDL-C changes. Thus, the treatment periods of 4 weeks in our study may have been too short to provide a reduction in LDL-C levels. Additionally, it may be more difficult to reduce LDL-C levels in non-dyslipidemic participants. Overall, our results confirm that HDL-C increases following anthocyanin intake. However, as Curtis *et al.*²² did not find similar effects on blood lipids after 500 mg of anthocyanins (cyanidin 3-glucoside) for 12 weeks, it is possible that different anthocyanin compounds may possess different bioactivities. Interestingly, a previous intervention study, in which various berries (about 60% bilberry and black-currant) were consumed for 8 weeks by subjects with cardiovascular risk factors, also reported an increase in HDL-C and no alterations in LDL-C.²³

Blood glucose was significantly higher following the anthocyanin period and insulin sensitivity (HOMA-IR) tended to be higher. However, insulin and long-term glucose assessed by HbA1c were not significantly different across treatment periods. The two previous Medox studies did not detect differences in fasting glucose levels after 3 or 12 weeks, respectively.^{14,15} Importantly, these studies differed from our study in two ways; they used half the anthocyanin dose and blood samples were collected after a strict fast. In our study, the capsules were ingested 1–3 h before blood samples were collected. Thus, we cannot rule out the possibility that high doses of anthocyanins may lead to a small unfavorable rise in fasting blood glucose. However, although both

placebo and anthocyanin capsules had a very low and relatively similar energy content, such carbohydrate composition differences between placebo and anthocyanin capsules may have contributed to the differences observed in blood glucose levels.

In the first Medox study,¹⁴ participants treated with 300 mg anthocyanins daily reached significantly lower levels of IL-8, IFN α and RANTES, after 3 weeks. It was suggested that the anti-oxidative potential of anthocyanins could explain these findings, and further assessment of anthocyanins role in prevention of chronic inflammatory diseases was recommended.¹⁴ In the present study, inflammatory variables (associated with atherosclerosis) were assessed, and no significant differences were found. Oxidative stress is thought to inversely affect the antioxidant potential of plasma (FRAP and modified FRAP) and to promote lipid peroxidation (dROM), but none of these oxidative stress markers differed. It was assumed that potential beneficial effects of anthocyanins on the endothelium, would be accompanied by a reduction of markers of endothelial dysfunction. Although most endothelial markers (ADMA, Arg/ADMA ratio and NOx) were similar following both treatment periods, von Willebrand factor, a marker of endothelial dysfunction that is associated with CVD,²⁴ was in fact significantly higher following the anthocyanin period. This may represent an unfavorable effect of anthocyanins, however the underlying mechanism and the biological impact on such an increase of von Willebrand factor in prehypertensive men is not known.

The absorption of anthocyanins is reported to be <1% of the ingested dose.²⁵ Thus, potentially beneficial health effects following anthocyanin intake may partly be due to the *in vivo* formation of bioactive anthocyanin metabolites that are more readily absorbed.²⁶ *In vitro*, anthocyanins metabolize to phenolic acids such as gallic acid, protocatechuic acid, vanillic acid and syringic acid. Whether these compounds are formed *in vivo* as well, is largely unknown. In this study, plasma concentrations of the above-mentioned compounds were significantly higher in the anthocyanin group compared with the placebo group in blood samples collected 1–3 h after ingestion of the morning dose of anthocyanins. Our results may indicate that these compounds are anthocyanin metabolites that have been formed *in vivo*. However, the capsules contained small amounts of several polyphenols, including the above-mentioned compounds. Thus, we cannot rule out that the observed increase in plasma polyphenols were partly due to the presence of phenolic acids in the capsules.

Our participants had elevated BP at screening, but did not have end-organ damage or established CVD. Such a group of

participants was considered ideal for this kind of study, as prehypertensive participants have a mildly increased risk without an urgent need for medical treatment. Importantly, Curtis *et al.*²² suggested that anthocyanin-containing intervention studies, in which participants with clinically diagnosed diseases were assessed, tended to demonstrate beneficial effects on the cardiovascular system, whereas studies of healthy subjects tended to show no effects. Furthermore, they suggested that low baseline levels of hsCRP could explain the lack of anti-inflammatory effects in their 12-week placebo-controlled trial. The first Medox study demonstrated reduced markers of inflammation in relatively healthy men and women, however, the hsCRP levels among these participants were in a high range compared with the relatively low hsCRP levels observed in our study.²⁷ Thus, the lack of anti-inflammatory and anti-oxidative effects in this study may be due to the feature that our participants were not characterized by inflammation or oxidative stress in the first place.

The amount of anthocyanin intake in the present study equals about 10 times the average consumption, however, it may be achievable by increasing intake of berries substantially.²⁸ As the bioavailability of anthocyanins is low,²⁵ a high intake was ensured in our study by using twice the dose of anthocyanins as compared with the dose used in the previously mentioned Medox studies.

In the present study, we aimed to maximize potential effects by instructing participants to ingest their morning dose before visit 2 and 4, and subsequently increase the possibility of capturing potential additional short-term changes in blood analyses. As a consequence, we do not know for sure that anthocyanin effects in this study are due to chronic consumption, they may also be due to acute effects following the morning dose. The highly significant increase in plasma polyphenols, in particular, was probably due to the morning dose of anthocyanins, and not the result of chronic intake. Blood samples were collected 1–3 h after ingestion of the morning capsules. This time interval from anthocyanin ingestion to blood collection may be justified, as plasma concentrations of anthocyanins generally peaks within the first hours²⁵ and as reduction of oxidative stress markers have been shown to peak about 2 hours after intake of anthocyanin compounds.^{6,29}

Few participants dropped out in our study and the capsule compliance was satisfactory. The study was carefully designed to avoid systematic bias. In order to conduct a placebo-controlled study, anthocyanins were provided in capsules. This enabled us to study effects of purified anthocyanins at high concentrations with little influence from other bioactive non-anthocyanin molecules. Given the quality control of the anthocyanin content of these capsules, we believe that the variation of anthocyanin content in these capsules is less than what we would expect if our participants ingested a particular weight of blueberries or black currants. However, natural sources may contain other potentially bioactive compounds that may influence CVD. Moreover, foods ingested concomitantly may influence degradation and subsequent absorption. All these considerations limit the possibility to generalize. Other limitations include limited number of patients, analyses of many parameters and relatively short intervention periods. Lack of effects on inflammation and oxidative stress markers may also be due to low statistical power, as power calculations were based on potential antihypertensive effects of anthocyanins.

In a crossover study, outcome measures are compared on an individual level, and this increases statistical power. The lack of dietary assessment may be considered a limitation of the current study. However, we did not assume any major intra-individual differences in dietary intake across treatment periods, given our instructions not to change eating habits. Moreover, if there was a tendency that our participants tended to eat more healthy in second treatment period compared with the first, this effect would be evened out by the randomization of treatment sequence.

Except for the restriction of blueberries, which are easily available and particularly rich in anthocyanins, we did not restrict the habitual intake of other habitual flavonoids or supplements. As we primarily wanted to assess the effects of high doses of anthocyanins, we considered the relatively low contribution of anthocyanins from a normal diet and/or even various supplements to be of less importance. However, as potentially beneficial anthocyanin effects may be mediated by general anti-inflammatory or anti-oxidative mechanisms, subjects with a high dietary flavonoid intake as well as subjects eating other presumably beneficial substances may be less likely to gain additional benefits from anthocyanin supplementation. An alternative approach that is commonly used in similar studies, is to restrict most habitual flavonoids and habitual supplements throughout the study. However, the latter approach may potentially eliminate benefits from 'competing' habitual flavonoids and thereby overestimate benefits caused by anthocyanin supplementation.

In conclusion, we have shown for the first time that purified anthocyanins may increase HDL-C in prehypertensive and non-dyslipidemic participants. Although the HDL-C increase was modest after 4 weeks, this effect may possibly increase following long-term consumption and may positively affect CVD development in the long term. However, possibly unfavorable rises of glucose and von Willebrand factor were also observed following the anthocyanin period. Importantly, increased levels of glucose have not been found by similar studies and may be related to the morning dose in our study, and von Willebrand factor have not been assessed at all in similar studies. Thus, future studies should include these parameters to assess whether similar effects are reproduced. Further, our results suggest that these anthocyanin supplements do not beneficially affect markers of inflammation, endothelial dysfunction, or oxidative stress in the short-term among prehypertensive men with low total CVD risk.

What is known about this topic

- Anthocyanins are present in berries, fruits, vegetables and red wine, and they are considered to possess antioxidative properties.
- Intake is believed to reduce risk of CVD.
- Some studies assessing anthocyanin-containing interventions have reported beneficial effects on CVD risk factors, whereas other studies have not.

What this study adds

- We performed a randomized double-blind crossover study of purified anthocyanin supplements on cardiovascular risk factors in prehypertensive men.
- Pure anthocyanins modestly increased the level of HDL after 4 weeks of treatment, but did not beneficially affect markers of inflammation or oxidative stress.
- Overall anthocyanin effects on factors related to CVD appear modest in the short-term among prehypertensive men with low total CVD risk.

CONFLICT OF INTEREST

Biolink AS provided the corresponding author SS Hassellund half his income for an year. They were encouraged to comment on the manuscript, however, the conduction, analysis and article preparation were investigator initiated and unfavorable results could not be restricted from publication. The other authors declare no conflict of interest.

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