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# Blood Orange Juice Consumption Increases Flow-Mediated Dilation in Adults with Overweight and Obesity: A Randomized Controlled Trial

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## ABSTRACT

**Background:** Epidemiological studies have indicated an inverse association between citrus fruit consumption and cardiovascular disease (CVD) risk. There is, however, a paucity of data concerning effects of blood orange juice (BOJ) intake on endothelial function and cardiovascular risk biomarkers.

**Objectives:** We examined short-term effects of BOJ on endothelial function, blood pressure, lipid profile, and inflammatory markers in healthy participants of European origin who were overweight or obese.

**Methods:** In a randomized, controlled, single-blind, crossover trial, 15 men and women (age: 28.7 ± 6.5 y; BMI: 28.3 ± 3.1 kg/m$^2$) consumed BOJ or a sugar-matched control drink (CD) (200 mL twice daily) for 2 wk with a washout period of 1 wk. Endothelial function, measured as flow-mediated dilation (FMD) (primary outcome), and the secondary outcomes blood pressure, anthropometric measures, lipid profile, inflammatory markers, markers of vasodilation and vasoconstriction, and urinary flavanone metabolites were evaluated prior to and at the end of each treatment period following an overnight fast. Changes between treatments over time were assessed using repeated-measures ANOVA.

**Results:** The results demonstrate a significant increase in FMD following BOJ consumption (pre: 8.15% ± 2.92%; post: 10.2% ± 3.31%; $P = 0.002$) compared with CD (pre: 8.11% ± 2.52%; post: 7.77% ± 2.43%; time × treatment interaction: $P = 0.001$). Concurrent significant increases in urinary hesperetin-3′-glucuronide and hesperetin-7-glucuronide were observed following BOJ supplementation only (time × treatment interaction: $P \leq 0.01$). Baseline blood pressure, lipid profile, high-sensitivity C-reactive protein, and endothelin-1 were generally within healthy ranges and unaffected by the intervention.

**Conclusions:** A 2-wk consumption of BOJ exerted favorable effects on endothelial function in healthy women and men who were overweight or obese, which is likely mediated by the combined actions of anthocyanin and flavanone metabolites on mechanisms that contribute to enhancing NO bioavailability. This trial was registered at clinicaltrials.gov as NCT03611114. *J Nutr* 2020;00:1–8.

**Keywords:** flavanones, blood orange juice, overweight/obese participants, endothelial function, flow-mediated dilation, shear rate, urinary flavanone metabolites

## Introduction

Epidemiological studies have suggested that a higher intake of citrus fruit is associated with reduced risk of ischemic stroke (1), lower levels of inflammation, and endothelial dysfunction (2). Bioactives in citrus fruits such as hesperidin and naringin have received considerable attention due to in vitro and in vivo evidence demonstrating anti-atherogenic effects (3, 4). Given the role of inflammation and endothelial dysfunction in the development of atherosclerosis and that endothelial function is a strong prognostic indicator for cardiovascular events (5, 6), the consumption of citrus fruit may have a significant impact.

However, evidence from randomized controlled trials (RCTs) investigating effects of orange juice on endothelial function and inflammatory markers is conflicting (7–9). A number of factors have been highlighted that may contribute to observed heterogeneity, including differences in trial design, food composition, flavonoid source, as well as volunteer-related differences (e.g., age, sex, and ethnicity) (10, 11).


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Indeed, the majority of previous RCTs have involved men or postmenopausal women. Issues associated with the inclusion of premenopausal women mainly relate to fluctuations in either exogenous or endogenous reproductive hormones. The increase in estrogen during the late follicular phase of the menstrual cycle has been seen to markedly increase endothelial function, as measured by flow-mediated dilation (FMD) (12, 13). Regulated by an upregulation in NO, FMD is a direct result of blood flow–enhanced shear stress along the endothelium. Thus, shear stress as the stimulus for FMD is crucial to the interpretation of FMD data (14) but has rarely been calculated and discussed in the context of polyphenol intervention studies.

Genetic variants have been highlighted as important contributors to interindividual differences (15). Lactase phlorizin hydrolase, for example, plays a pivotal role in the exclusive hydrolysis of some polyphenol glucosides prior to absorption (16), with low concentrations occurring in 5% of European and 90% of African and Asian adults. Variations in the enzyme aldehyde dehydrogenase, which contributes to nitrate conversion into NO (17), are highly prevalent among Asian populations (18) but relatively uncommon in Caucasians (19). However, details on participant ethnicity are frequently not provided in human intervention studies.

In comparison to blond orange juice, blood orange juice (BOJ) has received much less attention, yet it is an abundant source for bioactives such as anthocyanins, flavanones, hydroxycinnamic acids (20), and vitamin C (21). With the exception of the study by Buscemi et al. (22), which showed an increase in FMD in men with moderately increased CVD risk after 1-wk BOJ consumption, no other studies have demonstrated beneficial changes in CVD biomarkers after chronic exposure of up to 12 wk (23, 24).

The aim of this study was to investigate the effects of short-term consumption of BOJ on endothelial function and other CVD risk factors. We chose participants who were overweight or obese and of Caucasian heritage because higher BMI (in kg/m$^2$) and chronic low-grade inflammation are associated with a higher risk of coronary heart disease and impaired endothelial function (25–27).

# Methods

## Study population

Sixteen healthy men and premenopausal women, aged 20–45 y, were recruited according to the following eligibility criteria: Caucasians (of European origin), generally healthy with absence of any form of CVD, nonsmokers, BMI >25, no medications or dietary supplements (vitamins and antioxidants), and absence of lactose intolerance. Fifteen participants completed the study; 1 participant was excluded due to the use of medication during the intervention. The study was approved by Biological Sciences Faculty Research Ethics Committee, University of Leeds (Ethics Reference No. BIOSCI 15–030), in accordance with ethical principles of the Declaration of Helsinki. Written informed consent from all participants was obtained prior to study commencement.

## Study design

The study design was a randomized, controlled, single-blind, crossover trial (**Figure 1**). During two 2-wk periods, participants were asked to consume sugar-matched 400 mL of BOJ or control drink (CD) (200 mL with breakfast and 200 mL with dinner) daily, with a 1-wk washout period between each intervention. Block randomization was conducted to allocate drink sequences to participant codes. After enrollment into the study, participants visited the vascular laboratory at the University of Leeds on 4 separate occasions for measurements prior to and following each 2-wk period (January–June 2017). Participants were instructed to stay fasted and refrain from exercise for 12 h before measurements in the morning. After the participant was supine and comfortable for 15 min to reach a cardiovascular steady state, blood pressure was measured in triplicate with 2-min intervals. Endothelial function was evaluated via brachial artery FMD, with each measurement performed at the same time of the day and on the same area of the brachial artery, as explained later. Following FMD measurements, venous blood samples from the antecubital vein were collected, and participants were asked to provide a spot urine sample. Anthropometric measures were conducted at baseline and following each intervention. Participants were asked to maintain their lifestyle as usual throughout the study, including dietary routines and physical activity level, and they were asked to record the time of individual drink consumption on a separate sheet that was returned to the researcher after the 2-wk periods. Questionnaires on the habitual intake of citrus fruit/juice and other flavonoid sources were also collected. Researchers who conducted the measurements were blinded and only unblinded post data analysis.

Female participants started the intervention on specific days of the menstrual cycle (e.g. day 4 of a 28-d menstrual cycle) in order to avoid any measurements during the late follicular phase. For women consuming the oral contraceptive pill, as either combined estrogen and progesterone or progesterone only, all assessments were undertaken during the period of time the pill was being consumed (not in pill breaks).

## Intervention products

Commercially available BOJ (47 kcal/dL) and a low-flavonoid CD (41 kcal/dL) were obtained from Waitrose. The flavanone concentration of the juices was analyzed by HPLC-MS (28). Hesperidin and narirutin were 80.2 ± 2.7 and 9.5 ± 0.1 mg/dL for BOJ and 6.3 ± 0.2 and 1.0 ± 0.1 mg/dL for CD, respectively (both $P < 0.001$). The total anthocyanin concentration, analyzed using pH differential method (29), was 2.40 ± 0.13 mg/dL for BOJ, with no anthocyanins being detectable in CD. Sugar quantification was performed by Dionex ICS-5000 (30), and the total sugar concentration of BOJ and CD was calculated at 14.3 ± 0.6 and 14.7 ± 0.7 g/dL, respectively ($P = 0.591$). Test drinks for 2 wk of consumption were provided to all participants at the start of the intervention and stored in their home refrigerators until consumption.

## Endothelial function

The local protocol for assessment of brachial artery endothelial function via FMD (31, 32) was in accordance with established guidelines (14, 33) using duplex ultrasonography (Vivid E9 with XDclear, GE Healthcare). Following 15 min of rest in a supine position in a quiet and temperature-controlled vascular laboratory, the brachial artery was imaged above the antecubital fossa in the longitudinal plane. Resting brachial artery diameter was recorded for 20 s at 15 images/s using vascular imaging software (Vascular Imager; Medical Imaging Applications). Reactive hyperemia was created by inflating a pneumatic cuff on the forearm for 5 min at 220 mmHg. Post cuff deflation brachial artery diameter and blood flow were recorded for 180 s starting 30 s before cuff deflation. Brachial artery diameter and blood flow were assessed offline using Brachial Analyzer for Research version 6 (Medical Imaging Applications). Peak diameter was calculated from the

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Data described in the manuscript, code book, and analytical code will be made available upon request pending approval.
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Abbreviations used: AUCpeak, area under the shear rate curve to peak dilation; BOJ, blood orange juice; CD, control drink; cGMP, cyclic guanosine-5′-monophosphate; CVD, cardiovascular disease; FMD, flow-mediated dilation; hsCRP, high-sensitivity C-reactive protein; LOD, limit of detection; LOQ, limit of quantification; RCT, randomized controlled trial; UHPLC-QqQ-MS, ultra-HPLC coupled with triple quadrupole MS.

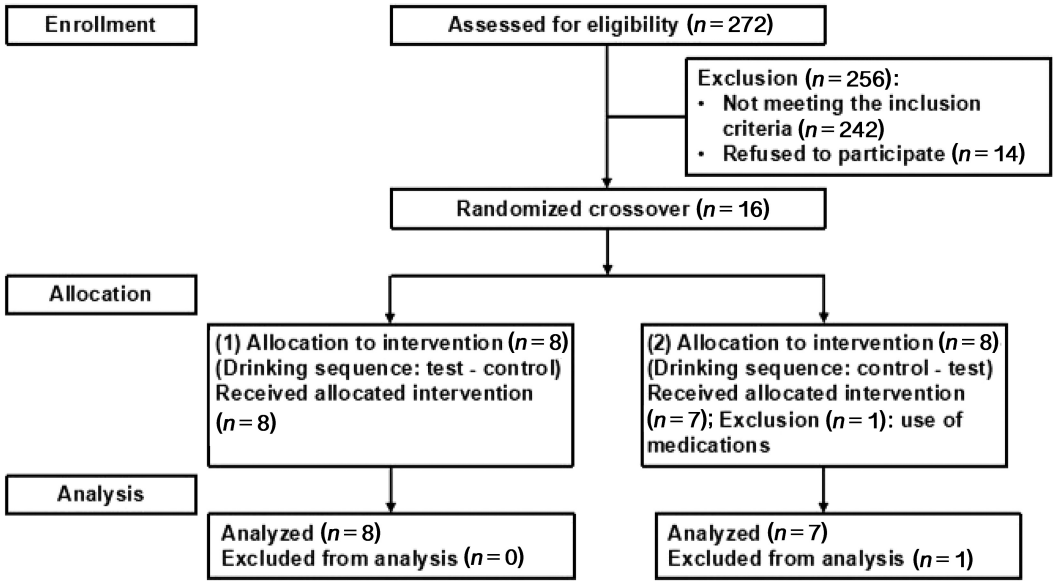


**FIGURE 1** Participant flow diagram. Simple randomization was used to determine the different groups (starting with test drink or control drink). Block randomization was conducted to randomly allocate participants into groups to ensure an equal number in each group.

maximum diameter of the moving averages of 3 consecutive diameters. Absolute FMD (mm; peak diameter – resting diameter) and relative FMD (%; absolute FMD/resting diameter × 100) were determined. Velocity time integral and the area under the shear rate curve (AUC) were calculated as previously described (34). AUC to peak diameter was calculated as an indication of the stimulus for FMD (14). Time to peak diameter was calculated as the time period starting from cuff deflation to peak diameter. Specifically, relative FMD was the primary outcome, and the other measures of FMD [resting diameter, Area under the shear rate curve to peak dilation (AUCpeak), absolute FMD, scaled FMD index, and time to peak diameter] were secondary outcomes. To determine reliability of ultrasound measurements, FMD was conducted on 10 healthy participants, with each individual examined twice on 2 consecutive days. Resting brachial artery diameter showed a CV of 0.4%, and CV of relative FMD was 6.12%.

### Plasma cardiovascular risk biomarkers

Serum and EDTA plasma were generated using standard procedures. Urine samples were centrifuged (2000 × *g*; 15 min; 4°C) and filtered (0.22-$\mu$m CA-CN syringe filter). Aliquots of serum, plasma, and urine were stored at –80°C prior to analysis. Lipid profile, high-sensitivity C-reactive protein (hsCRP), and estradiol were analyzed in serum using standardized assays by the Pathology Services (Leeds General Infirmary). Plasma endothelin-1 and cGMP were measured using commercially available immunoassays (R&D Systems).

### Urinary metabolite analysis

Major urinary metabolites were analyzed by ultra-HPLC coupled with triple quadrupole MS (UHPLC-QqQ-MS) as previously described (35). The limit of detection (LOD) was determined as the concentration of analytes with a signal-to-noise ratio of at least 3, and the limit of quantification (LOQ) was the lowest standard with a signal-to-noise ratio of at least 10. LOQs were 80 nM for hesperetin; 50 and 80 nM for hesperetin-7- and 3′-glucuronides, respectively; and 40 and 50 nM for hesperetin-7- and 3′-sulfates, respectively. LODs were 30 nM for hesperetin; 20 and 30 nM for hesperetin-7- and 3′-glucuronides, respectively; and 15 and 20 nM for hesperetin-7- and 3′-sulfates, respectively. The intraday repeatability of the UHPLC-QqQ-MS method was assessed from 10 consecutive chromatographic runs using a standard solution with 2.5 $\mu$M of every standard in MeOH:0.1% (v/v) formic acid. The interday repeatability of the method was assessed by analyzing the same standard solution on 2 consecutive days. The relative SD for peak area was in the range of 0.5–4.7% in the intraday test and 1.3–3.5% in the case of the interday test.

### Sample size and statistical analysis

To detect a 2.0-unit increase in relative FMD (the primary outcome of the current study), assuming an SD of 2.0 (based on FMD reliability data), with 80% power and at the 5% significance level, a total sample size of 10 participants was required to complete a 2-treatment crossover study. Data are presented as means ± SDs. Statistical analyses were conducted by using SPSS version 24 (IBM). Data were tested for normality by using the Shapiro–Wilk test with normality defined as $P > 0.05$. Differences in study outcomes between treatments were analyzed using a 2-factor repeated-measures ANOVA, with treatment and time (baseline and week 2 within each treatment period) as within-subject factors. The main effects of treatment and time, as well as the time × treatment interaction, were investigated. When significant time, treatment, and/or time × treatment effects were identified, post hoc comparisons were carried out by using Bonferroni correction for multiple comparisons. The effect of sex on relative FMD was investigated by using a mixed ANOVA, with treatment and time as within-subject factors and sex as the between-subjects factor, including a time × treatment × sex interaction. Differences in relative FMD adjusted for BMI were analyzed by using a repeated-measures ANOVA, with treatment and time as within-subject factors and BMI as the covariate, including a time × treatment × BMI interaction. Carryover effects on relative FMD were assessed by using a mixed ANOVA, with treatment and time as within-subject factors and treatment sequence as the between-subjects factor. Missing data were not imputed, and a complete case analysis was performed. The flavanone concentration in the different drinks was analyzed using an independent-samples *t* test. Significance was defined at $P < 0.05$. Correlation analyses were conducted by using Pearson's correlation coefficient.

## Results

Clinical characteristics of participants at screening are shown in Table 1. Among 16 enrolled participants, 15 participants (10 women and 5 men) completed all arms of the intervention. All 15 participants reported no major changes in diet and lifestyle during the intervention, which was confirmed by

**TABLE 1** Clinical characteristics of study participants at baseline[1]

| | Values |
|---|---|
| Age, y | 28.7 ± 6.5 (20–45) |
| BMI, kg/m$^2$ | 28.3 ± 3.1 (25.5–36.5) |
| SBP, mmHg | 110.0 ± 12.9 (91.0–128.7) |
| DBP, mmHg | 71.9 ± 9.5 (59.3–92.3) |
| Total cholesterol, mmol/L | 4.7 ± 0.6 (4.0–5.8) |
| HDL cholesterol, mmol/L | 1.5 ± 0.5 (0.8–2.1) |
| LDL cholesterol, mmol/L | 2.6 ± 0.4 (2.1–3.2) |
| Triglycerides, mmol/L | 1.3 ± 0.6 (0.5–2.8) |
| hsCRP, mg/L | 0.9 ± 0.9 (0.2–3.4) |

[1]Values are means ± SDs (ranges), $n = 15$ except for lipids and hsCRP, $n = 10$. DBP, diastolic blood pressure; hsCRP, high-sensitivity C-reactive protein; SBP, systolic blood pressure.

unchanged body weight (data not shown). The habitual intake of citrus fruit/juice was generally low (both <1 portion per week), and the total intake of tea and coffee was 2.4 ± 1.1 cups/d (1 cup: 200–250 mL). No carryover effects were observed ($P$-interaction = 0.20). Resting brachial artery diameter did not differ between treatments prior to the intervention ($P = 1.00$; **Table 2**); however, a time × treatment interaction ($P = 0.017$) was observed. Compared with baseline, relative FMD markedly increased only following the 2-wk consumption of BOJ (**Figure 2**A; time × treatment interaction: $P = 0.001$). AUCpeak (Figure 2B; time × treatment interaction: $P = 0.56$) and time to peak diameter did not change over time in either treatment (Table 2). To remove the influence of changes in resting artery diameter on FMD, FMD was scaled to resting artery diameter according to Atkinson (36). Analysis of the scaled FMD index also revealed an increase following the 2-wk consumption of BOJ only, compared with baseline ($P = 0.001$).

Plasma concentrations of estradiol in female participants did not differ during the trial periods (0.33 ± 0.27 and 0.28 ± 0.25 nmol/L for pre- and post-BOJ consumption, respectively; 0.27 ± 0.16 and 0.29 ± 0.21 nmol/L for pre- and post-CD consumption, respectively; time × treatment interaction: $P = 0.80$). Moreover, the effect of the drinks on relative FMD did not differ with sex (the effect of sex: $P = 0.28$; $P$-interaction = 0.70). Although the effect of BMI on relative FMD was not significant (the effect of BMI: $P = 0.61$; $P$-interaction = 0.56), there was a moderate inverse correlation between the BMI of participants and changes in relative FMD following 2-wk consumption of BOJ ($R = –0.42$, $P = 0.12$). Similarly, a moderate inverse correlation between the BMI of participants and changes in relative FMD was also observed following 2-wk consumption of CD ($R = –0.45$, $P = 0.09$).

All participants complied with dietary restrictions, confirmed by low (or not detectable) urinary concentrations of hesperetin-3′-glucuronide and hesperetin-7-glucuronide at baseline ($P = 0.42$ and $P = 0.39$, respectively; **Figure 3**). Urinary hesperetin-3′-glucuronide and hesperetin-7-glucuronide both increased following BOJ consumption from 0.17 ± 0.04 to 9.78 ± 2.52 μM ($P = 0.007$) and from 0.06 ± 0.02 to 2.71 ± 0.70 μM ($P = 0.009$), respectively, but not following the CD (hesperetin-3′-glucuronide from 0.30 ± 0.08 to 0.59 ± 0.15 μM and hesperetin-7-glucuronide from 0.11 ± 0.03 to 0.16 ± 0.04 μM). Both urinary hesperetin-3′-glucuronide ($R = 0.35$, $P = 0.007$) and hesperetin-7-glucuronide ($R = 0.32$, $P = 0.012$) were significantly correlated with relative FMD.

Blood pressure (systolic and diastolic), lipids (total cholesterol, triglycerides, and HDL cholesterol), hsCRP, and endothelin-1 were within healthy ranges (37–40) and not affected by the interventions, except for cGMP with a significant interaction and LDL cholesterol with a significant treatment effect (Table 2). No significant correlations were observed between these outcomes and relative FMD (data not shown).

**TABLE 2** Endothelial function, blood pressure, and circulating inflammatory markers, lipids, endothelin-1, and cGMP in healthy adults with overweight and obesity at baseline and following 2-wk consumption of blood orange juice or control drink in a random sequence[1]

| | Blood orange juice | | Control drink | | | | |
|---|---|---|---|---|---|---|---|
| | Basal | 2 wk | Basal | 2 wk | $P$-treatment[2] | $P$-time[3] | $P$-interaction[4] |
| Resting diameter, mm | 3.62 ± 0.56 | 3.64 ± 0.54 | 3.63 ± 0.57 | 3.62 ± 0.56 | 0.77 | 0.36 | 0.017 |
| AUCpeak, 1000 A.U. | 55.4 ± 19.1 | 57.3 ± 22.3 | 57.4 ± 27.4 | 56.6 ± 21.0 | 0.25 | 0.91 | 0.56 |
| Absolute FMD, mm | 0.29 ± 0.08$^b$ | 0.36 ± 0.09$^a$ | 0.29 ± 0.07$^b$ | 0.27 ± 0.07$^b$ | 0.001 | 0.06 | 0.001 |
| Relative FMD, % | 8.15 ± 2.92$^b$ | 10.2 ± 3.31$^a$ | 8.11 ± 2.52$^b$ | 7.77 ± 2.43$^b$ | 0.001 | 0.06 | 0.001 |
| Scaled FMD index | 1.23 ± 0.03$^b$ | 1.25 ± 0.03$^a$ | 1.23 ± 0.02$^b$ | 1.22 ± 0.02$^b$ | 0.001 | 0.06 | <0.001 |
| Time to peak diameter, s | 47.6 ± 13.6 | 47.5 ± 14.8 | 47.5 ± 17.1 | 46.5 ± 17.7 | 0.88 | 0.82 | 0.83 |
| SBP, mmHg | 108 ± 12 | 108 ± 11 | 108 ± 11 | 108 ± 11 | 0.97 | 0.70 | 0.97 |
| DBP, mmHg | 71 ± 8 | 69 ± 7 | 72 ± 9 | 70 ± 8 | 0.61 | 0.06 | 0.87 |
| hsCRP, mg/L | 0.59 ± 0.29 | 0.58 ± 0.35 | 0.87 ± 0.82 | 1.14 ± 1.47 | 0.15 | 0.64 | 0.63 |
| Total cholesterol, mmol/L | 4.63 ± 0.60 | 4.68 ± 0.56 | 4.55 ± 0.57 | 4.54 ± 0.77 | 0.24 | 0.82 | 0.81 |
| Triglycerides, mmol/L | 1.23 ± 0.77 | 1.28 ± 0.61 | 1.38 ± 0.68 | 1.28 ± 0.61 | 0.58 | 0.37 | 0.57 |
| LDL cholesterol, mmol/L | 2.62 ± 0.39 | 2.60 ± 0.23 | 2.39 ± 0.46 | 2.46 ± 0.46 | 0.005 | 0.78 | 0.74 |
| HDL cholesterol, mmol/L | 1.53 ± 0.49 | 1.57 ± 0.57 | 1.59 ± 0.57 | 1.50 ± 0.49 | 0.82 | 0.61 | 0.27 |
| Endothelin01, pg/mL | 1.08 ± 0.18 | 1.05 ± 0.27 | 1.15 ± 0.22 | 1.09 ± 0.29 | 0.51 | 0.40 | 0.83 |
| cGMP, pmol/mL | 61.2 ± 19.0 | 78.9 ± 29.1 | 72.4 ± 15.0 | 65.9 ± 16.5 | 0.84 | 0.37 | 0.043 |

[1]Values are means ± SDs, $n = 15$ except for lipids, endothelin-1, cGMP, and hsCRP, $n = 10$. Values with different superscript letters differ, $P < 0.05$. A.U., arbitrary units; AUCpeak, area under the shear rate curve to peak dilation; cGMP, cyclic guanosine-5′-monophosphate; DBP, diastolic blood pressure; FMD, flow-mediated dilation; hsCRP, high-sensitivity C-reactive protein; SBP, systolic blood pressure.
[2]Indicates the main effect of treatment, $P < 0.05$.
[3]Indicates the main effect of time, $P < 0.05$.
[4]Indicates the time × treatment interaction, $P < 0.05$.

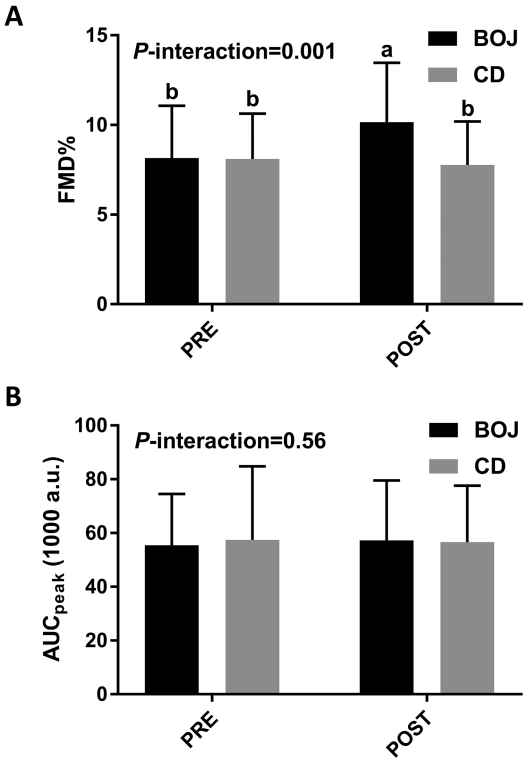


**FIGURE 2** Endothelial function in healthy adults with overweight and obesity prior to and following 2-wk consumption of blood orange juice or control drink (2 × 200 mL/d) in randomized order. (A) Relative FMD and (B) AUCpeak. Data are means ± SDs, $n = 15$. Labeled means without a common letter differ, $P < 0.05$. AUCpeak, area under the shear rate curve to peak dilation; BOJ, blood orange juice; CD, control drink; FMD, flow-mediated dilation.

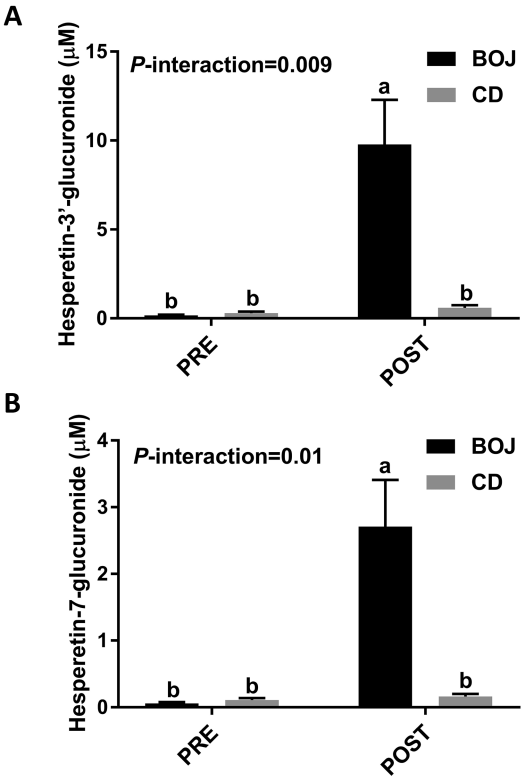


**FIGURE 3** Urinary flavanone metabolites hesperetin-3′-glucuronide (A) and hesperetin-7-glucuronide (B) in healthy adults with overweight and obesity prior to and following consumption of blood orange juice or control drink (2 × 200 mL/d) in randomized order. Data are means ± SDs, $n = 15$. Labeled means without a common letter differ, $P < 0.05$. BOJ, blood orange juice; CD, control drink.

## Discussion

This study demonstrates a significant 2.01% increase in FMD following consumption of anthocyanin-rich BOJ compared with a low-flavonoid CD, with a concurrent significant increase in urinary flavanone metabolites. Importantly, although the clinical use of FMD in the calculation of CVD risk has not been recognized, a 1% increase in FMD in large trials has been associated with a range of 8–13% reduction in CVD risk (6, 41). Furthermore, FMD is most successful to monitor effects of interventions, as seen in the current study. The increase in FMD following BOJ intake can be considered as relatively large, given that the chronic effect of flavonoids on FMD is only 0.73% (range: 0.17–1.30%), as demonstrated by a pooled analysis of flavonoid intervention trials (42). The effects of BOJ on FMD in the current study are comparable to those of cocoa powder (800 mg cocoa flavonoids/d for 1 wk) consumed by healthy individuals (43). To our knowledge, the crossover study by Buscemi et al. (22) is the only other currently published study on BOJ consumption (500 mL/d) that demonstrates a significant increase in endothelial function via FMD along with decreases in the inflammatory markers CRP, IL-6, and TNF-$\alpha$ after 1-wk supplementation in participants with augmented CVD risk. The few other studies on BOJ supplementation were not able to show effects on biomarkers of CVD risk in participants who were overweight (500 mL/d over 28 d) (23) or showed only a moderate reduction in LDL cholesterol concentrations in participants with obesity after a 12-wk supplementation with 500 mL BOJ/d (24). The current study, using a relatively high volume of 400 mL BOJ/d, did not demonstrate changes in blood pressure, lipid profiles, and markers of inflammation, likely due to all values being within a healthy range. Missing data on lipid profiles and markers of inflammation might have limited impact on the interpretation of the results because the participants were relatively young and healthy, and hence it is likely those outcomes were within a healthy range at baseline and did not change by the intervention. It cannot be excluded that spontaneous changes in the diet were made by the participants to compensate for the energy load in the drinks, as body weight remained unchanged during the study period. Also, because participants' habitual diet was low in citrus fruit/juice and not high in tea/coffee, and they were asked to maintain their diet during the study, the effects of compensatory changes in flavonoid-rich foods/drinks were considered to be negligible. This was confirmed by unchanged baseline values of urinary metabolites and endothelial function.

To our knowledge, this is the first study to report an impact of polyphenol-rich foods or drink consumption upon resting artery diameter. This indicates a small amount of remodeling of the brachial artery, which may be due to elevated concentrations of NO following the consumption of BOJ, evidenced by a 29% increase in plasma cGMP. Together with unchanged shear rate stimulus, it suggests a functional improvement in endothelial function probably through enhanced NO bioavailability due to anthocyanin and flavanone metabolites following BOJ consumption, and hence for the same shear stimulus, the reactivity of the vessel

is greater. Indeed, a positive correlation was demonstrated between plasma cGMP and FMD response following a 12-wk supplementation with anthocyanins in hypercholesterolemic individuals (44), with cGMP being considered as an indicator for plasma NO concentrations (45).

Large variations in study outcomes have been observed in previous flavonoid supplementation trials, particularly when healthy participants were recruited, making overall interpretation of supplementation effectivity difficult. Our approach was aimed at minimizing potential confounding effects of the female hormone estrogen by scheduling FMD measurements to avoid the late follicular phase. Thereby the reported improvements in endothelial function observed in the current study are likely due to the intervention and not fluctuations in hormone concentration. Hence, this study demonstrates that premenopausal women can be suitable participants for the evaluation of endothelial function under defined experimental conditions.

A further factor that may contribute to the conflicting evidence in the literature is shear rate, which if not carefully controlled might give rise to variations in the resultant FMD values and be mistaken as a "functional change" after an intervention. Present improvement in endothelial function following BOJ intake was induced under unchanged shear rate conditions, demonstrating a strong and significant correlation between shear rate and FMD. In support of these findings, a significant correlation has only been observed in younger (27 ± 6 y) but not older adults (58 ± 4 y) (46), which may indicate the loss of endothelial functionality during aging. Likewise, time from cuff deflation to peak diameter was not affected in the current study but is positively associated with increasing age (47). Given that the participants in the current study were young and healthy, the time to reach peak dilation following cuff deflation was relatively quick but consistent with previous research in participants of a similar age (47). Another novel finding of the current study is the differential responses of endothelial function to flavonoid-rich food consumption, depending on the BMI of participants, which to our knowledge have not been reported in previous studies. In support of this, BMI has been highlighted as a factor impacting the responsiveness of individuals in intervention trials. Azzini et al. (24) reported a lacking/abnormal response of total and LDL cholesterol in participants with obesity compared with lower BMI female participants when given BOJ supplementation.

We demonstrate here that the increase in FMD, following a 2-wk daily consumption of BOJ, is concurrent with urinary excretion of citrus flavanone metabolites hesperetin-3′- and hesperetin-7-glucuronides. Our results therefore provide compelling evidence that the in vivo FMD response is indeed linked to the presence of citrus flavonoids and/or their circulating metabolites. The availability of flavanones from orange juice (as a sum of small intestine and gut microbiota-derived compounds) is, despite high interindividual variation, considered high (48–50). Nevertheless, many orange juice studies have not demonstrated modulation of CVD risk biomarkers or endothelial function. Schär et al. (8) showed that citrus flavonoids from juice in comparison to a hesperidin supplement are much more available to humans. However, in their acute crossover RCT, neither orange juice nor hesperidin supplement were able to affect any of the outcome markers, such as reactive hyperemia–peripheral arterial tonometry, or CVD risk biomarkers. Although acute effects were not investigated in the current study, flavanone-rich citrus beverages have been reported to be effective at counteracting the negative impact of a double meal rich in fat on postprandial endothelial function measured by FMD at 7 h post intake (7). In comparison, the measurements in the current study were conducted ∼12 h following the final drink, indicating a prolonged effect of the bioactive compounds in BOJ.

Anthocyanins, as present in BOJ, are rapidly but poorly absorbed in the small intestine (51); as a consequence, we were not able to reliably detect anthocyanin metabolites in the urine samples of participants. However, the availability and molecular effects of anthocyanins toward CVD biomarkers have been documented in a number of studies. Indeed, Speciale et al. (52) suggested that anthocyanins prevent stress-induced endothelial dysfunction. Consumption of BOJ for 3 wk significantly increased plasma antioxidant concentrations (21), and the intake of blackcurrant juice, an abundant source of anthocyanins and other bioactives, for 6 wk resulted in a significant increase in FMD in healthy adults (53). It has been suggested that the beneficial effects of blood orange may be mediated by the synergistic effects of its different compounds (54).

There are several limitations to the current study. First, distinct differences in the color and taste of the 2 drinks made double-blinding impossible. However, the researchers who conducted the analyses of the biological samples and FMD data were blinded to which juice the participants were consuming. It is unlikely that the outcomes of this study (e.g., endothelial function, lipid profile, and hsCRP) were influenced by participants knowing which juice they were consuming. Second, although vitamin C concentration was not matched in the CD, it is unlikely the observed enhancement in FMD was due to vitamin C presence. Clinical data suggest that doses of vitamin C up to 500 mg do not alter endothelial function, both acutely and chronically (55), and vitamin C concentration in the BOJ ingested in this study was only 168 mg/d. In addition, given the short half-life of vitamin C (∼30 min), it seems unlikely that vitamin C exerted any effect on the markers determined after a 12-h overnight fast. Sex difference analysis was of course hampered by the small sample size. Although Bonferroni corrections were used, there is always a chance of making a type I error in any study testing multiple secondary outcomes.

In conclusion, we observed favorable changes in resting arterial tone and endothelial function in healthy Caucasian men and premenopausal women with overweight or obesity following the consumption of BOJ. Further studies are required to better understand the role and potential interactions of individual flavonoids and their metabolites in BOJ and their contribution to reducing CVD risk. The differential effects on FMD according to BMI warrant further confirmation in larger cohorts. In addition, future RCTs on specific participant groups (based on age, sex, ethnicity/genotype, BMI, and CVD risk) are needed to investigate the effects of polyphenol-rich products following long-term consumption.

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LL: drafted the manuscript; GKL, KMB, and CB: edited the manuscript; and all authors: read and approved the final manuscript.

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