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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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Abbreviations: ALDH2, aldehyde dehydrogenase; AUCpeak, area under the shear rate curve to peak dilation; BOJ, blood orange juice; CD, control drink; cGMP, cyclic guanosine monophosphate; CHD, coronary heart disease; CVD, cardiovascular disease; DBP, diastolic

blood pressure; ET-1, endothelin 1; FMD, flow-mediated dilation; HDL, high-density lipoprotein; hsCRP, high-sensitivity C-reactive protein; IL-6, interleukin 6; LDL, low-density lipoprotein; LPH, lactase phlorizin hydrolase; NO, nitric oxide; RCT, randomized controlled trial; RSD, relative standard deviation; SBP, systolic blood pressure; TNF-α, tumour necrosis factor-α; UHPLC-QqQ-MS, ultra-high performance liquid chromatography coupled with triple-quadrupole mass spectrometry; VTI, velocity time integral.

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Clinical Trial Registry number NCT03611114, registered at clinicaltrials.gov (with information on the full protocol); Data described in the manuscript, code book and analytical code will be made available upon request pending (application and approval, payment, other).

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.

Objective: 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 weeks with a washout period of 1 week. Endothelial function, measured as flow-mediated dilation (FMD) (primary outcome), and the following 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 to CD (pre: 8.11%± 2.52%, post: 7.77%± 2.43%) (time by treatment interaction: P=0.001). Concurrent significant increases in urinary hesperetin-3'-glucuronide and hesperetin-7-glucuronide were observed following BOJ supplementation only (time by treatment interaction: P≤0.01). Baseline blood pressure, lipid profile, hsCRP, and ET-1 were generally within healthy ranges and unaffected by the intervention.

Conclusions: A two-week consumption of BOJ exerts favourable 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.

29

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

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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 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 which may contribute to observed heterogeneity including differences in trial design, food composition, flavonoid source as well as volunteer related (such as age, sex, ethnicity) (10, 11). 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 nitric oxide (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 inter-individual differences (15). Lactase phlorizin hydrolase (LPH), for example, plays a pivotal role in the exclusive hydrolysis of some polyphenol glucosides prior to absorption (16) with low levels occurring in 5% of European and 90% of African and Asian adults. Variations in the enzyme aldehyde dehydrogenase (ALDH2), 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 Buscemi et al. (22) who showed an increase in FMD in men with moderately increased CVD risk after one-week BOJ consumption, none of the other studies were able to demonstrate beneficial changes in CVD biomarkers after chronic exposure of up to 12 weeks (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 as higher BMI and chronic low-grade inflammation are associated with a higher risk of coronary heart disease (CHD) 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, non-smokers, BMI over 25 kg/m^2, no medications or dietary supplements (vitamins, antioxidants), absence of lactose intolerance. Fifteen participants completed the study as one 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 Number: 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-week 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-week washout period between each intervention. Block randomization was conducted to allocate drink sequences to participant codes. After enrolment onto the study, participants visited the vascular laboratory at the University of Leeds on four separate occasions for measurements prior to and following each 2-week 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-minute 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 below. 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 which was returned to the researcher after the 2-week 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-day menstrual cycle) in order to avoid any measurements during the late follicular phase. For women consuming the oral contraceptive pill, either as 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 blood orange juice (BOJ) (47 kcal/dL) and a low flavonoid control drink (CD) (41 kcal/dL) were obtained from Waitrose, Leeds, UK. 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 calculated at 14.3 ± 0.6 and 14.7 ± 0.7 g/dL, respectively ($P=0.591$). Test drinks for 2 weeks of consumption were provided to each participant 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, US). 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, Iowa, USA). 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 off-line using Brachial Analyzer for Research (version 6, Medical Imaging Applications, Iowa, USA). Peak diameter was calculated from the maximum diameter of the moving averages of 3 consecutive diameters. Absolute FMD (mm; Peak diameter – resting diameter) and relative FMD (%; absolute FMD / resting diameter x 100) were determined. Velocity time integral (VTI) 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, AUCpeak, absolute FMD, scaled FMD index, 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 two consecutive days. Resting brachial artery diameter showed a coefficient of variation (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 x g at 4°C, 15 min) and filtered (0.22 µm CA-CN syringe filter). Aliquots of serum, plasma and urine were stored at -80°C prior to analysis. Lipid profile, hsCRP and estradiol were analysed in serum using standardized assays by the Pathology Services (Leeds General Infirmary). Plasma ET-1 and cGMP were measured using commercially available immunoassays (R&D Systems, Abingdon, UK).

Urinary metabolite analysis

Major urinary metabolites were analyzed by 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 intra-day repeatability of the UHPLC-QqQ-MS method was assessed from 10 consecutive chromatographic runs using a standard solution with 2.5 µM of every standard in MeOH:0.1% (v/v) formic acid. The inter-day repeatability of the method was assessed by analyzing the same standard solution on 2 consecutive days. The relative standard deviation (RSD) for peak

area was in the range of 0.5–4.7% in the intra-day test and 1.3–3.5% in the case of the inter-day test.

Sample size and statistical analysis

To detect a 2.0 unit increase in relative FMD (the primary outcome of the present study), assuming a standard deviation 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 two-treatment crossover study. Data are presented as means ± SDs. Statistical analyses were conducted by using Statistical Package for the Social Sciences (SPSS, version 24, IBM Corporation, USA). Data were tested for normality by using Shapiro-Wilk test with normality defined as $P>0.05$. Differences in study outcomes between treatments were analyzed using a two-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 by treatment interaction were investigated. When significant time, treatment, and/or time by 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 by treatment by 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 by treatment by 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 female participants and 5 male participants) completed all arms of the intervention. All 15 participants reported no major changes in diet and lifestyle during the intervention, which was confirmed by unchanged body weight (data not shown). The habitual intake of citrus fruit/juice was generally low (both less than one portion per week) and the total intake of tea and coffee were 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 by treatment interaction (*P*=0.017) was observed. Compared with baseline, relative FMD markedly increased only following the 2-week consumption of BOJ (**Figure 2A**: time by treatment interaction: *P*=0.001). Area under the shear rate curve to peak dilation (AUCpeak) (**Figure 2B**: time by 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-week 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 by 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-week 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-week 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).

Discussion

This study demonstrates a significant 2.01% increase in FMD following consumption of anthocyanin-rich blood orange juice as compared to a low flavonoid control drink, with a concurrent significant increase in urinary flavanone metabolites. Importantly, whilst 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 % (ranging from 0.17 to 1.30) as demonstrated by a pooled analysis of flavonoid intervention trials (42). The effects of BOJ on FMD in the present study are comparable to cocoa powder (800 mg cocoa flavonoids/d for 1 week) 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 can demonstrate a significant increase in endothelial function via FMD alongside decreases in the inflammatory markers CRP, IL-6 and TNF-α after 1 week 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 days) (23) or only a moderate reduction in LDL cholesterol levels in participants with obesity after a 12-week supplementation with 500mL BOJ/d (24). Our current study, using a relatively high volume of 400 mL BOJ per day, 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, since 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. Since their habitual diet was low in citrus fruit/juice and not high in tea/coffee, and participants 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 levels 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-week supplementation with anthocyanins in hypercholesterolemic individuals (44) with cGMP being considered as an indicator for plasma NO levels (45).

Large variations in study outcomes have been observed in previous flavonoid supplementation trials, in particular 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 present study are likely due to the intervention and not fluctuations in hormone levels. Hence, the present study demonstrates that premenopausal women can be suitable participants for the evaluation of endothelial function under defined experimental conditions.

A further factor which 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 in 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 present study but is positively associated with increasing age (47). Given that the present participants 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 to lower BMI female participants when given BOJ supplementation.

306 We demonstrate here that the increase in FMD, following a 2-week daily consumption of BOJ,
307 is concurrent with urinary excretion of citrus flavanone metabolites hesperetin-3'- and
308 hesperetin-7-glucuronides. Our results therefore provide compelling evidence that the *in vivo*
309 FMD response is indeed linked to the presence of citrus flavonoids and/or their circulating
310 metabolites. The availability of flavanones from orange juice (as a sum of small intestine and
311 gut microbiota derived compounds) is, despite high inter-individual variation, considered high
312 (48-50). Nevertheless, many orange juice studies are not demonstrating modulation of CVD
313 risk biomarkers or endothelial function. Schär et al. (8) has recently shown that citrus
314 flavonoids from juice in comparison to a hesperidin supplement are much more available to
315 humans. However, in their acute crossover RCT neither orange juice nor hesperidin
316 supplement were able to affect any of the outcome markers such as RH-PAT (reactive
317 hyperemia-peripheral arterial tonometry), or CVD risk biomarkers. Although acute effects were
318 not investigated in the present study, flavanone-rich citrus beverages have been reported to
319 be effective at counteracting the negative impact of a double meal rich in fat on postprandial
320 endothelial function measured by FMD at 7 h post intake (7). In comparison, the
321 measurements in the present study were conducted approximately 12 hours following the final
322 drink, indicating a prolonged effect of the bioactive compounds in BOJ.
323 Anthocyanins, as present in BOJ, are rapidly but poorly absorbed in the small intestine (51);
324 and as a consequence, we were not able to reliably detect anthocyanin metabolites in the
325 urine samples of participants. However, the availability and molecular effects of anthocyanins
326 towards CVD biomarkers have been documented in a number of studies. Indeed, Speciale et
327 al. (52) suggested that anthocyanins prevent stress-induced endothelial dysfunction.
328 Consumption of BOJ for 3 weeks significantly increased plasma antioxidant concentrations
329 (21) and the intake of blackcurrant juice, an abundant source of anthocyanins and other
330 bioactives, for 6 weeks resulted in a significant increase in FMD in healthy adults (53). It has
331 been suggested that the beneficial effects of blood orange may be mediated by the synergistic
332 effects of its different compounds (54).

There are several limitations to the present study. First, distinct differences in the colour and taste of the two 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 participant was consuming. It is unlikely that the outcomes of the present study (such as endothelial function, lipid profile, hsCRP) were influenced by participants knowing which juice they were consuming. Second, although vitamin C concentration was not matched in the control drink, 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 the present study was only 168 mg per day. In addition, given the short half-life of vitamin C (approximately 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 favourable changes in resting arterial tone and endothelial function in healthy Caucasian men and premenopausal women with overweight or obesity following the consumption of blood orange juice. 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, CVD risk) are needed to investigate effects of polyphenol-rich products following long term consumption.

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support; GKL and KMB scaled FMD data and conducted statistical analyses on scaled FMD index; JAMB, FV, FTB analyzed urinary flavanone metabolites; LL drafted the manuscript which was finally edited by GKL, CB and KMB. All authors have read and approved the final manuscript.

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Tables

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 mean ± SD (range), n=15 except for lipids and hsCRP, n=10. DBP, diastolic blood pressure; HDL, high-density lipoprotein; hsCRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein; SBP, systolic blood pressure.

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-week 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
Endothelin 1 (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 mean ± SD, n=15 except for lipids, endothelin 1, cGMP and hsCRP, n=10. Values with different superscript letters differ (P<0.05). AUCpeak, area under the shear rate curve to peak dilation; cGMP, cyclic guanosine monophosphate; DBP, diastolic blood pressure; FMD, flow-mediated dilation; HDL, high-density lipoprotein; hsCRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein; 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 by treatment interaction, P<0.05.

Legends for figures

Figure 1

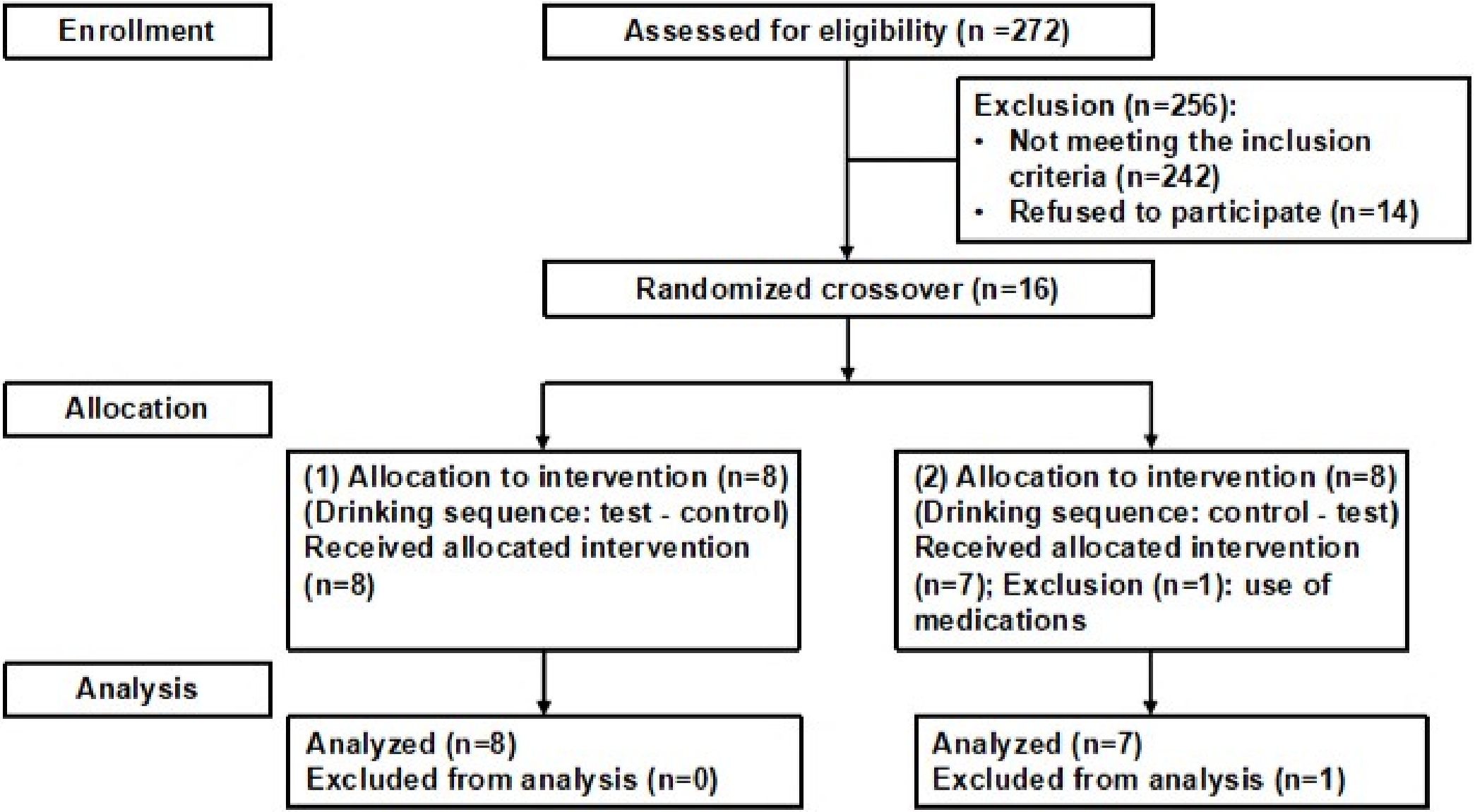
Participant flow diagram. Simple randomization was used to determine the different groups (starting with test drink or control drink). Block randomization was conducted to randomly allocate participants into groups to ensure an equal number in each group.

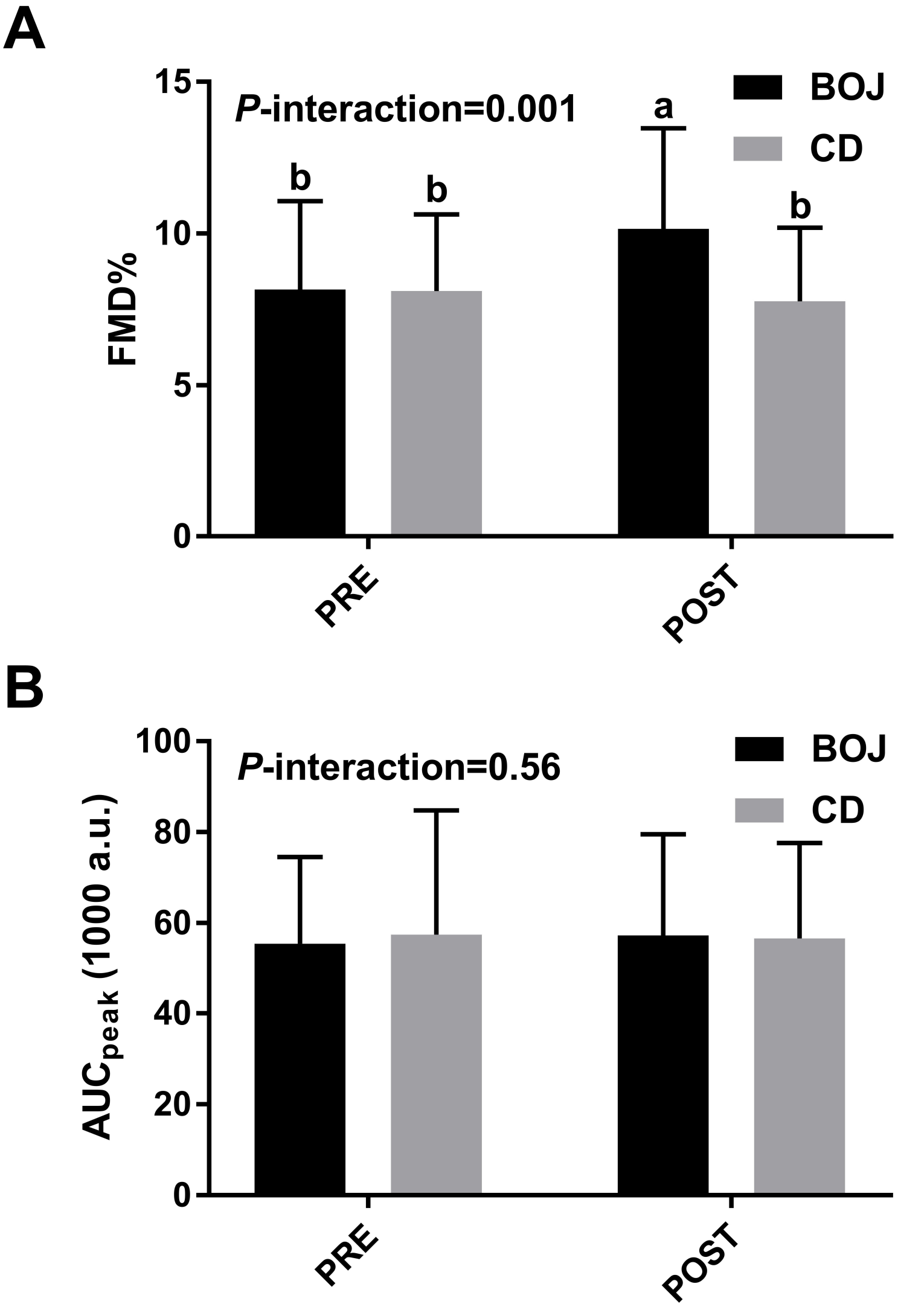
Figure 2

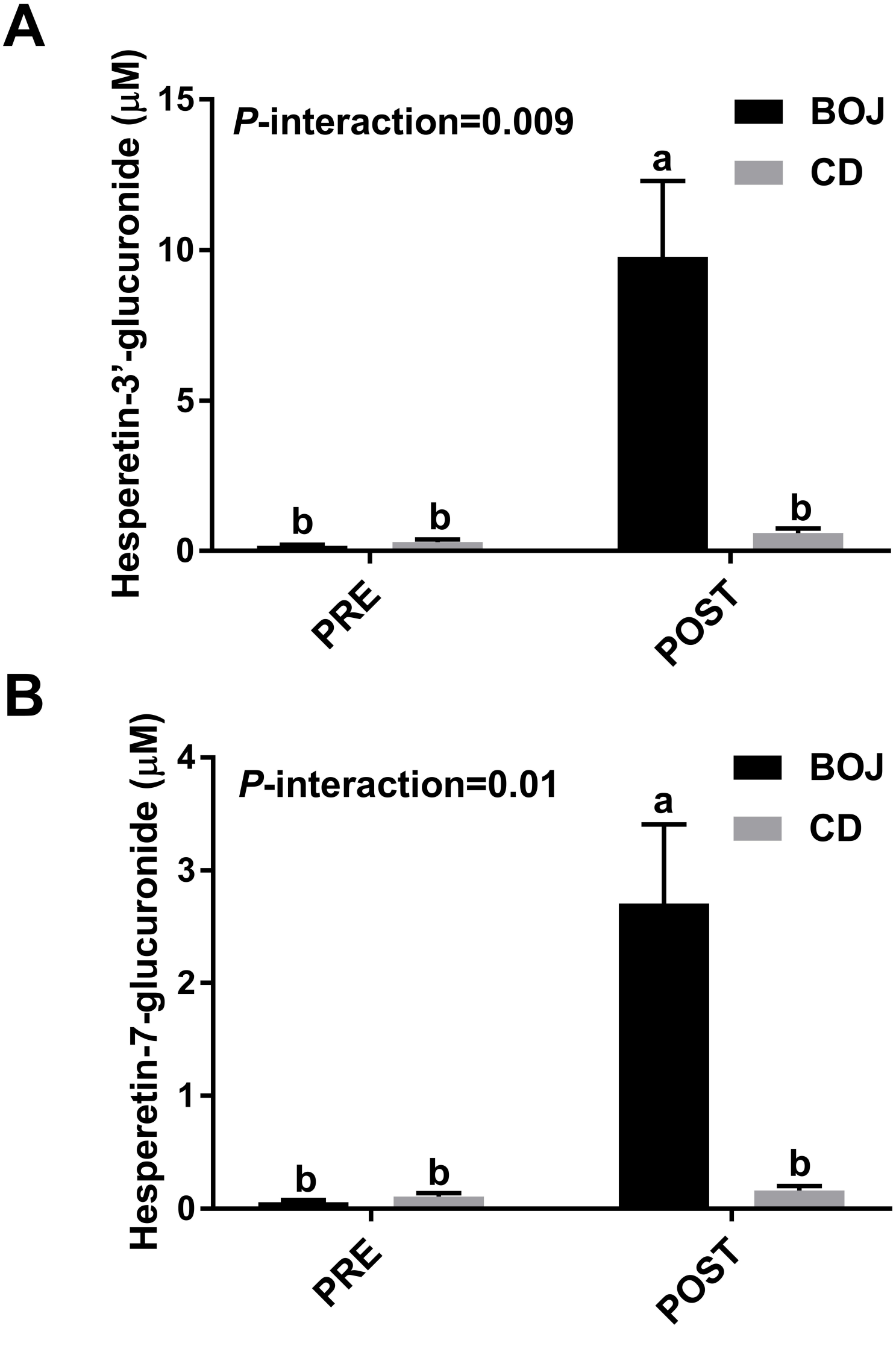
Endothelial function in healthy adults with overweight and obesity prior to and following 2-week consumption of blood orange juice or control drink (2 x 200 mL/d) in randomized order. (A) Relative FMD, (B) AUCpeak. Data are mean ± SD, n=15. Labeled means without a common letter differ, $P<0.05$. AUCpeak, 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 x 200 mL/d) in randomized order. Data are mean ± SD, n=15. Labeled means without a common letter differ, $P<0.05$. BOJ, blood orange juice; CD, control drink.


Enrollment
Assessed for eligibility (n =272)
Exclusion (n=256):
• Not meeting the inclusion criteria (n=242)
• Refused to participate (n=14)
Randomized crossover (n=16)
Allocation
(1) Allocation to intervention (n=8)
(Drinking sequence: test - control)
Received allocated intervention (n=8)
(2) Allocation to intervention (n=8)
(Drinking sequence: control - test)
Received allocated intervention (n=7); Exclusion (n=1): use of medications
Analysis
Analyzed (n=8)
Excluded from analysis (n=0)
Analyzed (n=7)
Excluded from analysis (n=1)


A
15
10
5
0
FMD%
P-interaction=0.001
BOJ
CD
b
b
a
b
PRE
POST
B
100
80
60
40
20
0
AUCpeak (1000 a.u.)
P-interaction=0.56
BOJ
CD
PRE
POST


A
Hesperetin-3'-glucuronide (μM)
15
10
5
0
P-interaction=0.009
BOJ
CD
a
b
b
b
PRE
POST
B
Hesperetin-7-glucuronide (μM)
4
3
2
1
0
P-interaction=0.01
BOJ
CD
a
b
b
b
PRE
POST