Cardiovascular Disease Risk Biomarkers and Liver and Kidney Function Are Not Altered in Postmenopausal Women after Ingesting an Elderberry Extract Rich in Anthocyanins for 12 Weeks\textsuperscript{1,2}

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Abstract
Growing evidence supports a cardio-protective role for anthocyanins; however, there is limited evidence on their efficacy and safety following the consumption of relatively high but dietarily achievable doses in humans. We conducted a parallel-designed, randomized, placebo-controlled study to examine the effect of chronic consumption of anthocyanins on biomarkers of cardiovascular disease (CVD) risk and liver and kidney function in 52 healthy postmenopausal women (\(n = 26\) in treatment and placebo groups). Volunteers (BMI, 24.7 \(\pm\) 3.6 kg/m\(^2\); age, 58.2 \(\pm\) 5.6 y) consumed 500 mg/d anthocyanins as cyanidin glycosides (from elderberry) or placebo for 12 wk (2 capsules twice/d). At the beginning (wk 0) and end of the 12-wk intervention, levels of anthocyanins and biomarkers of CVD (inflammatory biomarkers, platelet reactivity, lipids, and glucose) and liver and kidney function (total bilirubin, albumin, creatinine, alkaline phosphatase, alanine aminotransferase, and \(\gamma\)-glutyl transferase) were assessed in fasted blood. Anthropometric, blood pressure, and pulse measurements were also taken. In addition, postprandial plasma anthocyanins were measured (\(t = 1, 2, 3\) h) following a 500-mg oral bolus dose. After 12 wk of chronic exposure to anthocyanins, there was no significant change in biomarkers of CVD risk and liver and kidney function remained within clinically acceptable ranges. We observed no plasma accumulation of anthocyanins; however, postprandial metabolism increased (\(P = 0.02\)). In conclusion, these data suggest that chronic consumption of 500 mg/d of elderberry extract for 12 wk is apparently safe, but ineffective in altering biomarkers of CVD risk in healthy postmenopausal women. J. Nutr. 139: 2266–2271, 2009.

Introduction
Dietary flavonoids have emerged as potential candidates to protect against cardiovascular disease (CVD),\textsuperscript{6} with prospective studies (1,2) suggesting that higher intakes reduce the relative risk of CVD. Mechanistic studies also support beneficial effects of flavonoids on established biomarkers of CVD risk, including nitric oxide, inflammation, and endothelial dysfunction (3–5). In our recent meta-analysis of randomized controlled trials (6), flavonoids in chocolate and soy protein isolate had a positive impact on a number of CVD risk markers, including blood pressure and flow-mediated dilation; however, in agreement with a previous review of epidemiologic studies (7), insufficient evidence was available to determine the relative importance of several flavonoid subclasses on CVD risk, including anthocyanins (6).

Anthocyanins confer the red, blue, and purple colors to many fruits and vegetables, with levels particularly high in berries; e.g., 100 g of blackcurrants provide up to 900 mg of anthocyanins (8). Available data from prospective studies suggest that habitual daily intakes of anthocyanins may be relatively low, within the range of 3–200 mg/d (9–11); however, these levels may be unrepresentative of actual intake in part due to the lack of comprehensive nutrient databases, including quantitative levels of anthocyanins. Nonetheless, even at these low levels of intake, these cohort studies have reported a reduced risk of coronary heart disease (CHD), CVD, and total mortality with increased anthocyanin consumption (1).
To date, the limited number of human interventions with anthocyanin-rich extracts and foods has provided equivocal data. Whereas relatively low-dose anthocyanin interventions (19—100 mg/d) have been associated with significant reductions in ischemia (12), blood pressure and Intima media thickness (13), lipid levels (14), inflammatory status (15), and oxidative stress in participants with clinically diagnosed disease (e.g. CHD, hyperlipidemia), studies using similar doses in healthy individuals found little effect (16–18). However, 1 study by Karlesen et al. (19) showed significant improvements in plasma inflammatory biomarkers [interleukin (IL)-8 and chemokine ligand 5 (RANTES)].

Despite the extensive number of studies feeding anthocyanins, there are still limited data regarding the safety of prolonged consumption of physiologically relevant doses of anthocyanins. Therefore, to establish the effects of chronic consumption of anthocyanin on vascular, inflammatory, and lipid and lipoprotein responses and to address the paucity of data on safety biomarkers of liver and kidney function, we conducted a 12-wk randomized, parallel design, placebo-controlled trial in healthy postmenopausal women.

**Materials and Methods**

**Participants.** Fifty-seven healthy postmenopausal women were recruited by advertisement in the university area and in the local media. Postmenopausal women were selected because they have an elevated risk of CVD compared with younger women (20) but remain a relatively understudied population. Eligible participants were postmenopausal women (no menstruation for at least 12 mo) <70 y, not taking hormone replacement therapy for ≥6 mo, with BMI in the 20–22 kg/m² range and who were nonsmokers (i.e. never smokers or those ceasing ≥12 mo ago). During a telephone screening, participants were excluded if they had a history of 1 of the following: diabetes; hepatitis, renal, cardiac, pulmonary, digestive, hematological, neurological, thyroidal, or psychiatric disease; or taking concomitant therapy (e.g. antiinflammatory or steroidal medication, or vaccines and antibiotics). Those on therapeutic or weight-loss diets, using concomitant therapy (e.g. antiinflammatory or steroidal medication, or vaccines and antibiotics). Those on therapeutic or weight-loss diets, using antibiotics or being a smoking person. In addition, the following foods that potentially alter CVD risk biomarkers were also limited: dark chocolate, tea, and oily fish. A list of foods that could be freely consumed was supplied along with instructions to maintain normal exercise and lifestyle patterns. Compliance with the trial profile summarizing the flow of study participants during recruitment, treatment allocation, follow-up, and analysis. 1. Failed clinical screen (n = 24). 2. Passed clinical screen but withdrew before baseline (n = 5). 3. Treatment groups balanced for mean participant age, BMI, and years since menopause. 4. Lost to follow-up after cancer surgery (n = 1), poor compliance (n = 1). 5. Adverse events (n = 3); anal irritation (n = 1), recurrence of previously undisclosed condition (n = 1), lump found in routine breast scan (n = 1).

12-wk trial, participants were instructed to avoid consuming anthocyanin-rich fruit, berries, and vegetables (e.g. blueberries, raspberries, plums, red grapes, red cabbage, radish) and products derived from such items (e.g. fruit juices from berries, berry-rich jams and desserts, red wine, and sherry) and limit the intake of foods with a lower anthocyanin yield (e.g. red apples, nectarine, black olives, aubergine) (a list was provided). In addition, the following foods that potentially alter CVD risk biomarkers were also limited: dark chocolate, tea, and oily fish. A list of foods that could be freely consumed was supplied along with instructions to maintain normal exercise and lifestyle patterns. Compliance with the study protocol was approved by the Norfolk Research Ethics Committee and all participants provided written informed consent.

**Capsules.** An elderberry (Sambucus nigra) extract was encapsulated, with each opaque capsule containing 125 mg of anthocyanin (cyanidin-3-glucoside) (Artemis International). Identical placebo capsules were also produced. Participants received a 12-wk supply of capsules in 6-2 wk batches and participants and researchers were unaware of the treatment allocation. Quantification of anthocyanin capsule content was verified prior to commencement of the trial using HPLC.

**Study protocol.** In this double-blind, placebo-controlled, parallel study, 57 volunteers were stratified by age, number of years postmenopause, and BMI and randomized to the placebo (n = 29) or anthocyanin (n = 28) treatment group. Five of the 57 participants randomized to treatment did not complete the study (2 anthocyanin treatment, 3 placebo treatment) (Fig. 1): 1 discovered a lump on a routine breast scan, 2 were withdrawn by the researchers [1 for poor compliance (<85%), 1 for recurrence of a condition previously undisclosed], 1 experienced anal irritation, and 1 was lost to follow-up. The characteristics of the 52 participants who completed the study are shown (Table 1) (26 anthocyanin, 26 placebo). Participants consumed 4 capsules (2 × twice/d) each day for 12 wk; 2 in the morning and 2 in the evening. The anthocyanin capsules (4/d) provided a total of 500 mg/d anthocyanin (as cyanidin-3-glucoside), equivalent to the anthocyanin levels found in 25 g elderberries, 100 g blueberries, and 140 g blackberries. For 7 d preceding and during the

**TABLE 1** Baseline and end of study characteristics of healthy postmenopausal women completing 12 wk supplementation with anthocyanin (500 mg/d) or placebo1,2

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Anthocyanin</th>
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<tbody>
<tr>
<td>Age, y</td>
<td>58.3 ± 5.8</td>
<td>58.1 ± 5.5</td>
</tr>
<tr>
<td>Menopausal years, y</td>
<td>7.7 ± 5.5</td>
<td>8.0 ± 8.4</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>24.3 ± 3.4</td>
<td>25.1 ± 3.8</td>
</tr>
<tr>
<td>wk 12</td>
<td>24.4 ± 3.3</td>
<td>25.1 ± 3.5</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>130 ± 14</td>
<td>123 ± 15</td>
</tr>
<tr>
<td>wk 12</td>
<td>124 ± 15</td>
<td>124 ± 13</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>82 ± 11</td>
<td>78 ± 7</td>
</tr>
<tr>
<td>wk 12</td>
<td>80 ± 10</td>
<td>77 ± 7</td>
</tr>
<tr>
<td>Pulse, beats/min</td>
<td>61 ± 6</td>
<td>65 ± 8</td>
</tr>
<tr>
<td>wk 12</td>
<td>63 ± 9</td>
<td>64 ± 9</td>
</tr>
</tbody>
</table>

1 Values are means ± SD, n = 26/group.
2 No significant treatment effects were observed by univariate general linear ANCOVA. No significant differences were observed between treatment groups at baseline by 1-way ANOVA.
dietary restrictions was monitored via 4-d food diary records. Compliance with treatment was determined by counting the returned capsules at the end of the trial and measuring anthocyanin excretion in the first morning urine void at baseline and at the end of intervention.

Participants were assessed at the start (wk 0) and end (wk 12) of the intervention period and the 2 assessment days followed an identical schedule. Fasting blood samples were collected for assessment of plasma anthocyanins, CVD biomarkers (inflammatory biomarkers, lipids, glucose, platelets) and liver and kidney function [total bilirubin, albumin, urea, creatinine, alkaline phosphatase (ALP), alanine aminotransferase (ALT), and γ-glutyl transferase (GGT)]. They were then given a 500-mg bolus dose (equivalent to the daily dose administered within the study) of elderberry anthocyanins and provided with a standard breakfast consisting of white toast with spread and cereal. Further blood samples were then collected at 1, 2, and 3 h postbolus consumption. Plasma was obtained by centrifugation at 1500 × g for 15 min within 30 min of collection. To quantify anthocyanin levels in plasma, samples were acidified with HCl (to a final concentration of 1%) within 10 min of collection to stabilize the anthocyanins and subsequently stored at 4°C until later analysis on the day of collection. All other plasma samples were stored at −80°C until further analysis. For the platelet sample collection, whole blood (3.0 mL) was drawn into sodium citrate tubes under minimal pressure and without the use of a tourniquet. Body weight, height, blood pressure and pulse rate were measured in duplicate following standardized protocols. All measures were taken before the blood draw and in the case of blood pressure and pulse rate after a rest period of at least 5 min.

**Plasma biomarkers of CVD risk.** Plasma concentrations of C-reactive protein (CRP) [R&D Systems; minimum detectable level (MDL), 0.01 μg/L], IL-6 (R&D Systems; MDL, typically <0.7 ng/mL), tumor necrosis factor-α (TNFα) (Cayman Chemical; MDL, 3.9 ng/L), TNF receptor 1 (TNFR1) and TNFR-2 (both Hycult Biotechnology; MDL, 25 ng/L), endothelin-1 (R&D Systems; MDL, typically <1.0 ng/L), and RANTES (R&D Systems; MDL, 2.0 ng/L) were measured using commercially available ELISA kits according to the manufacturer’s protocol. Intra-assay precision ranged from 2.9% CV (for CRP) to 5.9% CV (for IL-6), interassay precision ranged from 2.7% CV (for CRP) to 8.0% CV (for IL-6). Fasting glucose and lipid analysis (cholesterol, HDL cholesterol, LDL cholesterol, and triglycerides) were analyzed using automated diagnostic equipment (Abbott Architect Instruments) following standard protocols at the pathology laboratory at the Norfolk and Norwich University Hospital.

**Platelet reactivity.** Platelet reactivity was assayed in response to stimulation with ADP and collagen according to previously published methods (21). Briefly, whole blood (2.5 μL) was incubated for 10 min at room temperature with Mg-HEPES buffer (1 mL pH 7.4 unstimulated control) and then 2 additional aliquots were stimulated with either ADP (10 μM) or collagen (4 μg/mL). Postincubation, 100-μL aliquots were transferred into tubes containing labeled monoclonal antibodies: procaspase activating compound-1 (Pac-1)-fluorescein isothiocyanate (Becton Dickenson), CD61-allophycocyanin (Invitrogen), and CD62-phycocerythrin (Invitrogen) and incubated at room temperature for 20 min. Samples were then fixed with 1% paraformaldehyde and stored in the dark until analysis. All samples were analyzed on the day of collection using a Beckman Coulter Cytomics FC500 MPL flow cytometer. Data analysis was performed using CXP software (Beckman Coulter V.2.1), with activated platelets defined as the percentage of CD61 positive events that expressed CD62 or Pac-1.

**Liver and kidney function.** Plasma concentrations of total bilirubin, albumin, urea, and creatinine together with enzyme activities of ALP, ALT, and GGT were determined using automated diagnostic equipment (Abbott Architect Instruments) following standard protocols at the Norfolk and Norwich University Hospital.

**Extraction and quantification of anthocyanins in plasma.** Identification and quantification of anthocyanins was accomplished using previously published methods (22). Briefly, prior to the extraction of anthocyanins, 50 μL delphinidin-3-glucose (Extrasyrosynth) was added to samples (per 200 μL plasma) as internal standards. Samples were vortexed and centrifuged (16000 × g at 4°C for 15 min) and supernatants were transferred directly into autosampler vials for analysis. Samples were analyzed on an Agilent 1100 HPLC system with UV-diode array and electrospay ionization-MS detection (Agilent Technologies). The chromatographic column was a Gemini C18 (3 μm; 3.0 mm i.d. × 150 mm; Phenomenex) and the mobile phase consisted of acidified (95:5; v/v formic acid) water and acetonitrile (Sigma-Aldrich). Plasma concentrations of total parent anthocyanin compounds were calculated by summing levels of cyanidin-3-sambubioside-5-glucoside, cyanidin-3-sambubioside, cyanidin-3-glucoside, and cyanidin in each hourly sample (0, 1, 2, and 3 h); similarly, total anthocyanin metabolites were calculated by summing the levels of cyanidin-3-glucuronides and cyanidin-3-sulfate.

**Statistical analyses.** Results were tested for normality using Shapiro-Wilk analyses and data that were not normally distributed were transformed (using log10 function) prior to statistical analyses. Differences between the treatment groups at baseline were determined using 1-way ANOVA. Analysis of treatment effect between placebo and anthocyanin groups was determined using univariate general linear ANCOVA model, with baseline levels as a covariate. Differences between the treatment groups for postprandial levels of plasma anthocyanins was determined at wk 12 using a repeated-measures general linear model (repeated measures; 0-, 1-, 2-, 3-h plasma levels). For the platelet analysis, data were split into 3 groups (ADP, collagen, and control) and CD62 and Pac-1 expression were analyzed using 2-way ANOVA with treatment and time as factor variables. For nonparametric data (GGT, glucose, IL-6, endothelin-1, TNFR1), Kruskal-Wallis tests were performed. Data were analyzed using SPSS 16.0 for Windows statistical analysis software and P ≤ 0.05 was considered significant.

**Results**

In this randomized, parallel-design, placebo-controlled trial, 57 postmenopausal women consumed 4 capsules/d containing 500 mg anthocyanins (total) for 12 wk. For the 52 participants that completed the study (Fig. 1), compliance with treatment was high, with 96.8 and 97.7% of the capsules consumed for the treatment and placebo groups, respectively.

The 12-wk intervention did not affect plasma levels of inflammatory biomarkers (CRP, TNFα, IL-6, TNF RI and RII, and RANTES), vascular activity (endothelin-1, platelet reactivity, blood pressure, pulse), plasma lipids and lipoproteins (total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides), or glucose concentrations, which were all within the anticipated physiological range (Table 2).

Potential effects of the intervention on liver and kidney functions were also assessed. There was no effect of the treatment on the kidney function markers; urea and creatinine or the liver function markers; albumin, ALT, GGT, or ALP. There was a change in plasma bilirubin in the treatment group compared with the placebo group (P < 0.05); however, the change was minimal (an increase of 1.0 mmol/L over 12 wk) and the mean level remained within the normal physiological range for this age group (0–22 mmol/L).

At the beginning (wk 0) and end of the study (wk 12), the level of anthocyanin metabolism was also assessed. At wk 12, the plasma concentration of anthocyanin metabolites of the treatment group was greater (P = 0.02) than that of the control, whereas plasma levels of parent compounds (anthocyanin) did not differ (P = 0.06) (Fig. 2).

**Discussion**

The objectives of this study were to establish whether anthocyanins from elderberries could beneficially affect biomarkers of...
CVD risk in postmenopausal women and to determine the relative safety of chronic ingestion of high, but dietarily achievable doses of anthocyanins, on liver and kidney function. Although a number of cohort and intervention studies have shown a potentially beneficial effect of anthocyanins on CHD and CVD (1,6), few trials have established the effect of prolonged exposure to physiologically relevant doses on liver and kidney function.

Unlike previous anthocyanin trials in patients with established CVD (13–15), our 12-wk dietary supplementation with 500 mg/d elderberry anthocyanins did not affect a range of established CVD risk biomarkers, including inflammation, vascular reactivity, and plasma lipid and lipoprotein profiles, suggesting limited efficacy in apparently healthy individuals. To this extent, the results of this study are consistent with 2 previous studies (16,18) using related, but not directly comparable, interventions with fruit juice (elderberry and cranberry juices, respectively), that also found no effect after feeding healthy volunteers fruit juices containing relatively low levels of anthocyanins (40 and 2.2 mg/d anthocyanins, respectively) over a shorter duration (both 2-wk interventions).

Our findings are, however, inconsistent with 2 recent studies conducted on healthy male and female volunteers. The first study observed a reduction in CVD biomarkers (CRP, RANTES, and nitric oxide) following a 28-d consumption of sweet cherries that contained ~100 mg/d anthocyanins (providing ~25% of the 400-mg total polyphenol content of the cherries) (23) and the second showed a reduction in inflammatory markers (IL-8 and RANTES) following a 3-wk intake of 300 mg/d of purified anthocyanins derived from bilberries and blackcurrants (19). Differences in study design may explain the differential effects we observed on biomarkers of CVD risk. A comparison of baseline inflammatory status shows that our participants had a lower mean CRP level (3.5 ± 0.7 mg/L) than average-risk individuals (5.4 ± 0.7 mg/L) (24). Although a number of cohort and intervention studies have shown a potentially beneficial effect of anthocyanins on CHD and CVD (1,6), few trials have established the effect of prolonged exposure to physiologically relevant doses on liver and kidney function.

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**TABLE 2** Plasma biomarkers of liver and kidney function as well as CVD risk in healthy postmenopausal women before and after 12-wk supplementation with anthocyanin (500 mg/d) or placebo.

<table>
<thead>
<tr>
<th>Biomarkers of CVD risk</th>
<th>Placebo</th>
<th>Anthocyanin</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>26/26</td>
<td>26/26</td>
</tr>
<tr>
<td>Albumin, mg/L</td>
<td>9.0 ± 0.9</td>
<td>9.1 ± 0.9</td>
</tr>
<tr>
<td>ALT, µ/L</td>
<td>17.6 ± 7.5</td>
<td>18.8 ± 5.8</td>
</tr>
<tr>
<td>GGT, µ/L</td>
<td>19.0 ± 11.5</td>
<td>18.3 ± 9.5</td>
</tr>
<tr>
<td>ALP, µ/L</td>
<td>37.0 ± 16.5</td>
<td>44.7 ± 23.1</td>
</tr>
<tr>
<td>Creatinine, mmol/L</td>
<td>0.9 ± 0.1</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>Bilirubin, mmol/L</td>
<td>11.6 ± 3.1</td>
<td>14.0 ± 5.4</td>
</tr>
<tr>
<td>Urea, mmol/L</td>
<td>4.9 ± 1.2</td>
<td>5.4 ± 1.4</td>
</tr>
</tbody>
</table>

**Notes:**
- Values are means ± SD, n = 26/group. No significant treatment effects were observed by univariate general linear model except for bilirubin. *Between-treatment effect at wk 12, P = 0.04. No significant differences between treatment groups at baseline by 1-way ANOVA.
- Expressed as % positive platelets.
- Values are means ± SD, n = 26/group. Differences between treatment groups for effect of the anthocyanin metabolites over 3-h period, P = 0.02.
-200 mg/d hydroxyxycinnamates were present in the 280 g/d cherries) that may have contributed to the antiinflammatory response observed (23).

Our lack of effect on inflammatory biomarkers also contradicts the observed epidemiological findings (1) where a significant inverse association between anthocyanin consumption and risk of CHD, CVD, and total mortality was observed; after multivariate adjustment, the relative risk of CHD and CVD for the highest compared with the lowest quintile of anthocyanin-rich foods was 0.88 (95% CI, 0.78–0.99) and 0.90 (95% CI, 0.86–0.95), respectively (1). Our study examined high levels of anthocyanin intake over a short period of time (12 wk), whereas the inverse associations observed in the prospective epidemiological study (1) focus on risk reduction following long-term exposure to anthocyanin-rich foods. It is likely that this difference in the relative periods of exposure to anthocyanin intake explain much of the variation in CVD risk between our randomized controlled trial and the available epidemiological data.

Chronic exposure to high but dietarily achievable levels of anthocyanins did not lead to clinically significant changes in plasma markers of liver and kidney function. Whereas there was an increase in bilirubin levels in the anthocyanin group (P = 0.04) following the 12-wk intervention, mean levels remained clinically normal (i.e., well within the clinically acceptable range for this age group). These results are consistent with 2 previous studies (25,26) that showed no clinically deleterious effects of anthocyanins at lower doses (200 and 250 mg/d, respectively) over shorter periods of time (8 and 4 wk, respectively). No anthocyanins were detected in the fasting plasma samples of those randomized to anthocyanin treatment for 12 wk, suggesting efficient clearance without accumulation. In addition, individuals chronically consuming anthocyanins for 12 wk had increased plasma levels of anthocyanin metabolites postprandially relative to the control group (acute consumption), thus suggesting improved metabolic efficiency and further implicating safety.

In conclusion, these data confirm that supplementation with a relatively high but dietarily achievable dose of anthocyanins for 12 wk is safe but does not provide added cardioprotective benefits in this group of postmenopausal women. Future investigations should therefore focus on establishing the differential cardiovascular benefits of anthocyanins in population groups with and without clinically confirmed disease (high vs. low risk) to establish their usefulness in either the prevention or treatment of CVD.

Acknowledgments
A.C., G.J., and P.A.K. designed the intervention study; P.J.C., W.J.H., R.W., and C.D.K. conducted research and laboratory measurements; P.J.C. analyzed data; P.J.C., P.A.K., C.D.K., and A.C. wrote the paper. All authors read and approved the final manuscript. A.C. had primary responsibility for final content.

Literature Cited

