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# Two apples a day lower serum cholesterol and improve cardiometabolic biomarkers in mildly hypercholesterolemic adults: a randomized, controlled, crossover trial

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## ABSTRACT

**Background:** Apples are rich in bioactive polyphenols and fiber. Evidence suggests that consumption of apples or their bioactive components is associated with beneficial effects on lipid metabolism and other markers of cardiovascular disease (CVD). However, adequately powered randomized controlled trials are necessary to confirm these data and explore the mechanisms.

**Objective:** We aimed to determine the effects of apple consumption on circulating lipids, vascular function, and other CVD risk markers.

**Methods:** The trial was a randomized, controlled, crossover, intervention study. Healthy mildly hypercholesterolemic volunteers (23 women, 17 men), with a mean  $\pm$  SD BMI  $25.3 \pm 3.7$  kg/m<sup>2</sup> and age  $51 \pm 11$  y, consumed 2 apples/d [Renetta Canada, rich in proanthocyanidins (PAs)] or a sugar- and energy-matched apple control beverage (CB) for 8 wk each, separated by a 4-wk washout period. Fasted blood was collected before and after each treatment. Serum lipids, glucose, insulin, bile acids, and endothelial and inflammation biomarkers were measured, in addition to microvascular reactivity, using laser Doppler imaging with iontophoresis, and arterial stiffness, using pulse wave analysis.

**Results:** Whole apple (WA) consumption decreased serum total (WA: 5.89 mmol/L; CB: 6.11 mmol/L;  $P = 0.006$ ) and LDL cholesterol (WA: 3.72 mmol/L; CB: 3.86 mmol/L;  $P = 0.031$ ), triacylglycerol (WA: 1.17 mmol/L; CB: 1.30 mmol/L;  $P = 0.021$ ), and intercellular cell adhesion molecule-1 (WA: 153.9 ng/mL; CB: 159.4 ng/mL;  $P = 0.028$ ), and increased serum uric acid (WA: 341.4  $\mu$ mol/L; CB: 330  $\mu$ mol/L;  $P = 0.020$ ) compared with the CB. The response to endothelium-dependent microvascular vasodilation was greater after the apples [WA: 853 perfusion units (PU), CB: 760 PU;  $P = 0.037$ ] than after the CB. Apples had no effect on blood pressure or other CVD markers.

**Conclusions:** These data support beneficial hypocholesterolemic and vascular effects of the daily consumption of PA-rich apples by mildly hypercholesterolemic individuals. This trial was registered at clinicaltrials.gov as NCT01988389. *Am J Clin Nutr* 2019;00:1–12.

**Keywords:** apple, polyphenols, flavanols, proanthocyanidins, fiber, lipid, cholesterol, vascular, bile acids, sex

## Introduction

Regular consumption of plant foods, including fruits, has been consistently associated with a lower risk of cardiovascular disease (CVD) (1). Apples represent 12.5% of all consumed fruits in the world (2), due to their taste and convenience, and possibly

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Data described in the article will be made available upon request pending application and approval.

Supplemental Methods, Supplemental Tables 1–8, and Supplemental Figures 1–5 are available from the “Supplementary data” link in the online posting of the article and from the same link in the online table of contents at <https://academic.oup.com/ajcn/>.

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Abbreviations used: AIx, Augmentation Index; BA, bile acid; C-AGPH, central augmentation pressure-to-pulse height ratio; CB, control beverage; CVD, cardiovascular disease; FXR, farnesoid X receptor; GUDCA, glycooursodeoxycholic acid; HR, heart rate; ICAM-1, intercellular cell adhesion molecule-1; LDI, laser Doppler imaging; LH, lithium heparin; NEFA, nonesterified fatty acid; PA, proanthocyanidin; PU, perfusion units; PWA, pulse wave analysis; SST, serum separator tube; TAG, triacylglycerol; TC, total cholesterol; VCAM-1, vascular cell adhesion molecule-1; WA, whole apple.

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also because of their purported health benefits. Epidemiological studies suggest that a frequent apple intake is inversely associated with acute coronary syndrome, total CVD mortality, and all-cause mortality (3, 4).

Apples are an excellent source of polyphenols (typically 110 mg/100 g) and fiber (typically 2–3 g/100 g) and these bioactive components may be responsible for the potential health effects (5). Apple polyphenols include flavanols [catechin and proanthocyanidins (PAs)] as the major class (71–90%), followed by hydroxycinnamates (4–18%), flavonols (1–11%), dihydrochalcones (2–6%), and anthocyanins (1–3%) which are found only in red apples (5). PAs and flavanol monomers, as extracts or within a food, have been shown to lower serum cholesterol, raise HDL cholesterol, inhibit LDL oxidation, activate endothelial nitric oxide synthase, prevent platelet aggregation, and block inflammatory responses in atherosclerosis, although data from human intervention studies are limited and sometimes contradictory (6–8). In addition, pectin, the main soluble fiber found in apples, is reported to affect transit time, gastric emptying, and nutrient absorption, affecting lipid and glucose metabolism (5). Pectin also appears to modulate the gut microbiota, a key determinant of bile acid (BA) chemical structure and thus signaling potential (5).

There is consistent evidence that apple consumption is associated with hypolipidemic effects, with reductions in total cholesterol (TC) and LDL cholesterol in animal models, but these studies cannot be directly extrapolated to humans (5). Human intervention studies also suggest potential lipid-lowering effects of apples but results lack consistency, perhaps due to differences in the apple products tested, which have included fresh whole apples (WAs) (9–16), dry apples (17, 18), apple skin (19), apple pomace (15), cloudy (15, 20, 21) and clear apple juice (11, 15), and pure apple polyphenols (8, 22, 23); dose and molecular characteristics of the active components; study duration; sample size; and the use of appropriate controls (5). The effects of apples on endothelial function have been observed in postprandial human studies (19, 24) but are inconsistent in longer-term interventions (4-wk treatment) (17, 19), whereas effects on systemic inflammation are limited (5).

In this 8-wk dietary intervention study, we examined the hypothesis that the supplementation of the habitual diet of 40 healthy mildly hypercholesterolemic subjects with 2 fresh WAs (Renetta Canada) daily for 8 wk would result in TC reduction and beneficial effects on vascular function and other markers of CVD, compared with a sugar-matched apple control beverage (CB). Renetta Canada is an apple variety rich in PAs and dihydrochalcones and was previously shown to possess beneficial effects on gut microbiota composition and activity *in vitro* (25).

## Methods

### Research ethics

The study (NCT01988389) was given a favorable ethical opinion for conduct by the University of Reading Research Ethics Committee [UREC Project No. 13/22 (AVAG/effect of Apples on cardioVascular risk And Gut health study)]. The study was conducted according to the guidelines laid down in the Declaration of Helsinki of 1975 as revised in 1983. Subjects

provided written informed consent before participating and a small remuneration was given for participation in the trial.

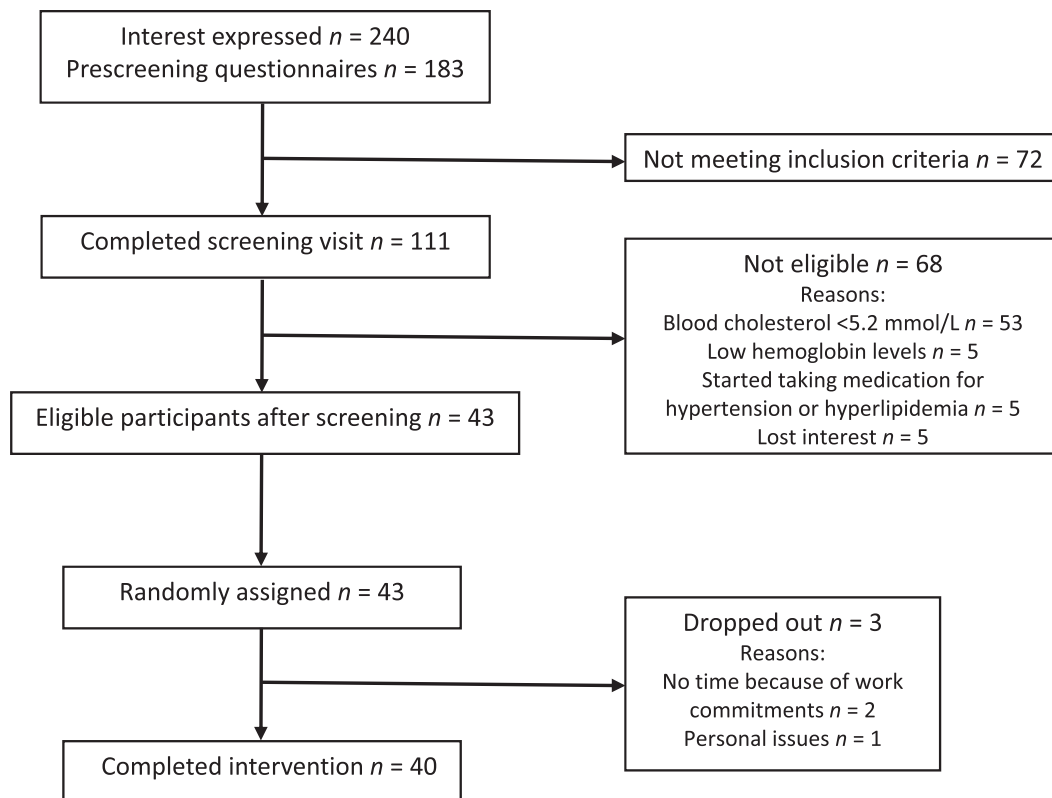
### Participants

Forty-three generally healthy volunteers (18 men, 25 women), aged 29–69 y, with BMI (in kg/m<sup>2</sup>) 19–33 and TC > 5.2 mmol/L (mildly hypercholesterolemic), were recruited from the general population of Reading, Berkshire, United Kingdom using human volunteer databases, posters, and adverts on social media (Figure 1). Subjects first completed a health and lifestyle questionnaire and then attended the Hugh Sinclair Unit of Human Nutrition at the University of Reading in a fasted state. After giving informed written consent, screening measurements were performed, such as height, weight, blood pressure, and blood measurements including lipid profile, liver and kidney function markers, and full blood count, 2–4 wk before commencing the trial. Subjects with an abnormal blood biochemistry profile, based on standard clinical cutoffs, were excluded. Further exclusion criteria included medical history of heart disease (or family history) and diabetes mellitus; kidney, liver, or pancreatic diseases; hematological and gastrointestinal disorders; and medication for hyperlipidemia, hypertension, hypercoagulation, inflammation, and gastrointestinal motility. Other exclusion criteria were pregnancy (current or planned) or lactation, smoking, history of alcohol misuse, antibiotic intake <3 mo before the study, food supplement use (including fish oil, vitamins, phytochemicals, probiotics, and prebiotics unless willing to stop 2 mo before study commencement), habitual intake of >1 apple/d, vegetarian and vegan diet, and planned weight loss regime.

The primary outcome was changes in serum cholesterol. All other outcomes were secondary and included the evaluation of endothelial function, measured by laser Doppler imaging (LDI), changes in blood pressure and arterial stiffness, measured with the use of an ambulatory blood pressure monitor and pulse wave analysis (PWA), respectively, and changes in serum lipids, BAs, markers of inflammation, endothelial function, glycemic control, and insulin resistance.

### Study design

The present trial was a randomized, controlled, crossover, dietary intervention study performed between November 2013 and August 2014. Forty-three volunteers were recruited onto the study; 3 volunteers dropped out due to personal reasons and 40 subjects (17 men, 23 women) completed the trial (Figure 1). For a 2-wk run-in period before the dietary intervention, volunteers followed their habitual diet but were required not to consume probiotics (e.g., live yogurts, fermented milk drinks), prebiotics (e.g., inulin, fructooligosaccharide), and any apples, apple juice, or apple-containing foods. Probiotics, prebiotics, and any other apple products (apart from the provided items) were also strictly avoided throughout the study period (20 wk). Volunteers were randomly allocated into 2 groups, using a minimization program (minim) (26), stratified by age [23–45 y (*n* = 13), 46–69 y (*n* = 27)], sex (17 men, 23 women), BMI [*<*25 (*n* = 20), *>*25 (*n* = 20)], and TC [5.2–6.2 mmol/L (*n* = 26), *>*6.2 mmol/L (*n* = 14)], to receive daily either 2 WAs or a sugar-matched CB



**FIGURE 1** Participant flow diagram of the AVAG (effect of Apples on cardioVascular risk And Gut health) study.

(Table 1, Figure 2). One group [ $n = 22$  (10 men, 12 women); mean  $\pm$  SD age:  $51 \pm 11$  y; BMI:  $25 \pm 3$ ; TC:  $5.9 \pm 0.7$  mmol/L] consumed 2 apples/d (340 g without core) for 8 wk, and then after a 4-wk washout period, consumed 500 mL of a sugar-matched apple CB (100 mL concentrate + 400 mL water) daily for a further 8 wk, whereas the other group [ $n = 18$  (7 men, 11 women); age:  $52 \pm 11$  y; BMI:  $26 \pm 4$ ; TC:  $6.0 \pm 0.6$  mmol/L] received the intervention foods in the reverse order (Figure 2). Volunteers were asked to incorporate the intervention products into their normal habitual diet, and understood that both products had potential benefit. Clear instructions were given to eat the apple skin and flesh and not to consume the apple core.

The study included 4 main visits before and after each intervention period (weeks 1, 8, 12, and 20) (Figure 2). Twenty-four hours before each study visit, volunteers were asked to collect their urine and refrain from intensive exercise, alcohol, oily fish, and caffeine intake (e.g., coffee, tea). The night before they consumed a standardized low-fat, low-flavonoid meal followed by an overnight fast consuming only water. The volunteers were asked to attend the Hugh Sinclair Unit of Human Nutrition in the morning of each of the 4 study visit days in a fasted state. During these visits, several measurements were taken, including anthropometrics (height, weight, waist circumference), body fat composition (body composition analyzer, Tanita BC-418 digital scale), blood pressure (30-min measurement), arterial stiffness (PWA), and microvascular reactivity (LDI with iontophoresis). Moreover, volunteers returned their 24-h urine collection and a fasted blood sample was collected before they were offered breakfast. Volunteers received the intervention products on the

visit day, and then every fortnight when they were also asked if there had been any changes to their lifestyle, such as diet, exercise levels, and medication. Finally, compliance was assessed with the daily completion of a tick sheet.

### Intervention products

Fresh apples from the same harvest (green variety “Renetta Canada”) grown in Val di Non, Trentino, Italy were used as the intervention food. All apples were kindly provided by Melinda SCA, Cles, Trentino. A total of 7337 apples were delivered to the University of Reading, United Kingdom in 7 batches between November 2013 and May 2014. There was no difference in the PA concentration of the apples during storage and between the different batches throughout the study (Supplemental Figure 1). After collection, the apples were stored in the dark at  $4^{\circ}\text{C}$  for  $\leq 4$ –6 wk. The CB was a commercially available apple juice squash consisting of 50% apple juice from concentrate and added sugar which matched the total sugar content of the apples. The daily dietary content of the intervention products is shown in Table 1. The apples’ nutritional composition was analyzed by Campden BRI laboratories, United Kingdom and the detailed polyphenol content was measured in the laboratory of Fondazione Edmund Mach according to the methods of Gris et al. (28) and Vrhovsek et al. (27).

### Diet diary analysis

Participants were asked to complete a detailed 4-d diary of all foods and drinks consumed (3 weekdays and 1 day at the

**TABLE 1** Nutritional composition of the intervention products (daily portion)<sup>1</sup>

| Component                                           | Whole apples <sup>2</sup> | Control beverage <sup>3</sup> |
|-----------------------------------------------------|---------------------------|-------------------------------|
| Energy, <sup>4</sup> kJ                             | 925                       | 699                           |
| Energy, <sup>4</sup> kcal                           | 221                       | 167                           |
| Fat, <sup>4</sup> g                                 | 0.7                       | <0.1                          |
| Protein, <sup>4</sup> g                             | 1                         | <0.1                          |
| Carbohydrate total, <sup>4</sup> g                  | 58                        | 42                            |
| Carbohydrate available, <sup>4</sup> g              | 49                        | 42                            |
| Total sugar, <sup>4</sup> g                         | 44                        | 41                            |
| Glucose, <sup>4</sup> g                             | 8                         | 18                            |
| Fructose, <sup>4</sup> g                            | 23                        | 20                            |
| Sucrose, <sup>4</sup> g                             | 12                        | 3                             |
| Total dietary fiber <sup>4</sup> (AOAC), g          | 8.5                       | <0.5                          |
| Soluble fiber <sup>4</sup> (AOAC), g                | 3.7                       | <0.5                          |
| Insoluble fiber <sup>4</sup> (AOAC), g              | 4.8                       | <0.5                          |
| Vitamin C, <sup>4</sup> mg                          | 21                        | <3                            |
| Chlorogenic acid, <sup>5</sup> mg                   | 83 ± 8.9                  | 0.9 ± 2.1                     |
| Cryptochlorogenic acid, <sup>5</sup> mg             | 0.8 ± 0.4                 | 0.1 ± 0.6                     |
| Ellagic acid, <sup>5</sup> mg                       | 0.2 ± 0.1                 | 0 ± 0                         |
| Phloridzin, <sup>5</sup> mg                         | 13.8 ± 3.5                | 0.3 ± 0.6                     |
| Quercetin-3- <i>O</i> -arabinoside, <sup>5</sup> mg | 2.3 ± 1.0                 | 0.03 ± 0.2                    |
| Quercetin-3- <i>O</i> -glucoside, <sup>5</sup> mg   | 2.3 ± 1.9                 | 0.03 ± 0.2                    |
| Quercetin-Glc-Rha (rutin), <sup>5</sup> mg          | 1.2 ± 1.5                 | 0.03 ± 0.2                    |
| Catechin, <sup>5</sup> mg                           | 5.3 ± 2.3                 | 0.01 ± 0.03                   |
| Epicatechin, <sup>5</sup> mg                        | 27 ± 6.5                  | 0.02 ± 0.09                   |
| Procyanidin B1, <sup>5</sup> mg                     | 23 ± 5.6                  | 0.01 ± 0.03                   |
| Procyanidin B2, <sup>5</sup> mg                     | 176 ± 26                  | 0.06 ± 0.32                   |
| Oligomeric PAs (DP > 2) <sup>6</sup>                | 854 ± 233                 | 1 ± 1.7                       |
| Mean DP <sup>6</sup>                                | 7.1                       | 3.5                           |
| Sum of polyphenols <sup>7</sup>                     | 990 ± 259                 | 2.5 ± 5.7                     |

<sup>1</sup>Values are means ± SDs for polyphenols.<sup>2</sup>Consists of a 340-g daily serving (2 apples; Renetta Canada) without the core including skin.<sup>3</sup>Consists of a 100-mL control beverage + 400-mL water daily serving (sugar-matched control).<sup>4</sup>Measured by Campden BRI laboratories, UK, mixture of 3 apples.<sup>5</sup>Quantified in house according to Vrhovsek et al. (27), measurement of 15 apples from December to April.<sup>6</sup>Quantified in house according to Gris et al. (28), measurement of 15 apples from December to April. DP, degree of polymerization; PA, proanthocyanidin.<sup>7</sup>Sum of all individual polyphenols and oligomeric PAs (DP > 2).

weekend), the week before each of the 4 main visits. The food diaries were analyzed using Dietplan6 (Forestfield Software Ltd.) to determine the macro- and micronutrient content of the participants' diet based on UK food databases (29). Flavonoid

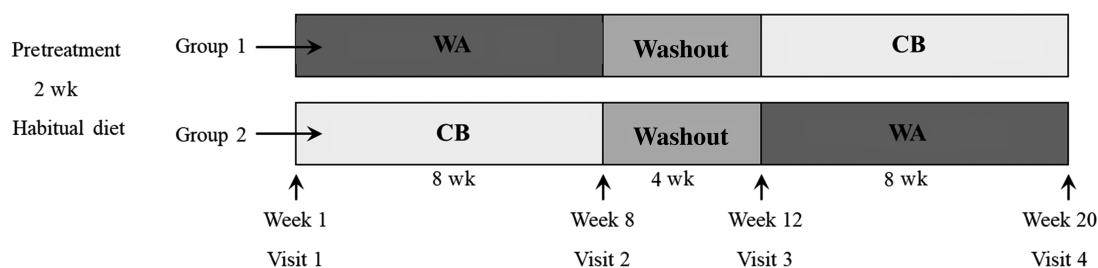
intake was assessed by using the USDA Flavonoid Database and by an "in-house" compositional analysis of fruits and vegetables as described by Chong et al. (30).

### Anthropometric, body fat composition, and blood pressure analysis

Height, weight, waist circumference, body fat percentage, and blood pressure were measured on 4 occasions, at the beginning and the end of the 2 intervention periods. Body fat composition (percentage) was measured with a Tanita body composition monitor (Tanita BC-418 digital scale, Tanita Europe) based on bioelectrical impedance technology. Measurements were performed under normal settings with participants wearing light clothing. Blood pressure was recorded using an ambulatory blood pressure monitor which was adjusted to take a measurement every 5 min. Participants were relaxed, seated in a quiet, light room for 15 min before the commencement of the measurement, which lasted for 30 min and included 6 measurements.

### Assessment of vascular function

All measurements were performed in a quiet temperature-controlled room (mean ± range: 22 ± 1°C), with participants in a supine position after a 30-min rest. Microvascular reactivity was measured using a moorLDI2 laser Doppler Imager with iontophoresis (Moor Instruments Ltd.) as previously described (31). Briefly, acetylcholine chloride (2.5 mL, 1% in 0.5% NaCl solution) for the measurement of endothelium-dependent reactivity and sodium nitroprusside (2.5 mL, 1% in 0.5% NaCl solution) for the measurement of endothelium-independent reactivity were delivered transdermally on the forearm via iontophoresis using ION6 chambers (Moor Instruments). Current delivery was controlled and repeated scans were taken, giving a total charge of 8 millicoulombs. Microvascular response was calculated by using the area under the flux-versus-time curve (AUC), measured as perfusion units (arbitrary). PWA was measured using a high-fidelity applanation tonometer to detect and capture electronically the shape of the radial arterial pulse (SphygmoCor system, AtCor Medical). The radial artery pressure waveform was then calibrated with the brachial systolic and diastolic pressure measurements (performed in the traditional manner at the brachial artery with an inflated cuff). The SphygmoCor system then converted this peripheral waveform to a central aortic pressure waveform using general transfer function



**FIGURE 2** Study design of a randomized, controlled, crossover study in which 40 healthy volunteers (Group 1:  $n = 22$ ; Group 2:  $n = 18$ ) received daily 2 WAs (340 g without core) or a CB (100 mL concentrated + 400 mL water) for a period of 8 wk each, separated by a 4-wk washout period. Fasted blood and a 24-h urine sample were collected on each study visit (weeks 1, 8, 12, and 20), and ambulatory blood pressure, vascular reactivity, and arterial stiffness were assessed. CB, control beverage; WA, whole apple.

algorithms (32). Moreover, several indexes were quantified from the peripheral and central waveforms: central systolic pressure, central diastolic pressure, central pulse pressure, central mean pressure, heart rate (HR), and central augmentation pressure-to-pulse height ratio (C-AGPH)—also known as the Augmentation Index (AIx) or arterial stiffness. Given that the AIx depends on HR, it was corrected for an HR of 75 beats per minute (C-AGPH-HR75). Measurements were performed in triplicate by a single trained operator.

### Blood and urine sample collection

For each volunteer, blood samples were collected into separate vacutainer tubes (Greiner Bio-One Ltd.), containing EDTA or lithium heparin (LH) anticoagulants for plasma and a clot activator for serum [serum separator tubes (SSTs)]. After collection, the EDTA and LH tubes were immediately put on ice for transportation to the laboratory. Cells were then removed from plasma by centrifugation at  $1800 \times g$  for 15 min at 4°C. Aliquots (0.5 mL) of the supernatant (plasma) were immediately transferred into clean cryogenic (polypropylene) vials and stored at  $-80^{\circ}\text{C}$ . To obtain serum, SSTs were left undisturbed at room temperature for 30 min after collection. The supernatant (serum) was then obtained by centrifugation at  $1800 \times g$  for 15 min at 21°C and aliquots were put into separate cryogenic vials for storage at  $-20^{\circ}\text{C}$ .

For the collection of the urine, volunteers were provided with a urine pot containing 10 mL 1 M hydrochloric acid, ice packs, and an isothermic bag and they were instructed to start collecting the sample the morning before the study visit and for 24 h. Once the urine was received in the lab, its pH was reduced to 4 with the further addition of 1 M hydrochloric acid, then it was centrifuged at  $1800 \times g$  for 10 min at 4°C, divided into aliquots, and stored at  $-80^{\circ}\text{C}$  until further analysis. Urine volume was also recorded. Analysis of blood and urine was performed when the intervention study was completed. All samples from each subject were analyzed together to reduce interbatch variation.

### Biochemical analysis

Concentrations of serum lipids [total cholesterol (TC), HDL cholesterol, triacylglycerol (TAG), and nonesterified fatty acids (NEFAs)], glucose, albumin, and uric acid were measured using an Automatic Analyzer ILAB 600 with appropriate enzyme-based kits (Instrumentation Laboratory Ltd.) and quality controls (Alpha Laboratories Ltd.). Controls were run with each batch and their values were within the range specified by the manufacturers. LDL cholesterol was estimated by using the Friedewald formula ( $\text{LDL cholesterol} = \text{TC} - \text{HDL cholesterol} - \text{TAG}/2.19$ ). ELISA kits were used for the determination of serum insulin (Dako Ltd.), adiponectin, and endothelin (R&D Systems Europe Ltd.) according to the manufacturer's instructions. An Adhesion Molecule Luminex Performance Assay 4-plex kit was used for the determination of E-selectin, P-selectin, vascular cell adhesion molecule-1 (VCAM-1), and intercellular cell adhesion molecule-1 (ICAM-1) (R&D Systems Europe Ltd.) using a flow cytometry-based Luminex 200™ System (Invitrogen, Thermo Fisher Scientific). A Milliplex MAP human cytokine kit (Merck-Millipore) was used for the determination of TNF- $\alpha$ , IL-1 $\beta$ , and

IL-6 according to the manufacturer's instructions. IL-1 $\beta$  and IL-6 were under the detection limit and are not reported here.

### BAs in blood

BAs were quantified by the isotopic dilution method described elsewhere (33) and modified for the purpose of the current sample set. Briefly, 100  $\mu\text{L}$  plasma was placed in Sirocco 96-well plates followed by 200  $\mu\text{L}$  internal standards in methanol and 200  $\mu\text{L}$  methanol:water (90:10, vol:vol) with 0.1% formic acid. Samples were filtered and the residue was extracted with 400  $\mu\text{L}$  acetonitrile:acetone (80:20, vol:vol) with 0.1% formic acid. The extract was evaporated until dryness and resolved with 200  $\mu\text{L}$  methanol:water (50:50, vol:vol) before injection. Samples were analyzed using a UHPLC (ultra-HPLC) UltiMate 3000 (Dionex) coupled with a Triple Quad 5500 (AB Sciex) mass spectrometer. Data were acquired according to multiple reaction monitoring methodology and results were expressed using an appropriate internal standard. Raw data were processed by AB Sciex Analyst 1.6.1 software. A detailed method description and validation can be found in the **Supplemental Methods**. The ursodeoxycholic acid was excluded from the statistical analysis because >30% of measures were missing. Missing values of glycolithocholic acid (20 measures, distributed among 8 subjects) and glyoursodeoxycholic acid (GUDCA) (4 measures, distributed among 2 subjects) were imputed with a random number ranging between 0 and the limit of quantification for each compound (Supplemental Methods, **Supplemental Tables 1–3**).

### Phloretin metabolites in urine

Urine samples were submitted to untargeted chemical analysis for the identification of phloretin metabolites as biomarkers of apple consumption (Supplemental Methods) (34). Phloretin glucuronide was not detected in 16% of measures and phloretin glucuronide sulfate in 61% of measures; these were detected almost solely in the presence of apple intake. Missing values were imputed with a random number ranging between 0 and 5,000, which is compatible with the electrical noise of the mass spectrometer.

### Statistics

The primary outcome of the study was to measure relative changes in serum TC and it was calculated that 38 volunteers were required to detect a 5% change ( $P < 0.05$  and 90% power), assuming a 6-mmol/L mean TC with a 0.41-mmol/L within-subject SD. On the basis of this power calculation, 43 volunteers were recruited to allow for a 10% dropout rate. Samples from each participant were recoded to protect volunteer identity and to mask treatment groups during the analysis.

Exploratory analysis on the study primary outcome (TC) was initially performed by calculating, for each individual, the difference ( $\Delta C$ ) in TC after the consumption of WAs and CB. Intervention-specific baselines were also taken into account [ $\Delta C = (C_{\text{apple}} - C_{\text{apple baseline}}) - (C_{\text{control}} - C_{\text{control baseline}})$ ]. The presence of a significant difference in the TC concentration was then tested by a 1-sample  $t$  test against a null hypothesis of  $\Delta C = 0$ . The dependence of  $\Delta C$  on the main study factors (age, BMI, and sex) was also assessed in this phase (**Supplemental Figures 2–4**).

Differences between the dietary components of apples and the CB were assessed using a *t* test, whereas the PA content of apples throughout storage was assessed using an ANOVA test using IBM SPSS version 21 (SPSS Inc.).

In order to test the difference in treatment response means, baseline and postbaseline data were jointly modeled applying mixed-effect linear modeling, accounting for subject variability (35). The model assumes that the effects of systematic bias typical of crossover studies, such as carryover, are negligible. As suggested for the 2-period 2-treatment design (35), a formal test on carryover was not performed. Restricted maximum likelihood was used to estimate the model parameters. To perform model optimization, the distribution of the conditional residuals of the models was inspected to assess normality and homoscedasticity, and exclude the presence of groups. To improve the distribution of skewed variables and the conditional residuals of the model,  $\log_{10}$  transformation was applied. For these variables, the model estimates for both baselines and treatments are presented as back-transformed values, which correspond to the median measured concentrations, in order to facilitate the interpretation of the results and allow their direct comparison with published clinical ranges. The uncertainty on the inference estimates was calculated with a bootstrap operation (500 repeats) and is indicated as (possibly back-transformed) 95% CIs.

In order to provide all the information to properly interpret the study findings, the treatment effect (computed as the difference between the value of a variable after WA intake and after CB consumption), as well as the estimated sequence and period effect, and the number of observations and subjects used for building each model are also explicitly reported (Supplemental Tables 4–7) (36). These results were not back-transformed from the logarithmic scale in order to improve interpretation. It is worth noting that with our modeling strategy the significance of the treatment effect can also be reliably assessed in the presence of period/sequence effects, even if the estimation of the effect is suboptimal (37).

The mixed linear models were built in R ([www.r-project.org](http://www.r-project.org)) version 3.5.0 by using packages *lme4* version 1.1-17 (38) and *lmerTest* version 3.0-1 (39), whereas the CIs were computed using the package *stats*. *P* values were computed using the Kenward-Roger approximation and 0.05 was chosen as the significance threshold.

The association between TC and BAs in women was tested without taking into account baselines. The predictors were chosen from among the BAs, which were log transformed, centered, and scaled using the R package *LaplacesDemon* version 16.1.0. The study design factors (age, treatment, sequence, period) were also included in the model and age was centered and scaled in the same way as the BAs. Both  $R^2$  and  $\Omega^2$  indicate that the model fit is good, being 0.961 and 0.960, respectively. The use of a mixed linear model also allowed us to take into account subject variability together with the grouping of the samples within the 2 sequences of the crossover design. In particular, subject nesting within the sequence was defined as a random effect.

## Results

### Nutritional composition analysis of intervention products

Table 1 presents the detailed nutritional composition analysis of the intervention products. Apples contained higher amounts

of polyphenols and fiber than the CB ( $P < 0.05$ ). In particular, a daily dose of 340 g apples provided 990 mg polyphenols (presented as the sum of the individual phenolic compounds), 854 mg PAs, and 8.5 g total fiber (3.7 g soluble) compared with 2.5 mg polyphenols, 1 mg PAs, and  $<0.5$  g total fiber from the daily intake of 500 mL sugar-matched CB (Table 1).

### Study population characteristics, dietary intakes, and compliance

Of the 43 volunteers who started the study, 40 completed all visits (23 women, 17 men). Three participants dropped out for personal reasons. No adverse effects were reported during the study. The volunteers' age range was 29–69 y (mean: 51 y) and BMI was between 18.4 and 33.4 kg/m<sup>2</sup> (mean: 25.3). Volunteers had mean TC above that considered physiologically normal (6.08 mmol/L compared with guideline values of  $<5.2$  mmol/L). Table 2 and Supplemental Table 4 show the baseline measurements before the 2 treatments. There was no effect of the intervention on body weight, BMI, waist circumference, and body fat percentage throughout the 20-wk period (Table 2, Supplemental Table 4). There was no change in daily energy and dietary carbohydrates, sugars, fat, and protein (Table 3, Supplemental Table 5). During the WA intervention, daily dietary fiber ( $P = 0.001$ ) and flavonoid intake ( $P = 0.001$ ) increased compared with the CB, suggesting that the participants complied with the dietary protocol (Table 3, Supplemental Table 5). Compliance was further confirmed by the higher concentrations of urinary biomarkers of apple intake in the WA group compared with the CB group ( $P < 0.05$ ). Figure 3 shows the  $\log_{10}$  value of intensities for urinary phloretin glucuronide (Figure 3A) and phloretin glucuronide sulfate (Figure 3B) measured in urine samples throughout the intervention. Phloretin glucuronide and phloretin glucuronide sulfate were detected almost exclusively after WA intake.

### Biochemical analysis

Table 2 and Supplemental Table 4 show the blood biochemical parameters. WA decreased serum TC ( $P = 0.006$ ), LDL cholesterol ( $P = 0.031$ ), and TAG ( $P = 0.021$ ) compared with the CB. The WA intervention increased uric acid ( $P = 0.020$ ) compared with the CB. There were no differences in the concentrations of circulating HDL cholesterol, NEFAs, glucose, insulin, TNF- $\alpha$ , albumin, adiponectin, and endothelin (Table 2, Supplemental Table 4). There was no effect of treatment on plasma BA concentrations (Table 4, Supplemental Table 6).

### Vascular function, blood pressure, arterial stiffness and endothelial function markers

The response to endothelium-dependent microvascular vasodilation (acetylcholine) was greater after the WA intake compared with the CB ( $P = 0.037$ ). There was no effect on blood pressure or other indexes calculated from peripheral and central pulse waveforms (PWA) (Table 5, Supplemental Table 7). WA consumption reduced plasma ICAM-1 ( $P = 0.028$ ) compared with the CB (Table 2, Supplemental Table 4). No differences

**TABLE 2** Anthropometric characteristics and blood biochemistry in mildly hypercholesterolemic participants BT (week 1) and AT (week 8) with WAs or CB<sup>1</sup>

| Parameters                | L | n/N    | WAs                    |                        | CB                     |                        | P value |
|---------------------------|---|--------|------------------------|------------------------|------------------------|------------------------|---------|
|                           |   |        | BT (95% CI)            | AT (95% CI)            | BT (95% CI)            | AT (95% CI)            |         |
| Anthropometrics           |   |        |                        |                        |                        |                        |         |
| Weight, kg                |   | 160/40 | 72.1 (67.66, 76.58)    | 72.3 (67.86, 76.78)    | 72.2 (67.74, 76.66)    | 72.4 (67.96, 76.88)    | 0.677   |
| BMI, kg/m <sup>2</sup>    |   | 160/40 | 25.3 (24.15, 26.51)    | 25.4 (24.22, 26.57)    | 25.3 (24.17, 26.53)    | 25.4 (24.27, 26.63)    | 0.500   |
| Waist, cm                 |   | 144/39 | 88.5 (85.01, 92.05)    | 88.5 (85.01, 92.05)    | 89.0 (85.51, 92.53)    | 88.5 (84.98, 92.00)    | 0.895   |
| Fat, %                    |   | 152/40 | 28.8 (26.49, 31.18)    | 29.2 (26.87, 31.55)    | 29.0 (26.68, 31.36)    | 29.0 (26.67, 31.35)    | 0.484   |
| Trunk fat, %              |   | 150/40 | 28.1 (26.01, 30.16)    | 28.6 (26.51, 30.67)    | 28.2 (26.09, 30.24)    | 28.2 (26.12, 30.27)    | 0.300   |
| Blood biochemistry        |   |        |                        |                        |                        |                        |         |
| Total cholesterol, mmol/L |   | 152/38 | 6.08 (5.84, 6.33)      | 5.89 (5.64, 6.14)      | 6.05 (5.80, 6.30)      | 6.11 (5.86, 6.36)      | 0.006   |
| LDL cholesterol, mmol/L   |   | 152/38 | 3.89 (3.67, 4.11)      | 3.72 (3.50, 3.95)      | 3.83 (3.61, 4.05)      | 3.86 (3.64, 4.08)      | 0.031   |
| HDL cholesterol, mmol/L   |   | 152/38 | 1.62 (1.49, 1.75)      | 1.58 (1.46, 1.71)      | 1.62 (1.50, 1.75)      | 1.61 (1.48, 1.74)      | 0.246   |
| TAG, mmol/L               | ✓ | 152/38 | 1.17 (1.04, 1.32)      | 1.17 (1.04, 1.32)      | 1.19 (1.06, 1.35)      | 1.30 (1.15, 1.46)      | 0.021   |
| NEFAs, μmol/L             | ✓ | 152/38 | 456.9 (402.23, 518.98) | 431.3 (379.74, 489.97) | 456.3 (401.72, 518.32) | 426.1 (375.09, 483.96) | 0.807   |
| Glucose, mmol/L           |   | 152/38 | 5.03 (4.88, 5.17)      | 5.04 (4.89, 5.18)      | 5.14 (5.00, 5.29)      | 5.09 (4.95, 5.24)      | 0.229   |
| Insulin, pmol/L           | ✓ | 160/40 | 33.8 (28.80, 39.79)    | 34.3 (29.15, 40.27)    | 35.9 (30.51, 42.16)    | 38.7 (32.89, 45.45)    | 0.051   |
| TNF-α, pg/mL              |   | 160/40 | 6.5 (5.69, 7.31)       | 6.1 (5.30, 6.92)       | 6.2 (5.44, 7.06)       | 6.5 (5.70, 7.33)       | 0.106   |
| Albumin, g/L              |   | 152/38 | 44.1 (43.32, 44.94)    | 43.3 (42.51, 44.13)    | 44.2 (43.43, 45.06)    | 43.8 (42.95, 44.57)    | 0.177   |
| Adiponectin, μg/mL        | ✓ | 152/38 | 6.4 (5.10, 8.11)       | 6.6 (5.20, 8.26)       | 6.4 (5.04, 8.01)       | 6.2 (4.92, 7.81)       | 0.071   |
| Endothelin, pg/mL         | ✓ | 160/40 | 1.1 (1.04, 1.26)       | 1.2 (1.06, 1.27)       | 1.1 (1.02, 1.23)       | 1.1 (1.03, 1.24)       | 0.561   |
| Uric acid, μmol/L         |   | 152/38 | 337.3 (313.65, 360.88) | 341.4 (317.75, 364.98) | 338.1 (314.46, 361.69) | 330.0 (306.41, 353.64) | 0.020   |
| VCAM-1, ng/mL             | ✓ | 152/38 | 696.6 (638.88, 759.55) | 665.7 (610.56, 725.88) | 682.6 (626.05, 744.30) | 690.8 (633.60, 753.28) | 0.090   |
| ICAM-1, ng/mL             | ✓ | 152/38 | 152.6 (133.04, 174.94) | 153.9 (134.22, 176.48) | 156.5 (136.44, 179.41) | 159.4 (139.04, 182.83) | 0.028   |
| E-selectin, ng/mL         | ✓ | 152/38 | 32.1 (28.82, 35.72)    | 32.8 (29.45, 36.50)    | 32.4 (29.11, 36.09)    | 33.3 (29.90, 37.07)    | 0.417   |
| P-selectin, ng/mL         | ✓ | 152/38 | 34.7 (32.16, 37.34)    | 34.7 (32.17, 37.35)    | 35.6 (33.06, 38.39)    | 35.2 (32.69, 37.95)    | 0.214   |

<sup>1</sup>Values are means (for nontransformed variables mean is equal to median) or medians (for the log-transformed variables) with (500) bootstrap 95% CIs estimated with a joint mixed-effect linear model on BT and AT data, adjusted for subject variability. The treatment effect is statistically significant when  $P < 0.05$ . AT, after treatment; BT, before treatment; CB, control beverage; ICAM-1, intercellular cell adhesion molecule-1; L, the model was built using the log10-transformed variable and the values are back-transformed in the original scale for the reader's convenience; n, number of observations; N, number of subjects; NEFA, nonesterified fatty acid; TAG, triacylglycerol; VCAM-1, vascular cell adhesion molecule-1; WA, whole apple.

were observed for the other adhesion molecules (Table 2, Supplemental Table 4).

### Exploratory association between TC response and BA changes

Our initial exploratory analysis showed that WA consumption reduced TC compared with the CB, but also suggested that the level of response to the intervention could be different for men

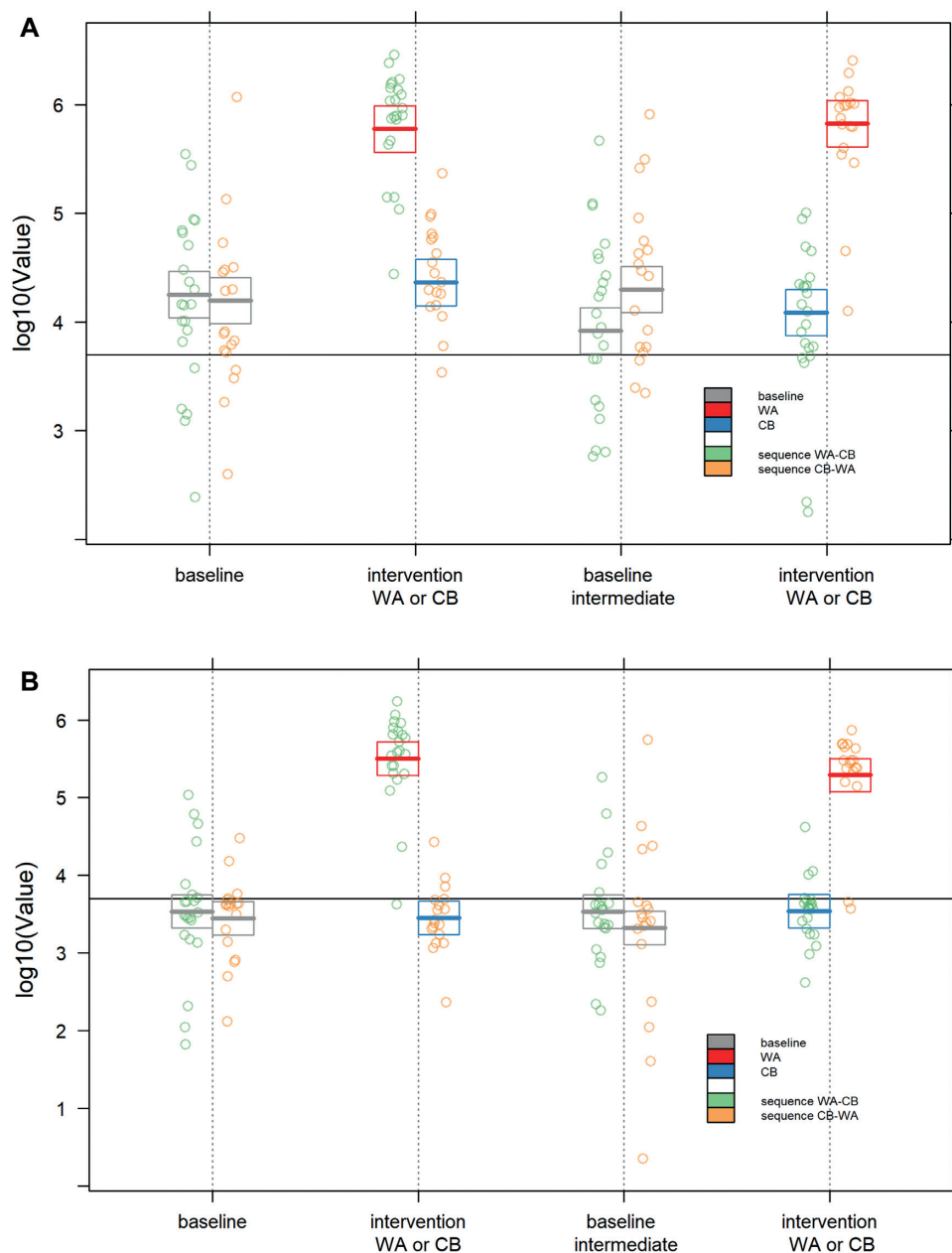
and women (Supplemental Figures 2–4). Although the study was not designed to test this hypothesis, exploratory analysis was performed which involved running our full statistical model for TC (primary outcome) separately for the 2 sexes, in order to provide new, even if not conclusive, evidence. TC was reduced after WA intake only in women (treatment effect:  $-0.33$ ; CI:  $-0.53, -0.12$ ;  $P = 0.002$ ).

Furthermore, a univariate mixed linear model, taking into consideration the volunteers' age in addition to the study design,

**TABLE 3** Dietary nutrient intake in mildly hypercholesterolemic participants BT (week 1) and AT (week 8) with WAs or CB<sup>1</sup>

| Parameters           | T | n/N    | WAs                |                      | CB                |                      | P value |
|----------------------|---|--------|--------------------|----------------------|-------------------|----------------------|---------|
|                      |   |        | BT (95% CI)        | AT (95% CI)          | BT (95% CI)       | AT (95% CI)          |         |
| Energy, kJ           | L | 156/39 | 7396 (6716, 8144)  | 8195 (7442, 9024)    | 7363 (6687, 8108) | 8097 (7353, 8917)    | 0.680   |
| Energy, kcal         | L | 156/39 | 1769 (1606, 1949)  | 1947 (1768, 2145)    | 1749 (1588, 1927) | 1926 (1749, 2122)    | 0.708   |
| Carbohydrates, g     |   | 156/39 | 194 (174, 214)     | 241 (221, 261)       | 198 (178, 218)    | 229 (209, 249)       | 0.144   |
| Total sugar, g       |   | 156/39 | 91.3 (79.8, 102.9) | 119.6 (108.1, 131.2) | 84.8 (73.3, 96.4) | 122.2 (110.7, 133.8) | 0.670   |
| Fat, g               | L | 156/39 | 67.8 (59.9, 76.7)  | 69.5 (61.4, 78.6)    | 66.2 (58.5, 74.9) | 69.5 (61.5, 78.7)    | 0.983   |
| Protein, g           | L | 156/39 | 71.9 (65.8, 78.7)  | 75.4 (69.0, 82.5)    | 72.9 (66.6, 79.7) | 74.1 (67.8, 81.0)    | 0.637   |
| Fiber (AOAC), g      |   | 156/39 | 20.6 (18.3, 22.8)  | 26.9 (24.6, 29.2)    | 20.5 (18.2, 22.8) | 20.3 (18.0, 22.5)    | 0.001   |
| Total flavonoids, mg | S | 156/39 | 438 (298, 603)     | 1534 (1263, 1833)    | 517 (365, 696)    | 426 (289, 589)       | 0.001   |

<sup>1</sup>Values are means (for nontransformed variables mean is equal to median) or medians (for the log- or square root-transformed variables) with (500) bootstrap 95% CIs estimated with a joint mixed-effect linear model on BT and AT data, adjusted for subject variability. The treatment effect is statistically significant when  $P < 0.05$ . AT, after treatment; BT, before treatment; CB, control beverage; n, number of observations; N, number of subjects; T, the model was built using a transformed version of the variable (L = log10, S = square root) and the values are back-transformed in the original scale for the reader's convenience; WA, whole apple.



**FIGURE 3** Measured values of urinary phloretin glucuronide (A) and phloretin glucuronide sulfate (B) for each visit, for both the treatments. Dots are individual values. Vertical dotted lines intersecting the  $x$  axis represent each visit; sequence WA–CB is plotted to the left of the vertical dotted lines and sequence CB–WA to the right of them. The boxplots represent the mean values and the 95% bootstrap CIs as computed by the mixed linear models of treatment effect. Red: estimates corresponding to WA intake; blue: estimates corresponding to CB intake; gray: estimates corresponding to both baselines (initial and intermediate). CB, control beverage; WA, whole apple.

confirmed the link between TC and circulating BAs [lithocholic acid (LCA) and GUDCA] (**Supplemental Table 8**; coefficient values represented in **Supplemental Figure 5**). In particular, the model showed that WA intake produced, on average, a significant decrease in TC and, given that the TC concentration increased with age, the apple intake attenuated this effect. Moreover, the increase in LCA was associated with an increase in TC and, after WA consumption, TC decreased as GUDCA increased. Similar results were obtained with other hydrophilic BAs including glycocholic acid and taurocholic acid (data not shown).

## Discussion

In the present randomized controlled trial, of 40 men and women with moderately raised serum TC, consumption of 2 fresh WAs (Renetta Canada, 340 g) daily decreased serum TC, LDL cholesterol, and plasma ICAM-1 with no detrimental effect on HDL-cholesterol concentrations compared with a sugar-matched control. Few human intervention studies have focused specifically on fresh apples (11–16). Two early studies reported that 2–3 apples/d decreased TC in moderately hypercholesterolemic participants from baseline, although there was no

**TABLE 4** Fasted circulating plasma bile acid concentrations in mildly hypercholesterolemic participants BT (week 1) and AT (week 8) with WAs or CB<sup>1</sup>

| Parameters                     | L | n/N    | WAs                  |                      | CB                   |                      | P value |
|--------------------------------|---|--------|----------------------|----------------------|----------------------|----------------------|---------|
|                                |   |        | BT (95% CI)          | AT (95% CI)          | BT (95% CI)          | AT (95% CI)          |         |
| Cholic acid, nM                | ✓ | 160/40 | 55.4 (36.9, 83.2)    | 41.5 (27.6, 62.3)    | 38.7 (25.8, 58.2)    | 49.0 (32.6, 73.6)    | 0.346   |
| Chenodeoxycholic acid, nM      | ✓ | 160/40 | 44.4 (29.7, 66.3)    | 34.0 (22.8, 50.8)    | 35.2 (23.6, 52.5)    | 40.0 (26.8, 59.7)    | 0.344   |
| Deoxycholic acid, nM           | ✓ | 160/40 | 93.9 (67.4, 130.7)   | 98.5 (70.7, 137.1)   | 99.1 (71.2, 137.9)   | 105.4 (75.7, 146.7)  | 0.603   |
| Lithocholic acid, nM           | ✓ | 160/40 | 5.9 (5.0, 6.9)       | 6.1 (5.1, 7.1)       | 6.1 (5.2, 7.2)       | 6.4 (5.4, 7.5)       | 0.523   |
| Glycocholic acid, nM           | ✓ | 160/40 | 86.8 (64.3, 117.2)   | 62.9 (46.6, 84.8)    | 73.0 (54.1, 98.5)    | 62.5 (46.3, 84.3)    | 0.967   |
| Glycochenodeoxycholic acid, nM | ✓ | 160/40 | 226.1 (164.5, 310.9) | 191.1 (139.0, 262.7) | 184.0 (133.9, 253.0) | 188.6 (137.2, 259.2) | 0.928   |
| Glycodeoxycholic acid, nM      | ✓ | 160/40 | 79.8 (54.0, 117.8)   | 76.9 (52.1, 113.6)   | 70.2 (47.6, 103.7)   | 66.0 (44.7, 97.5)    | 0.261   |
| Glycolithocholic acid, nM      | ✓ | 140/38 | 6.2 (4.7, 8.2)       | 5.1 (3.9, 6.7)       | 5.0 (3.8, 6.6)       | 4.9 (3.7, 6.4)       | 0.815   |
| Glycoursodeoxycholic acid, nM  | ✓ | 156/40 | 23.2 (15.4, 35.0)    | 20.2 (13.4, 30.5)    | 21.0 (13.9, 31.6)    | 21.7 (14.4, 32.6)    | 0.656   |
| Taurocholic acid, nM           | ✓ | 160/40 | 12.3 (9.0, 16.8)     | 9.4 (6.9, 12.8)      | 10.4 (7.6, 14.2)     | 9.3 (6.8, 12.7)      | 0.959   |
| Taurochenodeoxycholic acid, nM | ✓ | 160/40 | 25.8 (18.6, 35.6)    | 22.1 (16.0, 30.5)    | 23.8 (17.2, 32.9)    | 22.6 (16.4, 31.3)    | 0.847   |
| Taurodeoxycholic acid, nM      | ✓ | 160/40 | 13.8 (9.8, 19.4)     | 12.5 (8.9, 17.5)     | 12.2 (8.7, 17.2)     | 11.2 (8.0, 15.7)     | 0.424   |
| Taurolithocholic acid, nM      | ✓ | 160/40 | 2.3 (2.1, 2.7)       | 2.3 (2.0, 2.6)       | 2.2 (2.0, 2.5)       | 2.1 (1.8, 2.3)       | 0.235   |

<sup>1</sup>Values are means (for nontransformed variables mean is equal to median) or medians (for the log-transformed variables) with (500) bootstrap 95% CIs estimated with a joint mixed-effect linear model on BT and AT data, adjusted for subject variability. AT, after treatment; BT, before treatment; CB, control beverage; L, the model was built using the log10-transformed variable and the values are back-transformed in the original scale for the reader's convenience; n, number of observations; N, number of subjects; WA, whole apple.

control (9, 10). Later studies in normocholesterolemic (11), hypercholesterolemic (12), and elderly subjects (13) showed no effects, or reported adverse effects on blood lipids (14). A borderline significant decrease in serum TC and LDL cholesterol was found after consumption of fresh WAs (550 g/d) compared with a control period (15). More recently, and in support of our results, TC and LDL cholesterol decreased after the daily consumption of 2 Annurca apples (200 g/d) (16). The potential ability of apples to reduce serum TC has been attributed mainly

to the fiber fraction, pectin in particular; however, polyphenols may also play an important role (5). Pure apple polyphenols at daily doses between 600 (23) and 1500 mg (22) decreased TC and LDL cholesterol. In contrast, a cloudy apple juice (803 mg polyphenols) had no effects on blood lipids (20). A total of 990 mg polyphenols (854 mg oligomeric PAs) per day was provided by 2 Renetta Canada apples in our study, which supports the hypothesis that polyphenols play a role in cholesterol lowering. Furthermore, 6 g pectin/d has been reported to lower blood

**TABLE 5** Vascular function, blood pressure, and PWA indexes in mildly hypercholesterolemic participants BT (week 1) and AT (week 8) with WAs or CB<sup>1</sup>

| Parameters              | L | n/N    | WAs                  |                      | CB                   |                      | P value |
|-------------------------|---|--------|----------------------|----------------------|----------------------|----------------------|---------|
|                         |   |        | BT (95% CI)          | AT (95% CI)          | BT (95% CI)          | AT (95% CI)          |         |
| LDI                     |   |        |                      |                      |                      |                      |         |
| Ach AUC, PU             | ✓ | 132/33 | 821 (742.5, 908.8)   | 853 (771.0, 943.6)   | 784 (708.6, 867.3)   | 760 (686.9, 840.7)   | 0.037   |
| SNP AUC, PU             | ✓ | 132/33 | 881 (790.5, 982.6)   | 911 (816.8, 1015.3)  | 853 (765.5, 951.5)   | 829 (743.1, 923.8)   | 0.135   |
| ABPM                    |   |        |                      |                      |                      |                      |         |
| PP, mm Hg               | ✓ | 160/40 | 42 (39.5, 44.6)      | 41 (38.5, 43.4)      | 42 (39.4, 44.4)      | 41 (38.9, 43.8)      | 0.699   |
| Brachial SBP, mm Hg     | ✓ | 160/40 | 121 (117.0, 125.0)   | 120 (116.4, 124.3)   | 121 (117.0, 125.0)   | 122 (117.7, 125.7)   | 0.167   |
| Brachial DBP, mm Hg     |   | 160/40 | 79 (76.1, 81.3)      | 79 (76.7, 82.0)      | 79 (76.5, 81.7)      | 80 (77.6, 82.9)      | 0.367   |
| PWA                     |   |        |                      |                      |                      |                      |         |
| Central SBP, mm Hg      | ✓ | 148/37 | 114 (109.6, 119.6)   | 113 (108.7, 118.6)   | 115 (110.5, 120.5)   | 115 (109.8, 119.8)   | 0.388   |
| Central DBP, mm Hg      |   | 148/37 | 76 (73.2, 78.7)      | 75 (72.2, 77.7)      | 76 (73.2, 78.7)      | 75 (72.6, 78.0)      | 0.700   |
| Central PP, mm Hg       | ✓ | 148/37 | 38 (35.1, 41.5)      | 38 (35.1, 41.6)      | 39 (36.1, 42.7)      | 39 (36.2, 42.8)      | 0.168   |
| Central MP, mm Hg       |   | 148/37 | 93 (89.7, 96.6)      | 92 (88.6, 95.5)      | 94 (90.1, 97.1)      | 93 (89.4, 96.3)      | 0.447   |
| Central AP_HR75, %      |   | 148/37 | 6.8 (4.9, 8.6)       | 7.0 (5.2, 8.9)       | 7.3 (5.4, 9.2)       | 7.2 (5.3, 9.1)       | 0.642   |
| Central AGPH, AS %      |   | 148/37 | 24.8 (20.9, 28.6)    | 25.4 (21.6, 29.3)    | 25.9 (22.0, 29.7)    | 26.5 (22.6, 30.3)    | 0.217   |
| Central AGPH_HR75, AS % |   | 148/37 | 16.4 (12.4, 20.3)    | 17.6 (13.6, 21.5)    | 18.0 (14.1, 21.9)    | 18.4 (14.5, 22.4)    | 0.306   |
| Heart rate, beats/min   | ✓ | 148/37 | 20 (16.2, 24.7)      | 22 (18.1, 27.6)      | 23 (18.3, 28.0)      | 23 (19.0, 29.1)      | 0.451   |
| Ejection duration, ms   |   | 146/37 | 345.6 (340.5, 350.6) | 345.6 (340.5, 350.6) | 348.3 (343.2, 353.4) | 346.5 (341.4, 351.6) | 0.592   |

<sup>1</sup>Values are means (for nontransformed variables mean is equal to median) or medians (for the log-transformed variables) with (500) bootstrap 95% CIs estimated with a joint mixed-effect linear model on BT and AT data, adjusted for subject variability. The treatment effect is statistically significant when  $P < 0.05$ . ABPM, ambulatory blood pressure monitor; Ach, acetylcholine; AGPH, augmentation pressure/pulse height; AGPH\_HR75, heart rate-corrected central augmentation pressure/pulse height; AP\_HR75, heart rate-corrected augmented pressure; AS, arterial stiffness; AT, after treatment; BT, before treatment; CB, control beverage; DBP, diastolic blood pressure; L, the model was built using the log10-transformed variable and the values are back-transformed in the original scale for the reader's convenience; LDI, laser Doppler iontophoresis; MP, mean pressure; ms, milliseconds; n, number of observations; N, number of subjects; PP, pulse pressure; PU, perfusion units; PWA, pulse wave analysis; SBP, systolic blood pressure; SNP, sodium nitroprusside; WA, whole apple.

cholesterol according to the European Food Safety Authority (40). The daily intake of soluble fiber in the apple group in our study was 3.7 g. Pectin is the major component of soluble fiber in apples, therefore this amount might not be sufficient alone to lower TC and a major role of polyphenols, especially PAs, and/or a synergetic effect between apple polyphenols and pectin/soluble fiber cannot be excluded (41).

The matrix of a whole food, such as apples, can affect the release of nutrients, e.g., sugars, fiber, and polyphenols, as well as their fate and function in the body compared with a simple sugar beverage (5). Of the aforementioned studies, two have explored the effects of a clear apple juice on lipid metabolism in comparison with WAs. Ravn-Haren et al. (15) have shown that a clear apple juice (total polyphenols, 108 mg/d; total pectin, 0.03 g/d; total sugars, 63 g/d) intake for 4 wk, in 23 healthy participants, increased LDL cholesterol compared with WAs (total polyphenols, 239 mg/d; total pectin, 2.87 g/d; total sugars, 51 g/d). On the contrary, Hyson et al. (11) found no differences in blood lipid concentrations of 25 healthy participants after the intake of fresh WAs or an unsupplemented apple juice for 6 wk; however, this study did not include nutrition information about the tested products. In our study, although there was a tendency toward a significant increase in insulin after the CB, changes in glucose and adiponectin were not seen. However, potential further adverse effects on lipid and glucose metabolism related to the food matrix and glycemic index of the CB compared with the WA cannot be excluded.

Endothelial function can serve as an indicator of cardiovascular health. We found small but significant improvements in endothelium-dependent microvascular vasodilation (42) after the apple intake. To date, only 2 studies to our knowledge have assessed the long-term effects of apple intake on vascular function, showing beneficial effects with high-flavonoid apples (19) but not with dried apples (17). Improvement in vascular function has been mainly shown with a high daily dose of flavonoid monomers such as epicatechin (48–180 mg) and/or quercetin (184–195 mg), either postprandially (19, 24) or after a 4-wk treatment (19). The lower daily intakes of flavanol monomers in our study suggest a potential role for the apple PAs. Longer-term studies with PA-rich foods/beverages such as chocolate (43) and tea (44) have also reported beneficial effects on vascular function. Although PA bioavailability is low compared with the readily absorbed flavanol monomers, PAs with a degree of polymerization >4 reach the colon almost intact where they are degraded into phenolic acids by gut bacteria and absorbed into the circulation, where they may contribute to systemic health-promoting properties (45).

In addition, endothelial dysfunction has been related to a proinflammatory phenotype. Endothelial adhesion molecules such as VCAM-1, ICAM-1, E-selectin, and P-selectin are proinflammatory proteins which play a critical role in the adhesion of leukocytes to endothelial cells during the early stages of atherosclerosis (46). Their concentration is low in normal conditions, but they can increase when the endothelium is activated by stimuli such as proinflammatory cytokines and reactive oxygen species (46). In the present study, apple intake reduced plasma ICAM-1. ICAM-1 and VCAM-1 have been shown to decrease after a vitamin C-rich apple juice (21), but not after the intake of a cloudy apple juice (20). Nevertheless, PAs

from other sources such as red wine (47) have also been shown to decrease ICAM-1 and VCAM-1 in healthy humans.

Circulating BAs have been linked with regulatory functions in lipid and energy metabolism via binding to nuclear receptors and acting as signaling molecules (48). In our study, no differences were observed in blood BA concentrations. However, BA metabolism is complex and regulated by many parameters, which are still unclear (49). Interestingly, our exploratory analysis indicated differential TC and BA profile responses in men and women after apple consumption, with circulating LCA and GUDCA predicting the TC response in women. LCA is an agonist of farnesoid X receptor (FXR), activation of which reduces BA synthesis in the liver via fibroblast growth factor-19 signaling (50, 51), whereas GUDCA is an antagonist for FXR, recently shown to improve metabolic endpoints in humans related to diabetes (52). Significant sex differences in TC response after apple intake have been reported previously, with women showing the largest response (15). Similar sex differences have been shown to be dependent on BAs and their receptors in animal studies (53, 54). However, our study was not powered to explore sex differences; therefore, this warrants closer examination in specifically designed human intervention studies powered for comparison between sexes.

Strengths of this study include its adequately powered sample size, duration, crossover design, the use of a sugar- and energy-matched control, and the determination of a wide range of outcomes relative to other similar studies focusing on fresh apples (5). Another strength is the confirmation of good compliance using appropriate recognized biomarkers of apple intake in urine (phloretin metabolites). However, there were some limitations. The volunteers' diet was not restricted in terms of polyphenol-rich products such as fruits, vegetables, and beverages as in other similar studies (15). However, polyphenols and fiber dietary intake increased only after the intake of WAs and compliance to the treatments was confirmed. Another potential limitation was that volunteers were not blinded to the interventions, a common limitation when using whole foods (fresh apples). However, participants were told there may be benefit from either intervention and researchers in the study were blinded during analysis. In addition, the use of an apple beverage as a control, although appropriate to explore the potential beneficial effects of the polyphenol and fiber components of a whole food such as apple, does not take into account the effects of the food matrix as already discussed. Finally, this study was not powered to explore sex differences; these are only exploratory and need confirmation in studies adequately powered to explore sex effects.

In conclusion, our findings show clear cause and effect between inclusion of 2 Renetta Canada apples into normal diets and improved CVD risk factors, by reducing TC, LDL cholesterol, and ICAM-1 and increasing microvascular vasodilation, in healthy subjects with mildly raised serum cholesterol concentrations. Dose- and structure-response studies are necessary to explore the potential mechanisms, which are likely to involve BA signaling and/or small phenolic acids derived from apple polyphenols, both linked to gut microbiota metabolism.

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## References

- Hu FB. Plant-based foods and prevention of cardiovascular disease: an overview. *Am J Clin Nutr* 2003;78:544S–51S.
- Food and Agriculture Organization of the United Nations Statistics Division (FAOSTAT) [Internet]. Available from: <http://www.fao.org/faostat/en/#data/QC> (accessed 5 June 2019).
- Hansen L, Dragsted LO, Olsen A, Christensen J, Tjønneland A, Schmidt EB, Overvad K. Fruit and vegetable intake and risk of acute coronary syndrome. *Br J Nutr* 2010;104:248–55.
- Hodgson JM, Prince RL, Woodman RJ, Bondonno CP, Ivey KL, Bondonno N, Rimm EB, Ward NC, Croft KD, Lewis JR. Apple intake is inversely associated with all-cause and disease-specific mortality in elderly women. *Br J Nutr* 2016;115:860–7.
- Koutsos A, Tuohy KM, Lovegrove JA. Apples and cardiovascular health—is the gut microbiota a core consideration? *Nutrients* 2015;7:3959–98.
- Rasmussen SE, Frederiksen H, Struntze Krogholm K, Poulsen L. Dietary proanthocyanidins: occurrence, dietary intake, bioavailability, and protection against cardiovascular disease. *Mol Nutr Food Res* 2005;49:159–74.
- Aron PM, Kennedy JA. Flavan-3-ols: nature, occurrence and biological activity. *Mol Nutr Food Res* 2008;52:79–104.
- Hollands WJ, Tapp H, Defernez M, Perez Moral N, Winterbone MS, Philo M, Lucey AJ, Kiely ME, Kroon PA. Lack of acute or chronic effects of epicatechin-rich and procyanidin-rich apple extracts on blood pressure and cardiometabolic biomarkers in adults with moderately elevated blood pressure: a randomized, placebo-controlled crossover trial. *Am J Clin Nutr* 2018;108:1006–14.
- Gormley TR, Kevany J, Egan JP, McFarlane R. Effect of apples on serum cholesterol levels in humans. *Irish J Food Sci Tech* 1977;1:117–28.
- Sablé-Amplis R, Sicart R, Agid R. Further studies on the cholesterol-lowering effect of apple in human. *Biochemical mechanisms involved*. *Nutr Res* 1983;3:325–8.
- Hyson D, Studebaker-Hallman D, Davis PA, Gershwin ME. Apple juice consumption reduces plasma low-density lipoprotein oxidation in healthy men and women. *J Med Food* 2000;3:159–66.
- Conceição de Oliveira M, Sichieri R, Sanchez Moura A. Weight loss associated with a daily intake of three apples or three pears among overweight women. *Nutrition* 2003;19:253–6.
- Avci A, Atli T, Ergüder IB, Varli M, Devrim E, Turgay SAM, Durak I. Effects of apple consumption on plasma and erythrocyte antioxidant parameters in elderly subjects. *Exp Aging Res* 2007;33:429–37.
- Vafa MR, Haghighatjoo E, Shidfar F, Afshari S, Gohari MR, Ziaee A. Effects of apple consumption on lipid profile of hyperlipidemic and overweight men. *Int J Prev Med* 2011;2:94–100.
- Ravn-Haren G, Dragsted LO, Buch-Andersen T, Jensen EN, Jensen RI, Németh-Balogh M, Paulovicsová B, Bergström A, Wilcks A, Licht TR, et al. Intake of whole apples or clear apple juice has contrasting effects on plasma lipids in healthy volunteers. *Eur J Nutr* 2013;52:1875–89.
- Tenore GC, Caruso D, Buonomo G, D'Urso E, D'Avino M, Campiglia P, Marinelli L, Novellino E. Annurca (*Malus pumila* Miller cv. Annurca) apple as a functional food for the contribution to a healthy balance of plasma cholesterol levels: results of a randomized clinical trial. *J Sci Food Agric* 2017;97(7):2107–15.
- Auclair S, Chironi G, Milenkovic D, Hollman PCH, Renard CM, Mégnien J-L, Garipey J, Paul J-L, Simon A, Scalbert A. The regular consumption of a polyphenol-rich apple does not influence endothelial function: a randomised double-blind trial in hypercholesterolemic adults. *Eur J Clin Nutr* 2010;64:1158–65.
- Chai SC, Hooshmand S, Saadat RL, Payton ME, Brummel-Smith K, Arjmandi BH. Daily apple versus dried plum: impact on cardiovascular disease risk factors in postmenopausal women. *J Acad Nutr Diet* 2012;112:1158–68.
- Bondonno NP, Bondonno CP, Blekkenhorst LC, Considine MJ, Maghazal G, Stocker R, Woodman RJ, Ward NC, Hodgson JM, Croft KD. Flavonoid-rich apple improves endothelial function in individuals at risk for cardiovascular disease: a randomized controlled clinical trial. *Mol Nutr Food Res* 2018;62:1700674.
- Barth SW, Koch TCL, Watzl B, Dietrich H, Will F, Bub A. Moderate effects of apple juice consumption on obesity-related markers in obese men: impact of diet–gene interaction on body fat content. *Eur J Nutr* 2012;51:841–50.
- Soriano-Maldonado A, Hidalgo M, Arteaga P, de Pascual-Teresa S, Nova E. Effects of regular consumption of vitamin C-rich or polyphenol-rich apple juice on cardiometabolic markers in healthy adults: a randomized crossover trial. *Eur J Nutr* 2014;53:1645–57.
- Nagasako-Akazome Y, Kanda T, Ikeda M, Shimasaki H. Serum cholesterol-lowering effect of apple polyphenols in healthy subjects. *J Oleo Sci* 2005;54:143–51.
- Nagasako-Akazome Y, Kanda T, Ohtake Y, Shimasaki H, Kobayashi T. Apple polyphenols influence cholesterol metabolism in healthy subjects with relatively high body mass index. *J Oleo Sci* 2007;56:417–28.
- Bondonno CP, Yang X, Croft KD, Considine MJ, Ward NC, Rich L, Puddey IB, Swinney E, Mubarak A, Hodgson JM. Flavonoid-rich apples and nitrate-rich spinach augment nitric oxide status and improve endothelial function in healthy men and women: a randomized controlled trial. *Free Radic Biol Med* 2012;52:95–102.
- Koutsos A, Lima M, Conterno L, Gasperotti M, Bianchi M, Fava F, Vrhovsek U, Lovegrove JA, Tuohy KM. Effects of commercial apple varieties on human gut microbiota composition and metabolic output using an in vitro colonic model. *Nutrients* 2017;9(6):533.
- Taves DR. Minimization: a new method of assigning patients to treatment and control groups. *Clin Pharmacol Ther* 1974;15(5):443–53.
- Vrhovsek U, Masuero D, Gasperotti M, Franceschi P, Caputi L, Viola R, Mattivi F. A versatile targeted metabolomics method for the rapid quantification of multiple classes of phenolics in fruits and beverages. *J Agric Food Chem* 2012;60:8831–40.
- Gris EF, Mattivi F, Ferreira EA, Vrhovsek U, Pedrosa RC, Bordignon-Luiz MT. Proanthocyanidin profile and antioxidant capacity of Brazilian *Vitis vinifera* red wines. *Food Chem* 2011;126:213–20.
- Food Standards Agency, McCance and Widdowson's The Composition of Foods, Sixth summary edition. Cambridge: Royal Society of Chemistry; 2002.
- Chong MF, George TW, Alimbetov D, Jin Y, Weech M, Macready AL, Spencer JPE, Kennedy OB, Minihane A-M, Gordon MH, et al. Impact of the quantity and flavonoid content of fruits and vegetables on markers of intake in adults with an increased risk of cardiovascular disease: the FLAVURS trial. *Eur J Nutr* 2013;52:361–78.
- George TW, Paterson E, Waroonphan S, Gordon MH, Lovegrove JA. Effects of chronic consumption of fruit and vegetable puree-based drinks on vasodilation, plasma oxidative stability and antioxidant status. *J Hum Nutr Diet* 2012;25:477–87.
- Townsend RR, Black HR, Chirinos JA, Feig PU, Ferdinand KC, Germain M, Rosendorff C, Steigerwalt SP, Stepanek JA. Clinical use of pulse wave analysis: proceedings from a symposium sponsored by North American Artery. *J Clin Hypertens* 2015;17:503–13.
- Han J, Liu Y, Wang R, Yang J, Ling V, Borchers CH. Metabolic profiling of bile acids in human and mouse blood by LC-MS/MS in combination with phospholipid-depletion solid-phase extraction. *Anal Chem* 2015;87:1127–36.
- Saenger T, Hübner F, Humpf H-U. Short-term biomarkers of apple consumption. *Mol Nutr Food Res* 2017;61:1600629.
- Kenward MG, Roger JH. The use of baseline covariates in crossover studies. *Biostatistics* 2010;11:1–17.
- Li T, Yu T, Hawkins BS, Dickersin K. Design, analysis, and reporting of crossover trials for inclusion in a meta-analysis. *PLoS One* 2015;10:e0133023.
- Lawson J. Design and analysis of experiments with R. Boca Raton, FL: CRC Press; 2014.

38. Bates D, Mächler M, Bolker B, Walker S. Fitting linear mixed-effects models using lme4. *J Stat Softw* 2015;67:1.
39. Kuznetsova A, Brockhoff PB, Christensen RHB. lmerTest package: tests in linear mixed effects models. *J Stat Softw* 2017;82:13.
40. Brouns F, Theuvsen E, Adam A, Bell M, Berger A, Mensink RP. Cholesterol-lowering properties of different pectin types in mildly hyper-cholesterolemic men and women. *Eur J Clin Nutr* 2012;66: 591–9.
41. Aprikian O, Duclos V, Guyot S, Besson C, Manach C, Bernalier A, Morand C, Révész C, Demigné C. Apple pectin and a polyphenol-rich apple concentrate are more effective together than separately on cecal fermentations and plasma lipids in rats. *J Nutr* 2003;133: 1860–5.
42. Macready AL, George TW, Chong MF, Alimbetov DS, Jin Y, Vidal A, Spencer JPE, Kennedy OB, Tuohy KM, Minihane A-M, et al. Flavonoid-rich fruit and vegetables improve microvascular reactivity and inflammatory status in men at risk of cardiovascular disease—FLAVURS: a randomized controlled trial. *Am J Clin Nutr* 2014;99: 479–89.
43. Hooper L, Kay C, Abdelhamid A, Kroon PA, Cohn JS, Rimm EB, Cassidy A. Effects of chocolate, cocoa, and flavan-3-ols on cardiovascular health: a systematic review and meta-analysis of randomized trials. *Am J Clin Nutr* 2012;95:740–51.
44. Ras RT, Zock PL, Draijer R. Tea consumption enhances endothelial-dependent vasodilation; a meta-analysis. *PLoS One* 2011;6:e16974.
45. Ou K, Gu L. Absorption and metabolism of proanthocyanidins. *J Funct Foods* 2014;7:43–53.
46. Joseph SV, Edirisinghe I, Burton-Freeman BM. Fruit polyphenols: a review of anti-inflammatory effects in humans. *Crit Rev Food Sci Nutr* 2016;56:419–44.
47. Sacanella E, Vázquez-Agell M, Mena MP, Antúnez E, Fernández-Solá J, Nicolás JM, Lamuela-Raventós RM, Ros E, Estruch R. Down-regulation of adhesion molecules and other inflammatory biomarkers after moderate wine consumption in healthy women: a randomized trial. *Am J Clin Nutr* 2007;86:1463–9.
48. de Aguiar Vallim TQ, Tarling EJ, Edwards PA. Pleiotropic roles of bile acids in metabolism. *Cell Metab* 2013;17:657–69.
49. Gunness P, Williams BA, Gerrits WJJ, Bird AR, Kravchuk O, Gidley MJ. Circulating triglycerides and bile acids are reduced by a soluble wheat arabinoxylan via modulation of bile concentration and lipid digestion rates in a pig model. *Mol Nutr Food Res* 2016;60: 642–51.
50. Khan AA, Chow ECY, Porte RJ, Pang KS, Groothuis GMM. The role of lithocholic acid in the regulation of bile acid detoxication, synthesis, and transport proteins in rat and human intestine and liver slices. *Toxicol In Vitro* 2011;25:80–90.
51. Kliewer SA, Mangelsdorf DJ. Bile acids as hormones: the FXR-FGF15/19 pathway. *Dig Dis* 2015;33:327–31.
52. Sun L, Xie C, Wang G, Wu Y, Wu Q, Wang X, Liu J, Deng Y, Xia J, Chen B, et al. Gut microbiota and intestinal FXR mediate the clinical benefits of metformin. *Nat Med* 2018;24:1919–29.
53. Sheng L, Jena PK, Liu H-X, Kalanetra KM, Gonzalez FJ, French SW, Krishnan VV, Mills DA, Wan Y-JY. Gender differences in bile acids and microbiota in relationship with gender dissimilarity in steatosis induced by diet and FXR inactivation. *Sci Rep* 2017;7: 1748.
54. Vassileva G, Hu W, Hoos L, Tetzloff G, Yang S, Liu L, Kang L, Davis HR, Hedrick JA, Lan H, et al. Gender-dependent effect of *Gpbar1* genetic deletion on the metabolic profiles of diet-induced obese mice. *J Endocrinol* 2010;205:225–32.