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Research Article

Flavonoid-rich apple improves endothelial function in individuals at risk for cardiovascular disease: a randomised controlled clinical trial

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Abbreviations: AIx, augmentation index; **BP**, blood pressure; **CVD**, cardiovascular disease; **eNOS**, endothelial nitric oxide synthase; **FMD**, flow-mediated dilatation; **HFA**, high-flavonoid apple; **HFD**, high fat diet; **Hmox-1**, haem oxygenase-1; **HPLC**, high performance liquid chromatography; **HR**, heart rate; **LFA**, low-flavonoid apple; **MRM**, multiple reaction monitoring; **NO**, nitric oxide; **NO_2^-**, nitrite; **NO_3^-**, nitrate; **PP**, pulse pressure; **PWV**, pulse wave velocity; **TG**, triglycerides; **UGT**, urine-5'-diphosphate glucuronosyltransferases.

Abstract

Scope: The cardioprotective effects of apples are primarily attributed to flavonoids, found predominantly in the skin. This study aimed to determine if acute and/or chronic (4 weeks) ingestion of flavonoid-rich apples improves endothelial function, blood pressure (BP) and arterial stiffness in individuals at risk for CVD.

Methods and results: In this randomised, controlled cross-over trial, acute and 4 week intake of apple with skin (high flavonoid apple, HFA) was compared to intake of apple flesh only (low flavonoid apple, LFA) in 30 participants. The primary outcome was endothelial function assessed using flow-mediated dilation (FMD) of the brachial artery, while main secondary outcomes were 24 h ambulatory BP and arterial stiffness. Other outcomes included fasting serum glucose and lipoprotein profile, plasma haem oxygenase-1 (Hmox-1), F_2-isoprostanes, flavonoid metabolites, and plasma and salivary nitrate (NO_3^-) and nitrite (NO_2^-) concentrations. Compared to LFA control, the HFA resulted in a significant increase in FMD acutely (0.8%, $p<0.001$) and after 4 weeks chronic intake (0.5%, $p<0.001$), and in plasma flavonoid metabolites ($p<0.0001$). Other outcomes were not altered significantly.

Conclusion: A lower risk of CVD with higher apple consumption could be mediated by the beneficial effect of apple skin on endothelial function, both acutely and chronically.

1 Introduction

Apples are one of the most widely consumed fruits worldwide. In observational studies a higher apple intake has been associated with lower risk of abdominal aortic calcification [1], coronary heart disease, stroke [2-4], and all-cause mortality [5]. In human and animal studies, apples have been shown to have beneficial effects on several outcomes related to cardiovascular health, including vascular function, blood pressure (BP), lipids, inflammation and hyperglycaemia [6]. The cardioprotective effects of apples are primarily attributed to their high flavonoid content. Apples and other flavonoid-rich foods are a major research focus due to epidemiological studies showing an inverse association between high flavonoid intake and CVD risk [7-9]. Most apple varieties are rich in quercetin and (-)-epicatechin and anthocyanins are responsible for the red colour of particular varieties [10]. These flavonoids are concentrated in the apple skin [11].

Accumulating data indicate that certain dietary flavonoids, such as quercetin and epicatechin, positively impact on cardiovascular health via effects on nitric oxide (NO) bioavailability, endothelial function and blood pressure [2, 12]. Hypertension is the leading risk factor for CVD [13]; endothelial dysfunction is an early event in the development of hypertension and CVD [14]; and a reduced NO bioavailability is closely connected to endothelial dysfunction and hypertension [15]. We have previously shown an improvement in endothelial function, a decrease in BP and an increase in plasma NO after acute consumption of Cripps Pink apple with skin compared to apple flesh only, in healthy men and women [12]. Additionally, we have demonstrated in animal models a role of haem oxygenase-1 (Hmox-1) in this pathway [16]. The acute effects of flavonoid-rich apple consumption in a population at risk for CVD, and the effects of regular apple consumption on endothelial function and BP have yet to be determined.

Therefore, the aim of this study was to determine if consumption of flavonoid-rich apples could improve endothelial function, lower BP and reduce arterial stiffness, acutely (2 h) and over 4 weeks, in individuals with a mildly elevated risk for CVD. To address this aim we have conducted a randomised controlled 4-week cross-over intervention study in individuals with at least one risk factor

for CVD. We hypothesised that flavonoid-rich apples with skin would improve endothelial function, lower BP and reduce arterial stiffness compared with low-flavonoid apple flesh only. We also wished to determine if the high flavonoid apples with skin would decrease LDL cholesterol and markers of oxidative stress, increase NO production and increase expression of Hmox-1.

2 Materials and Methods

2.1 Participants

Thirty volunteers were recruited from the general population of Perth, Western Australia. Volunteers were included in the study if they had at least one of the following: elevated BP (120 mmHg< systolic BP <160 mmHg), moderately elevated blood sugar concentrations (5.6 mmol/L< glucose <6.5 mmol/L), raised fasting cholesterol (5 mmol/L< total cholesterol <8 mmol/L) or central obesity (measured by waist circumference; men >94 cm; women >80 cm). Exclusion criteria are described in the **Supplementary Material**. Participants were asked to reduce their intake of flavonoid-rich foods such as tea, red wine, dark chocolate and onions, for two weeks before and throughout the duration of the study (for more detail see Supplementary Material). The study was carried out in accordance with the Declaration of Helsinki and was approved by the University of Western Australia Human Research Ethics Committee (RA/4/1/5880). Participants provided written informed consent before inclusion in the study. The trial was registered with the Australian New Zealand Clinical Trials Registry (ACTRN12615000361505).

2.2 Study design and interventions

The randomised, controlled crossover trial was performed between April and December 2015. The acute and longer-term (4 weeks) effects of two apple treatments, varying in flavonoid content and administered in random order, were compared. The high-flavonoid apple (HFA) treatment was one whole Cripps Pink apple plus the skin only of a second Cripps Pink apple blended with water. The flesh only of one Cripps Pink apple blended with water was used as a low-flavonoid apple (LFA)

control treatment. Both treatments were consumed twice per day, once in the morning and once in the evening. Each subject completed four visits, as shown in **Figure 1** (see Supplementary material for further detail on study design).

2.5 Extraction and analysis of phenolic compounds

The concentration of total quercetin glycosides, (-)-epicatechin, phloridzin, chlorogenic acid and anthocyanins in Cripps Pink apple samples were determined using high performance liquid chromatography (HPLC) (see Supplementary Material).

2.6 Analysis of plasma quercetin metabolites

As quercetin is the most abundant flavonoid found in the skin of the Cripps Pink apple, plasma concentrations of quercetin aglycone and its metabolite, isorhamnetin, were measured before and after acute and chronic consumption of the apple treatment as an indicator of absorbance and compliance, respectively. Plasma quercetin metabolites were measured by LCMSMS as described previously [17] (see Supplementary material).

2.7 Assessment of endothelial function

In order to assess endothelial function, flow-mediated dilatation (FMD) of the brachial artery was calculated in accordance with a published protocol [18] by a trained ultrasonographer blinded to the interventions used. Responses were calculated as the percentage change in brachial artery diameter from baseline. The FMD was measured at 10 sec intervals for 4 min after cuff deflation. The primary outcome measured was the mean percent FMD over the 4 min, post cuff deflation [12, 17]. In addition we assessed the maximum artery diameter post cuff deflation and calculated the peak percentage FMD.

2.8 24 h ambulatory blood pressure

Twenty-four hour ambulatory BP was measured with a Spacelabs monitor (90207, NSW, Australia) fitted by a trained researcher, as described previously [19]. Readings were aggregated for each hour, and mean BP was determined for the 24 h period.

2.9 Office blood pressure and arterial stiffness

Office BP was assessed using the SphygmoCor Xcel (AtCor Medical, Sydney, Australia) (see Supplementary Material).

2.10 Plasma and salivary nitrate and nitrite analysis

Saliva samples were obtained before, 2 h after the acute intervention, and after 4 weeks of intake of each apple intervention. Plasma samples were obtained by venepuncture before, 3 h after the acute intervention, and after 4 weeks of intake of each apple intervention, by venipuncture (see Supplementary Material).

2.11 Detection of plasma haem, biliverdin and bilirubin

Fasting plasma concentrations of haem, biliverdin and bilirubin were determined using LCMSMS following extraction of the analytes by methanol (see Supplementary material).

2.12 Other biochemical analyses

Systemic oxidative stress was determined by measuring plasma F_2-isoprostane concentrations as previously described [20] (see Supplementary Material). Standard biochemical analyses of fasting serum total cholesterol, HDL-cholesterol (HDL-C), triglycerides (TG), and LDL-cholesterol (LDL-C) were performed in the PathWest laboratory at Fiona Stanley Hospital, Western Australia (see Supplementary Material).

2.13 Statistics

Sample size was calculated with FMD as the primary endpoint. Thirty participants provided >90% power (at α=0.05) to detect a 1.0% difference in mean percent FMD (0 to 240 sec) between the LFA

control and the HFA treatment. This assumed a within-group SD of 3% based on our previous studies [12], a correlation between measures of 0.6, a correlation between mean FMD measurements at each time point of 0.6, and a minimum of 20 FMD measurements post ischaemia. Thirty participants also provided >90% power (at α=0.05) to detect a 3 mmHg difference in mean 24 h ambulatory systolic blood pressure and a 2.0% difference in peak percent FMD. Statistical analyses were performed using SPSS 21.0 (SPSS, Chicago, IL, USA) and SAS® 9.2 (SAS institute, Cary, NC, USA). Participant characteristics are presented as means ± SD. All other results are presented as mean ± SEM or mean and 95%CI. Differences in primary and secondary outcomes post treatment were analysed by a mixed model ANOVA (PROC MIXED command) with additional adjustment for outcomes pre-treatment. The subject was included as a random factor in each model. Fixed effects included the treatment (LFA or HFA), order, and hour (for ambulatory BP) or minute post ischaemia (for FMD). A 2-sided type 1 error rate of $p<0.05$ was the level of significance used for all hypothesis testing.

3 Results

3.1 Baseline data

Recruitment began on 26 February 2015 and final data collection ended on 25 November 2015. Thirty participants (10 males, 20 females) completed the study (**Figure 2**). The baseline characteristics of the study participants are shown in **Table 1**. There was no significant change in weight after either the HFA treatment or the LFA control ($p>0.05$). Both apple treatments were well-tolerated by the participants and no adverse events were reported. Based on intervention diary recordings, compliance was 98.9%.

3.2 Phenolic concentrations of apple treatments

We measured the concentration of several key apple phenolics (quercetin, epicatechin, phloridzin, chlorogenic acid and anthocyanins) in apple flesh only and apple skin only and calculated the concentration in the two apple interventions. We observed higher concentrations of total quercetin

glucosides and epicatechin in the HFA intervention when compared to the LFA control. Phloridzin concentration was similar between interventions. Anthocyanins were only found in the skin of the apple. We also measured chlorogenic acid, which was found only in the apple flesh. By measuring the weight of the flesh only and skin only of 5 Cripps Pink apples and using the average concentration of phenolics determined from 2 Cripps Pink apples, we estimated daily consumption as shown in **Table 2**. We calculated that participants on the LFA intervention were receiving approximately 92 mg/day and participants on the HFA intervention were receiving approximately 306 mg/day of total phenolic compounds that were measured.

3.3 Plasma quercetin metabolites

Plasma concentrations of quercetin metabolites were determined before (baseline), 3 h post-ingestion and after 4 weeks of daily ingestion of each intervention. As participants were asked to fast for at least 10 h prior to their visits, and taking into account 2 h of clinic visit procedures the blood sample after the chronic intervention period would have been taken at least 12 h after their last ingestion of apple intervention. There was very little quercetin (23.9 ± 1.9 nM) and isorhamnetin (10.7 ± 0.5 nM) found in the plasma at baseline. There was a significant increase in plasma concentrations of quercetin and isorhamnetin after both acute and 4-week ingestion of the HFA intervention compared to the LFA control (**Figure 3**).

3.4 Endothelial function

The mean percent FMD over 4 min post cuff deflation at 1 h (n=15) and 2 h (n=30) post-acute apple consumption, are presented in **Figure 4**. There was a significant improvement in FMD (adjusted mean) after the HFA intervention in comparison to the LFA control at both 1 h [1.0%, 95% CI: 0.8, 1.3 ($p<0.001$)] and 2 h [0.8%, 95% CI: 0.6, 0.9 ($p<0.001$)] post intervention. Peak FMD is the highest FMD (%) measured during the 4 min post occlusion and occurs at a different time point for each individual, hence is higher than the ‘peak’ on the FMD time course graph which is the average FMD at that time point. When looking at differences in peak FMD after acute apple intervention (Figure 4),

there was a significant difference between treatments at the 2 h time point [1.2%, 95% CI: 0.2, 2.2 (p=0.02)] but not at the 1 h time point [1.0%, 95% CI: 0.4, 2.4 (p=0.17)].

The adjusted mean percent FMD over 4 min post cuff deflation after the 4-week intervention (n=30) are presented in **Figure 5**. There was a significant improvement in FMD after the HFA intervention in comparison to the LFA control [0.5%, 95% CI: 0.4, 0.7 (p<0.001)] after 4 weeks. When looking at differences in peak FMD after chronic apple intervention (Figure 5), there was no significant difference between treatments [0.2%, 95% CI: 0.9, 1.2 (p=0.72)].

3.5 Blood pressure and arterial stiffness

Mean clinic brachial systolic BP, brachial diastolic BP, central diastolic pressure, heart rate (HR), AIx75 and PWV before and after acute and 4 weeks apple consumption are presented in **Table 3**. There was no significant difference between apple treatments in any of these measurements after acute or chronic (4 weeks) ingestion. In addition, we found no significant difference between apple treatments for 24 h ambulatory systolic BP, diastolic BP, pulse pressure (PP) or HR (**Table 4**).

3.6 Plasma and salivary nitrate and nitrite

We measured plasma and salivary NO_3^- and NO_2^-, end products of NO metabolism, as markers of NO status. There was no significant effect of treatment on NO_3^- or NO_2^- in either the saliva or plasma (Table 5).

3.7 Plasma and urine biochemistry

Plasma concentrations of haem, biliverdin and bilirubin were measured as surrogate markers of Hmox-1. Consumption of the HFA for 4 weeks resulted in significantly lower plasma biliverdin concentrations, relative to the LFA control (**Table 6**). There was no significant effect of 4 weeks of apple consumption on plasma haem or bilirubin concentrations.

Plasma glucose, cholesterol, TG, LDL-C and HDL-C and urinary creatinine, potassium and sodium measured at baseline and after 4 weeks are presented in **Table 7**. We found no significant difference

between apple treatments on any of these markers. Between-group differences in these markers remained non-significant when looking at subgroups with elevated baseline glucose (>5 mmol/L, n=23), cholesterol (>5.5 mmol/L, n=23), TG (>1 mmol/L, n=21), LDL-C (>3.3 mmol/L, n=23) and low baseline HDL-C (<1 mmol/L, n=3) separately (data not shown).

The measurement of F_2-isoprostane in the plasma by GCMS was used as an indication of systemic oxidative stress. We found no significant difference between apple treatments in plasma F_2-isoprostane concentrations after 4-week consumption of the HFA intervention in comparison to the LFA control (Table 7).

4 Discussion

Few human trials have assessed the effects of apple consumption on outcomes related to cardiovascular health [12, 21-25]. We found significant improvements in endothelial function after both acute and 4-week ingestion of high flavonoid apple in individuals with at least one risk factor for CVD. This improvement was accompanied by a significant increase in plasma quercetin metabolites, but no statistically significant change in any other measured parameter related to endothelial function.

A healthy endothelium is central to cardiovascular health. Endothelial dysfunction is a precursor to CVD and CVD risk factors including pre-hypertension, hypertension, atherosclerosis and stroke [26, 27]. To date, only one other study has assessed the effects of longer-term apple consumption on FMD [24]. In that study 30 hypercholesterolaemic participants were asked to consume 2 bags of either high polyphenol (Marie Ménard) or low polyphenol (Golden Delicious) lyophilised apples per day for 4 weeks, equating to approximately 2 fresh apples per day. They found no significant difference in FMD between low polyphenol and high polyphenol lyophilised apples. The authors suggested that polyphenol bioavailability may have been influenced by the freeze-drying of the apples. The improvements in FMD found in our study are comparable to those seen in a study by Nakayama *et al*., where a 1.6% significant improvement in post-prandial FMD was seen after longer-term (30 days) intake of quercetin-rich onion extract [28].

The improvement in endothelial function with HFA in the present study was observed with a concomitant increase in plasma quercetin metabolites. Most apple varieties are rich in quercetin glycosides, epicatechin and anthocyanins (found in red apples), which are concentrated in the skin [11]. In the present study we measured the phenolic content of Cripps Pink apples and estimated that when on the HFA intervention, the participants were consuming approximately 16 times more quercetin glycosides compared to the LFA control. The significant increase in circulating quercetin metabolites 3 h after ingestion of apples with skin is consistent with the idea that the mediating compounds are likely to be those which are absorbed in the upper intestinal tract, such as the quercetin glycosides, anthocyanins, and epicatechin [29]. Interestingly, in a previous study we saw no acute improvement in endothelial function after administering any dose of pure quercetin-3-O-glucoside (ranging from 50 – 400 mg) in 15 healthy individuals [17]. Although quercetin 3-O-glucoside is one of the main forms in which quercetin is found in apples [30], the absorption of quercetin depends on the food matrix in which it is found [6]. It appears as though the improvements in endothelial function observed after the HFA intervention cannot be replicated with a pure quercetin glycoside alone. Additionally, we estimate that participants were consuming approximately 2 times more epicatechin with the HFA treatment. Similar improvements in endothelial function have been shown after acute (1-4 h) administration of both epicatechin-rich cocoa and pure epicatechin [31].

The benefits of flavonoids, such as quercetin and epicatechin, on endothelial function are thought to be due, in part, to effects on NO, a molecule that regulates vascular tone [2, 12]. These flavonoids can enhance NO bioavailability, possibly by stimulating endothelial NO synthase (eNOS) activity, protecting NO from consumption by superoxide, and/or inhibiting the synthesis of endothelin-1. We have previously shown an increase in plasma NO status acutely, after a HFA intervention compared to a LFA control, assessed by measuring *S*-nitrosothiols plus other nitrosylated species by chemiluminescence [12]. As this method requires fresh plasma samples, it was not feasible to measure plasma NO status by chemiluminescence in the present study. As an alternative we measured plasma and saliva NO_3^- and NO^-_2, end-products of NO metabolism [32], by GCMS which may not have been sensitive enough to detect small changes in NO_3^- and NO_2^- concentrations.

We have previously shown that mice on a high fat diet (HFD) supplemented with quercetin had preserved endothelial function, measured *ex vivo* by relaxation of abdominal aortic rings in response to acetylcholine, increased eNOS activity and increased aortic Hmox-1 protein expression, compared to mice on a HFD alone [16]. Additionally, quercetin was unable to attenuate endothelial dysfunction in arteries of Hmox-1 gene knockout mice on a HFD, indicating the critical role of Hmox-1 in this pathway. Hmox-1 degrades haem to biliverdin which is then converted to bilirubin by biliverdin reductase [33]. Bilirubin is subsequently glucuronidated to bilirubin diglucuronide by urine-5'-diphosphate glucuronosyltransferases (UGTs). In the present study we saw a decrease in plasma haem after the HFA intervention, consistent with an increase in Hmox-1, however this did not reach significance. Additionally, we saw a significant decrease in plasma biliverdin with no change in plasma bilirubin after the HFA intervention. It is important to note that plasma bilirubin concentrations are primarily determined by UGT1A1 rather than Hmox-1 activity [34]. Polyphenols in apples have been shown to induce UGT1A1 [35], which plays an important role in flavonoid glucuronidation [36]. This activity could possibly impact on plasma concentrations of haem and its metabolites. Therefore and unfortunately, no definitive conclusions about Hmox-1 activity can be drawn from our results.

The health benefits of polyphenol-rich foods, such as apples, have been primarily attributed to their antioxidant activity [22, 37]. While apple polyphenols have been shown to be excellent antioxidants *in vitro* [38], ingestion of large amounts of apples by humans does not appear to result in equivalent antioxidant effects [22]. In the present study we saw no significant changes in plasma F_2-isoprostanes, an *in vivo* biomarker of oxidative stress [39].

In a previous study we have shown that systolic BP was significantly lower after the HFA active intervention relative to LFA control [−3.3 mmHg, 95% CI−4.9,−1.8 ($P<0.001$)] [12]. Although the concentrations of quercetin in the active apple treatment were comparable to the levels in our HFA treatment, we saw no changes in office or 24 h BP, either acutely or chronically. Our findings are in agreement with a study by Ravn-Haren *et al.,* where Shampion apples and processed Shampion apple products (4 week intervention) had no effect on BP [21].

There is evidence from human, animal and *in vitro* studies that apples can ameliorate elevated plasma cholesterol, a risk factor for CVD [40, 41]. It is hypothesised that there is a synergistic relationship between apple pectin and apple polyphenols, as they are more effective together than individually in reducing cholesterol [24, 42]. We saw a decrease in LDL-C only after the HFA intervention, although this did not reach statistical significance. In a dietary crossover study evaluating the effects of clear and cloudy apple juices, whole apples and apple pomace on plasma lipids, there was a small decrease in total cholesterol and LDL-C in the whole apple intervention group, that did not reach statistical significance when compared to the control [21]. While the present study did not show any significant decrease in fasting plasma glucose, a recently published study by Cicero *et al.*, demonstrated a decrease after 8 weeks supplementation with an apple polyphenol extract [43].

Although we have focused on the flavonoids found in apple skin, it is possible that other bioactive phytochemicals and nutrients found in apple skin may contribute to observed effects. Apple skin contains damascone-related norisoprenoid aroma compounds, which have been shown to induce Nrf2 and hence may possibly mediate the induction of Hmox-1 [44], soluble fibers such as oligogalacturonic acid [45] and minerals such as potassium and magnesium. A limitation of the present study is that it was not feasible for participants to be blinded to the treatment they were receiving. In order to minimise the bias, volunteers were not told the hypothesis of the clinical trial and all research assistants, lab technicians and statisticians were blinded to the interventions when analysing the results. In addition, flavonoid intake from the background diet was not assessed and volunteers may have reduced their intake of other foods to compensate for the apple treatments which were rich in soluble fibre and pectin and known to supress food intake [25, 46]. As the difference between the two apple interventions was equivalent to only 4 apple skins, we assume that any change in diet, such as a reduction in flavonoid consumption, would have been comparable during both treatment periods, particularly since this was a cross-over study with the same participants being assessed under each condition. Another limitation of our study is that the broad inclusion criteria of at least 1 of 4 risk factors for CVD meant that the study population was heterogeneous. Effects may be different for each risk factor and the study was not sufficiently powered to detect differences in each

of these subpopulations. Finally, a washout period of two weeks means that women were studied during a different timeframe in their menstrual cycle; however, 18 out of the 20 women in the study were over the age of 50 and were most likely peri- or post-menopausal. Additionally, we attempted to minimise this effect by adjusting for the order in which they received the treatments, which was not found to be significant.

In conclusion, the lower risk of CVD with higher apple consumption is most likely due to the high concentration of flavonoids in the skin which improve endothelial function, both acutely and chronically. The mechanism behind this requires further investigation.

Author contributions: NPB, CPB, NCW, JMH and KDC were responsible for the project conception; NPB, LCB, GM and RS conducted the research; NPB, JMH and RJW analysed the data; NPB prepared the manuscript; all authors critically reviewed the manuscript.

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The authors have declared no conflicts of interest.

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Figure Legends:

Figure 1. Schematic diagram of trial design and stages. HFA, high-flavonoid apple; LFA, low-flavonoid apple.

Figure 2. Participant flow from recruitment through screening and randomization to trial completion.

Figure 3. Acute (3 h) and chronic (fasting) changes in plasma quercetin and isorhamnetin concentration post intervention. Results are presented as baseline adjusted means with standard error bars. Differences between interventions were assessed by mixed models ANOVA with adjustments for baseline and treatment order. Significant difference between interventions ($^{\#}p=0.031$); (*$p<0.0001$). HFA, high-flavonoid apple; LFA, low-flavonoid apple.

Figure 4. Acute changes in flow-mediated dilatation (FMD) over 240 sec at 1 h (**A**) and 2 h (**C**) post intervention and comparisons of peak FMD at 1 h (**B**) and 2 h (**D**) post intervention. Results are presented as adjusted means ± SEM (1 h, n=15; 2 h, n=30). Differences between interventions were assessed by mixed models ANOVA with adjustments for baseline and treatment order. Significant difference between interventions ($^{\#}p<0.05$); (*$p<0.0001$).HFA, high-flavonoid apple; LFA, low-flavonoid apple.

Figure 5. Changes in flow-mediated dilatation (FMD) after chronic (4 weeks) ingestion of either apple flesh or apple flesh + skin over 240 s (**A**) and comparisons of peak FMD (**B**). Results are presented as adjusted means ± SEM (n=30). Differences between interventions were assessed by mixed models ANOVA with adjustments for baseline and treatment order. *Significant difference between interventions ($p<0.0001$). HFA, high-flavonoid apple; LFA, low-flavonoid apple.

Table 1. Baseline characteristics of study participants

Characteristic	Mean ± SD
Age (years)	57.8 ± 10.6
Weight (kg)	77.7 ± 13.4
BMI (kg/m^2)	27.5 ± 3.9
SBP (mmHg)	124.6 ± 14.1
DBP (mmHg)	72.0 ± 7.2
Total cholesterol (mmol/L)	5.6 ± 0.9
Triglyceride (mmol/L)	1.1 ± 0.5
LDL-C (mmol/L)	3.7 ± 0.8
HDL-C (mmol/L)	1.4 ± 0.4
Fasting plasma glucose (mmol/L)	5.2 ± 0.3
Mean FMD over 4 min (%)	3.7 ± 3.2
Peak FMD (%)	7.2 ± 2.9
AIx75 (%)	20.8 ± 11.2
PWV (m/s)	7.6 ± 1.2
Plasma quercetin (nM)	0.03 ± 0.02
Plasma isorhamnetin (nM)	0.01 ± 0.00
Plasma heme (μM)*	1.6 ± 1.2
Plasma bilirubin (μM)*	7.5 ± 3.4
Plasma biliverdin (nM)*	30.9 ± 11.4

n=30; males=10, females=20
*n=13
AIx75; augmentation index standardised to a heart rate of 75 bpm; DBP, diastolic blood pressure; FMD, flow-mediated dilatation; HDL-C, HDL- cholesterol; LDL-C, LDL-cholesterol; PWV, pulse wave velocity.

Table 2. Daily phenolic consumption

	Total quercetin glucosides (mg/d)	Epicatechin (mg/d)	Phloridzin (mg/d)	Chlorogenic acid (mg/d)	Anthocyanins (mg/d)
Low-flavonoid apple	12.5	25.2	2.2	52.1	ND
High-flavonoid apple	195.3	48.0	5.1	52.1	6.0

Approximate weight of phenolics consumed per day (mg). Assuming one Cripps Pink apple without skin weighs 163.6 ± 3.3 g and one Cripps Pink plus an extra skin weighs 180.5 ± 12.5 g and volunteers were having each intervention twice per day. Values were calculated from the average phenolic concentration of 2 Cripps Pink apples. ND, not detected.

Table 3. Measures of blood pressure and arterial stiffness

	Acute			Chronic		
	Low-flavonoid apple	High-flavonoid apple	p-value	Low-flavonoid apple	High-flavonoid apple	p-value
SBP (mmHg)	126.9 ± 1.6	127.7 ± 1.6	0.66	126.4 ± 1.3	129.4 ± 1.3	0.09
DBP (mmHg)	74.7 ± 0.9	75.2 ± 0.8	0.66	76.8 ± 0.8	77.7 ± 0.8	0.34
Central DP (mmHg)	75.0 ± 0.8	76.14 ± 0.8	0.26	76.7 ± 0.7	78.2 ± 0.7	0.07
HR (bpm)	57.7 ± 1.3	57.5 ± 1.3	0.87	57.8 ± 0.9	57.6 ± 0.9	0.89
AIx75 (%)	17.5 ± 0.9	16.8 ± 0.9	0.52	21.1 ± 1.8	22.4 ± 1.9	0.65
PWV (m/s)	7.6 ± 0.1	7.7 ± 0.1	0.53	8.9 ± 0.9	7.7 ± 0.9	0.37

Changes in measurements of blood pressure and arterial stiffness after acute (2 h) and chronic (4 weeks) intake of high-flavonoid apple or low-flavonoid apple. Results are presented as adjusted means ± SEM (n=30). Differences between interventions were assessed by mixed models ANOVA with adjustments for baseline and treatment order.

AIx75; augmentation index standardised to a heart rate of 75 bpm; DBP, diastolic blood pressure; DP, diastolic pressure; HR, heart rate; PWV, pulse wave velocity; SBP, systolic blood pressure.

Table 4. Mean 24hr ambulatory blood pressure

	Low-flavonoid apple	High-flavonoid apple	p-value
SBP	118.9 ± 0.9	119.5 ± 0.9	0.57
DBP	72.3 ± 0.6	72.3 ± 0.6	0.99
PP	46.6 ± 0.6	47.1 ± 0.5	0.47
HR	70.7 ± 0.9	70.2 ± 0.9	0.70

Mean blood pressure over a 24 h period after chronic (4 weeks) consumption of high-flavonoid apple or low-flavonoid apple. Results are presented as adjusted means ± SEM (n=30). Differences between interventions were assessed by mixed models ANOVA with adjustments for baseline and treatment order.
DBP, diastolic blood pressure; HR, heart rate; PP, pulse pressure; SBP, systolic blood pressure.

Table 5. Plasma and salivary concentrations of nitrate and nitrite

		Acute			Chronic		
		Low-flavonoid apple	High-flavonoid apple	p-value	Low-flavonoid apple	High-flavonoid apple	p-value
Plasma	Nitrite	1.6 ± 0.1	1.7 ± 0.1	0.61	1.8 ± 0.2	1.9 ± 0.2	0.89
	Nitrate	23.1 ± 1.0	20.9 ± 1.0	0.11	23.1 ± 1.3	23.0 ± 1.3	0.94
Saliva	Nitrite	86.2 ± 12.3	84.7 ± 12.3	0.92	73.6 ± 16.5	81.3 ± 16.8	0.75
	Nitrate	201.5 ± 29.1	205.1 ± 29.1	0.89	167.2 ± 44.8	193.9 ± 45.6	0.68

Comparison of saliva nitrite and nitrate after acute and chronic ingestion of either high-flavonoid apple or low-flavonoid apple. Results are presented as adjusted means ± SEM (n=30). Differences between interventions were assessed by mixed models ANOVA with adjustments for baseline and treatment order.

Table 6. Plasma haem, biliverdin and bilirubin concentrations

	Low-flavonoid apple	High-flavonoid apple	p-value
Haem (µM)	2.0 ± 0.4	1.6 ± 0.4	0.21
Biliverdin (nM)	39.6 ± 7.2	28.8 ± 7.2	0.02*
Bilirubin (µM)	7.0 ± 0.9	6.0 ± 0.8	0.34

Plasma haem, biliverdin and bilirubin concentrations after long-term (4 weeks) consumption of high-flavonoid apple (HFA) or low-flavonoid apple (LFA). Results are presented as adjusted means ± SEM (n=14). Differences between interventions were assessed by mixed models ANOVA with adjustments for baseline and treatment order. *Significant difference between LFA and HFA ($p<0.05$).

Table 7. Biochemistry analysis of plasma and urine after 4-week intervention

	Low-flavonoid apple	High-flavonoid apple	P-value
Glucose (mmol/L)	5.3 ± 0.04	5.3 ± 0.04	0.55
Cholesterol (mmol/L)	6.0 ± 0.1	5.8 ± 0.1	0.18
TG (mmol/L)	1.3 ± 0.1	1.3 ± 0.1	0.99
LDL-C (mmol/L)	4.0 ± 0.1	3.8 ± 0.1	0.14
HDL-C (mmol/L)	1.4 ± 0.02	1.4 ± 0.02	0.31
F_2-Isoprostanes (pmol/L)	728.3 ± 18.6	690.5 ± 18.6	0.14
Creatinine (mmol/L)	7.3 ± 0.9	8.2 ± 0.9	0.33
Urinary potassium (mmol/L/mmol creatinine)	7.2 ± 0.5	6.6 ± 0.5	0.38
Urinary sodium (mmol/L/mmol creatinine)	12.5 ± 1.3	11.6 ± 1.3	0.62

Changes in measurements of plasma glucose, cholesterol, and F_2-isoprostanes and urinary creatinine and salts after chronic (4 weeks) consumption of high-flavonoid apple or low-flavonoid apple. Results are presented as adjusted means ± SE (n=30). Differences between interventions were assessed by mixed models ANOVA with adjustments for baseline and treatment order.

HDL-C, HDL-cholesterol; LDL-C, LDL-cholesterol; TG, triglycerides.