EFFECTS OF REGULAR CONSUMPTION OF VITAMIN C-RICH OR POLYPHENOL-RICH APPLE JUICE ON CARDIOMETABOLIC MARKERS IN HEALTHY ADULTS: A RANDOMIZED CROSS-OVER TRIAL

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Abstract

Purpose: The aim of the present study was to investigate the effects of the consumption of two cloudy apple juices with different polyphenol and vitamin C content on antioxidant status, cardiometabolic and inflammation markers in healthy young adults. *Methods:* Twenty subjects, aged 21–29 years, completed a randomized cross-over study. At each 4-wk. intervention period the volunteers randomly consumed 2 glasses (2 x 250mL/day) of either a vitamin C-rich apple juice (VCR) (60 mg/L vitamin C and 510 mg catechin equivalent/L) or a polyphenol rich (PR) juice (22 mg/L vitamin C and 993 mg catechin equivalent/L). Blood and urine samples were collected throughout the study and markers of antioxidant status, glucose metabolism, lipid profile and inflammation, were measured. *Results:* The comparison of the post-intervention minus pre-intervention change revealed differential results in HOMA index, total cholesterol, ICAM-1 and VCAM-1 ($P<0.05$) across juices. During the VCR period, plasma antioxidant activity (FRAP) increased ($P=0.031$) while ICAM-1 and total cholesterol showed a trend to decrease ($P=0.060$ and $P=0.094$, respectively). During the PR period, plasma insulin and HOMA increased, and total glutathione decreased ($P<0.05$). *Conclusions:* A joint consumption of apple juice natural antioxidants such as vitamin C and polyphenols might provide mild favorable effects on cardiometabolic markers, as compared to apple polyphenols alone.

Key words: apple juice, vitamin C, polyphenols, antioxidant activity, cardiometabolic markers.

Introduction

Several epidemiological studies have shown that fruit and vegetable-rich diets may decrease the risk of chronic diseases, such as cardiovascular diseases [1,2], metabolic syndrome [3-5], diabetes [6] and different types of cancer [7-9]. The beneficial effects of fruits and vegetables have been attributed to various plant polyphenols [10], as well as to vitamins, like vitamin E and C [11-13], carotenoids, and fiber. Specifically flavonoids, one of the polyphenol subclasses, have been shown to present a large list of beneficial effects on human health [14]. These properties have been attributed to their chemopreventive, immunoregulatory and antioxidant activity. In population studies, the higher quartiles of Vitamin C plasma levels or citrus fruit intake have also been associated with a lower risk of CVD compared to the lowest quartiles [13] and this effect might be related to its antioxidant activity, its potential ability to improve endothelial function [15], or to antiatherogenic mechanisms, including inhibition of LDL oxidation [16], or decreasing the expression of intercellular adhesion molecules [17].

Among plant foods, apples have attracted a good deal of interest regarding food contribution to health preservation [4]. A 28% reduction in diabetes risk for women consuming more than one apple/day has been reported in a prospective cross-sectional study [18]. Moreover, moderate evidence has been found suggesting that apple juice might induce a decrease of body fat percentage in obese individuals [19]. Scientific studies have shown that apple and apple juice have a wide range of biological activities, mainly attributed to their high polyphenol content [20]. In this sense, antiproliferative and apoptosis-inducing activity [21], as well as inhibition of pro-inflammatory gene expression [22] have been demonstrated in vitro for apple extracts and dimeric procyanidins. Vrhovsek et al. [23] reported the average content of total polyphenols in eight of the most widely cultivated apples varieties in western Europe to be between 66.2 and 211.9 mg/100 g of fresh weight depending on the variety, with flavanols (catechin and proanthocyanidins) as the major class of apple polyphenols (71–90%), followed by hydroxycinnamates (4–18%), flavonols (1–11%), dihydrochalcones (2–6%) and, in red apples, anthocyanins (1–3%). The Phenol-Explorer Database gives a mean total polyphenol value of 130.9 mg/100g resulting from 8 different scientific references [24]. Only one reference was available for apple juice and the total polyphenol amount was 33.9 mg/100mL. Furthermore, flavan-3-ols and procyanidins have been reported to have the highest correlation with antioxidant activity measured by ferric reducing antioxidant power (FRAP) and β-CLAMS activity [25]. Although apples are not a significant source of vitamin C [26], most commercially available apple juices are fortified to contain one or more Reference Dietary Intake

(RDI) daily values of vitamin C (1 RDI = 60 mg/day) [27]. However, the final content of these nutrients vary with the apple variety and the post-harvest storage conditions [28].

Few studies have been carried out so far investigating the effects of apple juice consumption on antioxidant activity and cardiovascular biomarkers in diverse populations [19,29]. Since polyphenols and vitamin C have been reported to increase plasma antioxidant activity and to protect against CVD, we hypothesized that regular apple juice consumption might provide benefits on antioxidant status and cardiometabolic markers in healthy subjects. Therefore, the aim of the present study was to investigate the effects of the consumption of two cloudy apple juices, with different polyphenol and vitamin C content, on antioxidant status, and cardiometabolic and inflammation markers in healthy young adults.

Experimental methods

Chemicals

6-Hydroxy-2,5,7,8,-tetramethylchroman-2-carboxylic acid 97%, 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$), 2,4,6-tris (2-pyridyl)-1,3,5-triazine (TPTZ), iron (III) chloride hexahydrate, acetate buffer saline, L(+) ascorbic acid, *p*-dimethylaminocinnamaldehyde (DMACA), metaphosphoric acid, diethylenetriaminepentaacetic acid (DTPA), (+)catechin, (-)-epicatechin, quercetin dihydrate, quercetin-3-ß-glucoside, phloretin, apigenin and the phenolic acids: chlorogenic, gallic, syringic, protocatechuic, vanillic, sinapic, homogentisic, 4-hydroxybenzoic, phloroglucinol, 3-(20,50-dimethoxybenzoil) propionic (DMB propionic), *p*-coumaric, ferulic and caffeic acids were purchased from Sigma-Aldrich Quimica S.A. (Madrid, Spain). Methanol, acetonitrile and formic acid (HPLC grade) were acquired from Fisher (Madrid, Spain). Ethanol absolute 99%, sulphuric acid 96%, hydrochloric acid 37%, phosphoric acid and dimethyl sulfoxide (DMSO) were obtained from Panreac (Barcelona, Spain).

Apple juices

Two cloudy apple juices, which differed in the vitamin C and polyphenol content as well as in the apple cultivation technique, were selected as intervention foods. Both were 100% pure freshly squeezed juices, without concentrate addition and no added sugar. The vitamin C-rich (VCR) juice was obtained from conventionally grown apples and had a vitamin C and polyphenol content of 60 mg/L and 510 mg epicatechin equivalents/L, respectively, while the polyphenol-rich (PR) juice was

elaborated from organically grown apples and had a vitamin C and polyphenol content of 22 mg/L and 993 mg epicatechin/L, respectively. The apple varieties in both cases were Cox Orange, Jonagold, Elstar, Gala, Braeburn, Delicious and Idared, cultivated in Germany. Both juices had a similar carbohydrate (and sugars) content per 100g of product (10.8 and 11.0 g/100g, respectively), and a similar fiber content (0.1 g/100g and 0.2g/100g in VCR juice and PR juice, respectively). The phenolic compounds in the analyzed juices are shown in table 1. Throughout the study, the juice bottles were stored under refrigeration. Vitamin C content was checked twice in both juices, at the beginning and in the end of the study, and no differences were found.

Study subjects

Twenty healthy volunteers (twelve women and eight men) aged between 21 and 29 years were included in the study. Exclusion criteria were: BMI >27.5 kg/m^2, illness diagnosed, chronic or current medical treatment, being smoker or vegetarian, dietary supplements or antibiotic treatment in the previous month, menopausal women, endurance sport training or more than 3 hours/week of vigorous physical activity and alcohol consumption superior to one drink/day for woman and two drinks/day for men. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and the Spanish law 14/2007 on Biomedical Research. All procedures involving human subjects were approved by the Ethics Committee of the Puerta de Hierro-Majadahonda University Hospital and the Bioethics committee of CSIC. Written informed consent was obtained from all subjects. No drop outs were registered in this study.

Study design

A randomized cross-over design was carried out during 11-weeks. The participants followed a one-week low-polyphenol diet prior to the beginning of the intervention. During this period, participants were instructed to avoid polyphenol-rich foods (chocolate, tea, apple, apple juice, red wine, red fruits, plumes, apricots, green pulses and onions). The study participants were randomly allocated into 2 groups (n=10 per group) consuming 500 ml/day of either a PR apple juice or a VCR apple juice during 4 weeks. After a washout period of 2 weeks (with low-polyphenol diet on the second week) the volunteers consumed the other apple juice for another 4 weeks. During both periods of apple juice consumption, participants were instructed to have 2 servings (250 ml each) at two different moments of the day, preferably in the morning and afternoon and to always shake the bottle before serving. They were also asked to follow their habitual diet. For random allocation

assignment into the two sequence groups a stratified randomization procedure was employed, using sex, age and BMI as potential covariates.

Fasting blood samples were collected at 8.00-9:00 a.m. on the day after the run-in period (basal 1) and then after 4, 6 (basal 2) and 10 weeks. Urine was collected over a 24 h period on the same time points as blood sampling and included the first morning void of the sampling day. Urine was collected in one or two 2L plastic containers with 2g boric acid as preservative. The volume of urine excreted was measured and aliquots were stored at -80º C.

Preparation of blood samples

Peripheral blood was withdrawn by antecubital vein puncture from 12-hour fasted subjects into three types of tubes: 1) ethylenediamine tetraacetic acid (EDTA) coated tube, 2) heparin containing tube and 3) gel-containing tube for serum fraction separation **(**BD Vacutainer® SST™**)**. Plasma or serum was generated by tube centrifugation at 1,300 *g* during 15 min at 4 °C. Three aliquots of 100 µL plasma from the heparin tube were acidified with 100 µL of cold 6% (w:v) metaphosphoric acid containing 1 mmol/L of the metal ion chelator diethylenetriaminepentaacetic acid (DTPA) and thoroughly mixed. The tubes were immediately stored at -80 °C until analysis of vitamin C by HPLC. Heparin plasma was also used for polyphenol analysis. Briefly, two aliquots (1 mL) of plasma were acidified with 30 µL of 50% aqueous formic acid and 100 µL of 10 mmol/L ascorbic acid and samples were frozen at -80 °C until analysis. Remaining heparin plasma was aliquoted (300 µL) and stored at -80 °C for determination of antioxidant activity. Likewise, plasma from EDTA tube was subdivided into aliquots that were stored at -80 °C before analysis of adhesion molecules (ICAM-1, VCAM-1, E-selectin), PAI-1, inflammatory cytokines (IL-6, IL-8 and MCP-1) and IL-10. The buffy coat interface from the EDTA tube was collected and mixed with cold lysis buffer (150 mM NH_4Cl, 10 mM $NaHCO_3$, 10 mM EDTA, pH 7.2) for red cell elimination. After 15 min incubation on ice, peripheral blood mononuclear cells (PBMC) were isolated by centrifugation (250 *g* for 15 min at 4 °C). Supernatant was discarded and cells were washed twice in PBS. Two aliquots (500 µL) were stored at -80 °C for glutathione analysis. Serum was used for glucose, insulin, cholesterol (total, HDL- and LDL-cholesterol), triglycerides, iron, ferritin and high sensitive C-reactive protein (hsCRP) analysis.

Samples preparation for polyphenol analysis

Plasma samples were analysed for polyphenols before and after ß-Glucuronidase/sulfatase treatment. Portions of 0.5 mL of plasma samples containing 15 µL of internal standard apigenin 1mM were extracted twice with 0.5 mL of pure acetonitrile containing 1% formic acid and mixed for 30 min to ensure complete protein precipitation. After centrifugation (5,000 *g*, for 5 min), supernatants were pooled and solvents were evaporated using a SpeedVac Concentrator at room temperature. The residue was redissolved in 150 µL of formic acid 1%/ acetonitrile (90:10), filtered through a 0.45 µM filter and analysed by HPLC–DAD and HPLC-MS to determinate polyphenols metabolites. Standard calibration curves were prepared in the same conditions and injected in the HPLC/HPLC-MS. Portions of 0.5 mL serum were treated with ß-glucoronidase (50 µL, 250 U) and sulfatase (50 µL, 100 U) at 37º C for 3 h. The reaction was stopped by the addition of 0.5 mL of acetonitrile containing 1% formic acid. Samples were then treated as described above.

Urine samples were centrifuged (5000 *g* for 5 min) and a volume of 5.4 mL was taken and filtered through a 0.45 µM PVDF filter (polyvinylidene difluoride). Then, samples were acidified with 0.6 mL of 85% (v/v) phosphoric acid to pH 1.5 and centrifuged (5000 g for 5 min). The supernatants were subjected to solid-phase extraction (OASIS HLB, 3cc, 60 mg adsorbent; Waters, Milford), preconditioned, and activated sequentially with 2 mL methanol and 2 mL 0.01% (v/v) HCl. The cartridges were loaded with 5 mL of the urine plus 10 µL of internal standard apigenin 1 mM , and each washed with 2 mL 0.01% (v/v) HCl, dried, and then eluted with 2 mL HCl (0.01% v/v) in methanol into glass tubes. The solvent was then evaporated using SpeedVac Concentrator at room temperature, for 2.30 h. The residue was quantified by redissolving it in 500 µL of 70% (v/v) methanol in water containing 1% (v/v) acetic acid and then analysing samples by HPLC–DAD and HPLC-MS.

Polyphenols analysis by HPLC

Polyphenols in both, VCR and PR apple juices were quantified by HPLC-DAD with an Agilent 1200 series liquid chromatograph with a quaternary pump and a photodiode array detector. Briefly, after centrifugation (5000 *g* for 5 min) supernatant filtered (pore size 0.45 µM) juice was injected into the HPLC system. HPLC chromatographic separation was carried out as in Hidalgo *et al*. [30].

Plasma and urine levels of polyphenols metabolites were determined after filtration of both samples through a 0.45 µm filter and analysed by HPLC-DAD in the same way as apple juices. The peaks were identified by comparing their retention times and UV spectra with those of authentic

standards, and with previously reported metabolites [31]. Phenolic compounds in juices and urine samples were quantitatively analysed by comparing the areas of identified phenolic compounds with calibration curves prepared with the corresponding standard phenolic compounds, wherever possible. Good linearity for the assay (r^2>0.995) was found over the investigated calibration range of 10-1000 ng/mL for all the compounds. The detection and quantification limits were in every case below 5 ng/mL.

Polyphenols analysis by HPLC-MS

In order to confirm the identity of the recorded phenolic compounds and to detect new compounds that could not be detected with HPLC-DAD, additional analysis were performed by HPLC-MS. Mass spectrometry was performed using an Agilent 1100 series liquid chromatograph equipped with an API source and employing an ESI (electrospray ionization) interface as in Hidalgo *et al*. [30].

Determination of total flavanol content

The total flavanol content in organic and conventional juices, plasma or urine was estimated using the *p*-dimethylaminocinnamaldehyde (DMACA) colorimetric method which provides a high sensitivity and specificity. After centrifugation (5000 *g* for 5 min) a volume of 30 µL of each supernatant sample was placed in a 96-well microplate and 150 µL of DMACA solution (DMACA in 1.5 M H_2SO_4 (2:1, w:v) in methanol) was added. The mixture was slightly shaken and allowed to react at room temperature and in the dark for 30 min. The absorbance at 640 nm was then read against a blank prepared similarly without DMACA. The concentration of total flavanols was estimated from a calibration curve with epicatechin (1.5-100 µg/mL). Results are expressed as µg epicatechin equivalent/mL sample.

Vitamin C analysis by HPLC

For ascorbic acid determination, plasma samples were thawed at room temperature and analysed by HPLC as described previously [32].

Ferric reducing antioxidant power (FRAP) assay

The total antioxidant status in plasma and juices was determined with FRAP as previously described [33]. A potential antioxidant reduces the ferric ion (Fe3+) to the ferrous ion (Fe2+) at low pH; the latter forms a blue complex (Fe2+/TPTZ), measured at 593 nm. The assay was carried out by placing 10 µL of plasma sample or juice in a 96-well microplate and then adding 290 µL of FRAP reagent (0.3 M acetate buffer (pH 3.6), 10 mM TPTZ in 40 mM HCl and 20 mM $FeCl_3$ in a proportion 10:1:1 (v/v/v)). After 15 min of incubation at 37º C and shaking, absorbance was read at 593 nm. All samples were run in triplicate. Results were compared with a standard curve prepared daily with different concentrations of Trolox and were expressed as µM trolox equivalents (TE).

$DPPH^{\bullet}$ radical scavenging capacity assay

Radical-scavenging capacity was measured using the $DPPH^{\bullet}$ method reported by Brand-Williams [34] with some modifications [35]. All samples were run in triplicate. Results were compared with a standard curve prepared daily with different concentrations of Trolox and were expressed as µM trolox equivalents (TE).

Determination of Glutathione

Total glutathione (GSH) in buffy coat samples was determined with a commercial detection kit (BioVision, CA, USA). Sample analysis was performed according to the protocol specifications. Following reduction of oxidized glutathione (GSSG), total GSH reacts with *o*-phthalaldehyde which forms a fluorescent isoindole derivative which was determined after 40 min of incubation at room temperature in the darkness. Costar black 96-well plates were used to run the assay (Fisher Scientific, Madrid, Spain) and a fluorescence plate reader set at λ_{ex}= 360 nm and λ_{em} = 460 nm (Synergy Mx, Biotek) was employed. Protein in the same sample preparation was measured by the method of Bradford [36]. Total GSH is expressed in nmol GSH per mg of protein.

Analysis of cardiometabolic markers and biochemical parameters

Glucose, insulin, cholesterol (total, HDL, LDL), triglycerides, iron, ferritin and high sensitive C-reactive protein (CRP) were analysed in serum by standard techniques in an accredited medical diagnostic laboratory (Unilabs, Madrid, Spain).

Determination of inflammatory markers

Plasma from EDTA-tube was used for quantification of Interleukin (IL)-8, IL-6, IL-10, MCP-1, tPAI-1, E-selectin, VCAM-1 and ICAM-1. These determinations were performed by multiple analyte assays using two Millipore kits and xMAP technology from Luminex® Corporation. All analyses were performed in different aliquots to avoid freeze- thawing repetitions. The sensitivity of these measurements was as follows: IL-8, 0.50 pg/ml; IL-6, 0.67 pg/ml; IL-10, 0.30 pg/ml; MCP-1, 0.59 pg/ml; tPAI-1, 1.0 pg/mL; E-selectin, 79.0 pg/mL; VCAM-1, 16.0 pg/mL and ICAM-1, 9.0 pg/mL. All measurements from the same subject corresponding to the different time points were always analysed in the same batch to avoid inter-assay variability within the subject.

Dietary intake

A 3-day food record was completed by each participant during each consumption period with specification of every food consumed, ingredients and use of household measurements (spoons, cups, serving size, etc.) for quantities estimation. One of these 3 days was a weekend day. Energy and nutrient intakes were calculated with the CESNID (Centre d'Ensenyament Superior de Nutrició i Dietètica) program of the University of Barcelona.

Organoleptic properties

Organoleptic properties, such as flavour, colour, smell, texture, sweetness, acidity, sensation of satiety after drinking the juice, and global score, were assessed by means of a visual analogue scale (VAS), where the score (ranging from 0 to 10) was measured in cm.

Statistical Analysis

The sample size was calculated taking FRAP as the main outcome, assuming a standard deviation of the differences between pre-intervention and post-intervention equal to or lower than 80 and considering a 10% mean change over the values in the population as relevant (Mean value in previous studies: 511 μM TE). For a statistical power of 80% and an alpha error of 0.05, the minimum number of measures to perform is 40. This is 20 subjects measured in pre-intervention and post-intervention.

A mixed model analysis of variance was performed in order to examine the effect of the fixed factors, intervention product (comparison of the outcomes after the consumption of the PR juice vs. VCR juice) and sequence (first PR juice or first VCR juice), and the interaction between them, on

each study outcome. Basal values were also included in the model as a covariate. In addition, in order to check any possible effect of factors not controlled for, the baseline values before each juice consumption were also compared by the mixed model analysis. Normality of the variable's distribution was checked (Kolmogorov-Smirnov test). The repeated measures comparison of means (pre-intervention *vs*. post-intervention) was performed with the Student paired-t-test for normally distributed variables and the Wilcoxon test for non-normally distributed variables. The changes (post-intervention minus pre-intervention) under PR and VCR consumption periods were calculated and the differences assessed also with the Student paired t-test. In those variables where a product*sequence interaction was found in mixed model only data from the first arm of the study were analysed. In this case, the differences between the mean changes (post-intervention minus pre-intervention) were assessed by the Student t-test for independent samples. Statistical analyses were performed with the Statistical Package for the Social Sciences (IBM® SPSS® Statistics, version 19.0). Statistical significance was set at α=0.05 and all tests were two-sided.

Results

As both groups were matched for demographic characteristics, no differences appeared for age or BMI between the participants in each sequence group (age: 23.7 years [SD: 2.3] in the group starting with PR juice and 23.3 [2.0] in the group starting with VCR juice; BMI: 21.8 [2.1] and 22.4 [2.0] in both groups, respectively; both, $P>0.05$). Moreover, no basal differences were observed in blood biochemical markers between sequence groups, except for ferritin which was significantly lower in the group starting with VCR juice (24 ng/mL [SD: 30]) than in the group starting with PR juice (65 [SD: 68]) ($P=0.024$). The product*sequence interaction was only significant for ferritin and iron ($P<0.05$). Thus, ferritin and iron were assessed considering only the first arm of the study. Consequently, the 10 subjects consuming PR in the first period were evaluated as an independent group from the 10 subjects consuming VCR in the first period. No differences were found between basal values of any biomarker measured prior to VCR consumption compared to values prior to PR consumption.

No significant differences in dietary intake were observed regarding energy, macronutrients, and fiber between the VCR consumption period and the PR period (Energy: 1992 [SD: 488] *vs*. 1993 [577] Kcal/day; Total carbohydrates: 243 [48] *vs*. 263 [72] g/day; Fiber: 15.8 [6.0] *vs*. 15.3 [5.2] g/day; VCR *vs*. PR, respectively; all $P>0.705$). Despite the higher vitamin C content in the VCR

juice, total vitamin C intakes were not significantly different between periods (94 [SD: 54] *vs.* 90 [127] mg/day in VCR and PR juices, respectively; *P*=0.905).

The analysis of the organoleptic characteristics of both juices revealed that the PR juice obtained a higher flavour score than the VCR juice (7.7 [SD: 2.0] *vs.* 5.8 [SD: 1.3]; dif.: -1.9; 95% CI: -3.0 to -0.9; *P*=0.001), and was also considered to have a better flavour than other apple juices tasted in the past. In addition, the total score of the PR juice was significantly higher than the VCR juice (8.0 [SD: 1.8] *vs.* 5.9 [SD: 1.4]; dif.: -2.1; 95% CI: -3.1 to -1.1; *P*<0.001).

Polyphenols in juices, plasma and urine

Total flavanols estimated by DMACA method and expressed as equivalents of epicatechin (µg/mL) showed a double amount of flavanols in PR juice (56.42 ± 4.21 mg epicatechin equivalents (ECE)/L) than in VCR juice (23.94 ± 3.64 mg ECE/L), showing correlation to the flavanol content measured by HPLC (993 and 510 mg ECE/L respectively). This difference in the flavanols levels between juices is mainly due to procyanidin B2 and epicatechin. However, after drinking PR or VCR juice, no substantial differences in the flavanol amount in plasma and urine were found neither comparing with baseline values or between juices (table 2). One possible explanation for the lack of differences is the fact that epicatechin reaction with DMACA could be more effective than that of epicatechin metabolites formed after juice ingestion.

The analysis of plasma samples revealed that, over a 12-h period, only traces of two polyphenol metabolites could be detected and their identities confirmed by HPLC-ESI-MS. Consequently, we analysed and quantified the polyphenol metabolites excreted in urine samples by congruent retention times and UV-vis spectra in HPLC and by their molecular ions in the negative ionization mode by HPLC-ESI-MS. A summary of the polyphenol metabolites detected in plasma and urine is shown in table 3. Uptake of PR or VCR juice resulted in average maximum increases in urine of free and conjugated caffeic acid and phloretin, conjugated epicatechin and a small amount of methyl-quercetin-glucuronide ($[M-H]^-$ =491) when comparing with baseline values and showing in all increments a coefficient of inter-individual variation higher than 56%. The comparison among the mean increases in urine metabolites between the subjects consuming the PR and VCR juices showed a significantly higher increase of 4´-O-methyl-epicatechin ($[M-H]^-$ = 303]; *P*=0.009) and phloretin-2'-glucuronide ($[M-H]^-$ = 449; *P*=0.014) and a small trend to an increase of phloretin ($[M-H]^-$ = 273; *P*= 0.058) after the VCR juice consumption. On the other hand, caffeic acid 3-O-sulfate

([M-H]$^-$ = 259; *P*=0.05) and caffeic acid 4-O-sulfate ([M-H]$^-$ = 259; *P*=0.044) exhibited a significantly higher increase after the PR juice consumption. Additionally, a non-significant increase of caffeic acid ([M-H]$^-$ = 273; *P*=0.098) and phloretin-glucuronide-sulfate ([M-H]$^-$ = 529; *P*=0.062) was observed after the PR juice consumption with respect to the VCR juice consumption.

Antioxidant, cardiometabolic and inflammation markers

Table 2 summarises the results regarding antioxidant status. A significant decrease of total glutathione was observed after the PR juice consumption (mean difference: -96.13 nmoles/mg of protein; 95% CI: -148.91 to -43.34; *P*=0.001) and a significant increase for FRAP after the VCR juice consumption (mean difference: 45.59; 95% CI: 4.72 to 86.46; *P*=0.031). Although there were not significant changes in plasma vitamin C after consuming neither the PR nor the VCR juices (both *P*>0.05), the direction of the differences was inverse, with the PR juice decreasing the vitamin C plasma levels and the VCR juice increasing the levels. The between-juices comparison did not reach statistical significance (*P*=0.066), but the post-intervention vitamin C values were significantly higher after juice VCR than after juice PR (*P*<0.001). No differences between juices, either as intervention-induced changes or post-intervention values, were found regarding DPPH measurement of antioxidant activity.

The results regarding lipid, glucose and iron metabolic profile are presented in Table 4. A significant increase of insulin (mean difference: 1.49 µUI/ml; 95% CI 0.12 to 2.85; *P*=0.034) and HOMA (mean difference: 0.37; 95% CI 0.03 to 0.71; *P*=0.034) was observed after the PR juice consumption. On the other hand, the post- minus pre-intervention change between juices was only significant for total cholesterol (*P*=0.037) and for HOMA (*P*=0.037). In relation with cholesterol, a non-significant trend for a decrease after the VCR juice consumption was observed (*P*= 0.094) and the final values after VCR were almost significantly lower than values after PR (*P*=0.055). The analysis of ferritin and iron (with data from the first arm of the study only) did not show any significant change after the consumption of neither of the apple juices.

The results regarding inflammation related parameters showed no significant effects of PR or VCR consumption when comparing with the data before intervention (Table 5). However, the mean change (post-intervention minus pre-intervention) for ICAM-1 and VCAM-1 was significantly different when consuming PR *vs*. VCR juice (*P*=0.033 and *P*=0.021, respectively). The differences

for ICAM might be attributable to an almost significant post-intervention decrease observed after VCR juice consumption (mean difference: -12.03; 95% CI: -24.6 to 0.6; *P*=0.060).

Discussion

The present study assessed the effects of two cloudy apple juices, differing in the vitamin C and polyphenol content, on antioxidant activity and cardiometabolic markers in healthy young adults. Our results suggest that a higher content of vitamin C relative to the polyphenol content in the apple juice provides mild favorable effects on cardiometabolic markers, such as plasma antioxidant capacity, adhesion molecules, total cholesterol and insulin levels as compared to polyphenols alone.

The juices used in this study were cloudy and thus showed a polyphenol content in the highest range of the values reported for commercial apple juice. Nonetheless, both juices were commercial high quality apple juices.

A rapid uptake and metabolism of polyphenols was observed, since by 12 h the plasma flavonoid levels were below the limit of detection, in agreement with other authors [37]. Food analyses have shown that the contribution of polyphenols to the antioxidant potential of apple juice is higher than the contribution of vitamin C [38-40], and several association studies have also shown a higher correlation between total polyphenols rather than vitamin C with the antioxidant activity of apple [25] and apple juice [41,42]. However, in vivo studies measuring plasma antioxidant activity after the ingestion of apple juice have shown controversial findings. We observed no plasma antioxidant activity changes after the PR juice consumption which is in agreement with one study in elderly subjects that also found no effect of apple juice consumption on plasma antioxidant status after 4 weeks of intervention [29], and also with animal studies showing a similar lack of effect [43,44]. By contrast, Yuan et al. [45] found an increase of plasma antioxidant activity after 2 weeks of apple and grape juice consumption (600 ml/day; 101.7 ±16.3 mg of polyphenols /100ml). Interestingly, one study examining the acute effects of ingesting one litre apple juice showed an increase in the plasma antioxidant activity with no increase in the plasma polyphenol concentration and it also showed that the increase was associated with the fructose-induced rise in uric acid [46]. The PR juice consumption in our study significantly reduced total GSH levels in PBMCs. Although the blood GSH levels have been previously shown to increase [47] or be unchanged [48] in two berry juice intervention studies in healthy subjects, the pro-oxidative capacity of plant polyphenols is strongly debated. Dual antioxidant and pro-oxidant activities for a variety of plant-derived

polyphenols has been noted and intracellular glutathione depletion has been suggested as a research strategy to ascertain oxidative damage linked to polyphenol biological activities [49]. Our results suggest that the PR juice decreases total GSH, likely to levels that do not compromise the cell redox status, although it would deserve further study.

We observed a significant increase of plasma antioxidant activity after the VCR juice consumption, although no significant differences were seen in plasma vitamin C during this period. However, a trend for inverse effects of the VCR and PR juices on plasma vitamin C levels was observed. Total cholesterol was also differentially affected during both periods, and a 4% decrease after VCR period was found. Therefore, our results suggested that VCR apple juice consumption might contribute to the decrease of total cholesterol, in agreement with Bhatt et al. [50], who found a reduction of 9.5% in total cholesterol after 500 mg/d of vitamin C supplementation for 3 months in diabetic subjects. On the other hand, an increase in total cholesterol in non-smoking healthy subjects has been reported after a 26-day intervention consisting of one apple, one pear and one orange juice consumption a day, while the same intervention decreased total cholesterol in smokers [51].

Regarding adhesion molecules, which have previously been shown to respond to dietary interventions with antioxidant supplementation, a significantly different effect of both apple juices was found in the current study, with ICAM-1 showing a ~10% reduction compared to baseline values after VCR juice consumption. Similar results were obtained by Rayment el al. [17] in healthy subjects with low plasma vitamin C receiving a vitamin C supplement during 6 weeks. They demonstrated that dietary vitamin C can modulate ICAM-1 monocyte gene expression and suggested that a similar effect might be exerted on the endothelial expression of ICAM-1, being this one possible mechanism mediating the beneficial effects of antioxidants on health. On the other hand, the PR juice did not significantly modify adhesion molecule levels in the volunteers, which is in agreement with the study by Barth et al. [19], who did not find any effect of a cloudy apple juice in endothelial markers of inflammation (sICAM-1 and sVCAM-1) over a period of 4 weeks. In this sense, the beneficial effects of apples or apple polyphenols on endothelial function are not consistent in human RCT. A higher flow-mediated vasodilation was observed in healthy subjects after the ingestion of an apple based meal [52], but no effect was reported in this measurement of endothelial function after an intervention with high-polyphenol lyophilised apple during 4 weeks in hypercholesterolemic adults [53]. Perhaps the differences in the study populations and protocols can

account for the different findings. Our results support the role of vitamin C contained in apple juice, for moderately improving the antioxidant status and endothelial function in healthy young adults. It might be that polyphenols and vitamin C work in a synergistic way and that the coexistence of both types of antioxidants is necessary to show a beneficial health effect.

The different effect observed on HOMA index with the two different apple juices is difficult to explain. The significant increase in insulin concentration and HOMA observed with the PR juice suggests that apple polyphenols might stimulate pancreatic beta cells. Indeed, in vivo studies in animal models and *in vitro* cell culture models have shown that different polyphenolic compounds enhance insulin secretion [54]; moreover, the evidences gathered support a beneficial effect of polyphenols and foods or beverages rich in polyphenols on glucose metabolism by attenuating postprandial glycemic responses and fasting hyperglycemia, improving acute insulin secretion and insulin sensitivity [54] and also on the amelioration of pathological damage of pancreatic beta-cells in diabetic mice models [55]. Since our subjects are non-diabetic and have no glucose tolerance problems, the biological relevance of the significant increment in basal insulin levels and HOMA is difficult to establish and complementary postprandial measurements might have clarified this issue.

This study had several limitations. Due to the limited sample size, the study might have been underpowered to detect significant differences in some variables. The number of subjects, however, is adequate regarding our "a priori" estimations in sample size calculation and is in agreement with other similar cross-over studies published in the literature [56,57]. The multiple comparisons performed in the present study might have increased the probability of finding hazardous outcomes, and therefore, our results should be regarded as preliminary results. Seasonal or time-frame related changes might have influenced our results. However, this is unlikely since this was a short time study (10 weeks), conducted along the spring season of a single year and the fact that the subjects were randomized between the two intervention periods minimizes the seasonal or time-frame related changes to be expected. In support of this assertion, no differences were observed between the first (post run-in) and second (post wash-out) baseline measurements in any studied outcome. Nonetheless, further research in subjects with an incipient pathological condition (i.e. prediabetic patients) is necessary to clarify the beneficial role of the antioxidant compounds in apple juice, in the context of a balanced diet, on the metabolic markers of disease

In conclusion, a joint consumption of apple juice natural antioxidants such as vitamin C and polyphenols might provide mild favorable effects on cardiometabolic markers, as compared to

apple polyphenols alone. However, further research is necessary to determine the biological relevance of the present results in disease conditions.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Table 1. Content of the main phenolic compounds quantified in the juices.

Compounds	**VCR juice** (mg/l)	**PR juice** (mg/l)
Chlorogenic acid*	83.8 ± 4.5	79.7 ± 3.0
4-caffeoylquinic	4.6 ± 0.5	5.9 ± 0.3
4-p-Cumaroylquinic acid	7.2 ± 0.1	11.9 ± 2.0
Cinnamic acid	2.5 ± 0.6	5.6 ± 0.2
Epicatechin	15.1 ± 3.0	33.5 ± 1.4
Procyanidin B2	17.3 ± 4.0	41.5 ± 2.3
Quercetin-3-O-glucoside	n.d.	1.7 ± 0.1
Quercetin-3-O-rhamnoside	1.0 ± 0.0	1.3 ± 0.1
Phloretin	9.7± 4.5	9.9 ± 0.4
Phloretin-2'-O-xyloglucoside	17.6 ± 2.4	34.3 ± 1.0
Phloretin-2'-O-glucoside (Phoridzin)	13.3 ± 1.3	30.1 ± 2.3

*As the sum of 3- and 5-caffeoylquinic acid. n.d.: non detected.

Table 2. Effect of the two intervention juices on antioxidant status.

	Juice PR							Juice VCR							P^b	P^c
	Pre-intervention		Post-intervention		Difference	95% CI	P^a	Pre-intervention		Post-intervention		Difference	95% CI	P^a		
	Mean	SD	Mean	SD				Mean	SD	Mean	SD					
Vitamin C (µmol/L)	46.72	(18.19)	40.27	(22.97)	-6.45	-17.10 , 4.20	0.220	51.84	(20.16)	63.05	(35.65)	11.21	-7.34 , 29.76	0.221	0.066	*<0.001*
FRAP (µM TE)	478.70	(102.63)	499.00	(120.53)	20.30	-5.38 , 45.96	0.114	479.36	(105.60)	524.95	(134.00)	45.59	4.72 , 86.46	*0.031*	0.203	0.248
DPPH (µM TE)	405.95	(85.90)	396.15	(72.04)	-9.80	-43.27 , 23.67	0.547	397.25	(66.45)	413.60	(102.92)	16.35	-26.16 , 58.86	0.431	0.388	0.371
DMACA (µg/mL ECE)	9.63	(5.00)	8.97	(3.82)	-0.66	-3.27 , 1.97	0.607	9.48	(3.48)	8.55	(5.46)	-0.93	-3.31 , 1.45	0.424	0.816	0.689
Total Glutathione (nmoles/mg of protein)	529.07	(176.76)	432.95	(135.89)	-96.13	-148.91 , -43.34	*0.001*	548.70	(168.51)	497.38	(182.58)	-51.32	-161.03 , 58.4	0.340	0.354	0.246

Data are expressed as mean and standard deviation (SD). [*]All measures refer to plasma except DMACA, which refers to urine. PR, polyphenol rich apple juice; VCR, vitamin C rich apple juice. [a] Paired t-test assessing the post-intervention minus pre-intervention difference. [b] Paired t-test assessing the between juices comparison of the post-intervention minus pre-intervention difference. [c] Effect of the factor "Product" in the mixed model analysis. FRAP, ferric reducing antioxidant power; DPPH, 2,2-diphenyl-1-picrylhydrazyl radical; DMACA, *p*-dimethylaminocinnamaldehyde; TE, trolox equivalents; ECE, epicatechin equivalents.

Table 3. Identification of polyphenol metabolites in plasma and urine (24h) collected after the ingestion of PR or VCR juice and quantification of those found in urine expressed as nmol/day.

Peak	t_R (min)	Compounds	$[M-H]^-$ (m/z)	Major fragment ions (m/z)	Urine	
					VCR	PR
1	8.6	3´-O-methyl-epicatechin*	303	137	n.d.	n.d.
2	11.7	4´-O-methyl-epicatechin	303	137	1.07±0.61	1.22±0.57
3	13.5	caffeic acid 4-O-sulfate	259	179, 135	0.18±0.08±	0.24±0.17
4	19.2	caffeic acid*	179		0.21±0.11	0.41±0.31
5	24.7	caffeic acid 3-O-sulfate	259	179, 135	0.12±0.10	0.13±0.10
6	34.5	hydroxycinnamic acid	163		0.09±0.06	0.16±0.08
7	36.9	3´-O-methyl-epicatechin-O-sulfate	383	303, 285, 259, 137	0.77±0.47	1.02±0.61
8	37.6	phloretin-2'-O-glucuronide	449	273	0.15±0.09	0.14±0.07
9	38.9	phloretin-O-glucuronide-O-sulfate	529	353, 449, 273	0.24±0.13	0.23±0.15
10	39.2	phloretin	273		0.05±0.02	0.10±0.04
11	39.8	methyl-quercetin-glucuronide	491	315	0.02±0.01	0.02±0.01

*Metabolites present in plasma in trace amounts. n.d.: non detected.

Table 4. Effect of the two intervention juices on lipid profile, glucose metabolism and iron status.

	Juice PR							Juice VCR							P[b]	P[c]
	Pre-intervention		Post-intervention		Difference	95% CI	P[a]	Pre-intervention		Post-intervention		Difference	95% CI	P[a]		
	Mean	SD	Mean	SD				Mean	SD	Mean	SD					
TG (mg/dl)	77.10	(28.50)	93.10	(43.86)	16.00	-1.07 , 33.07	0.065	73.60	(29.62)	80.65	(34.55)	7.05	-2.14 , 16.24	0.125	0.331	0.208
TC (mg/dl)	171.15	(30.83)	176.00	(31.29)	4.85	-4.01 , 13.71	0.266	180.90	(32.44)	174.05	(30.28)	-6.85	-14.97 , 1.27	0.094	*0.037*	0.055
HDL-c (mg/dl)	65.55	(13.89)	66.35	(12.34)	0.80	-2.36 , 3.96	0.602	70.20	(16.58)	67.70	(12.56)	-2.50	-6.22 , 1.22	0.175	0.120	0.268
LDL-c (mg/dl)	90.25	(29.17)	91.05	(32.80)	0.80	-5.10 , 6.70	0.780	95.95	(33.97)	90.10	(28.97)	-5.85	-13.66 , 1.96	0.133	0.096	0.172
TC/HDL-c	2.72	(0.73)	2.75	(0.73)	0.03	-0.08 , 0.14	0.566	2.71	(0.82)	2.67	(0.63)	-0.05	-0.16 , 0.06	0.376	0.202	0.131
LDL-c/HDL-c	1.47	(0.61)	1.46	(0.64)	-0.01	-0.12 , 0.10	0.857	1.49	(0.72)	1.39	(0.53)	-0.10	-0.22 , 0.03	0.120	0.114	0.260
Glucose (mg/dl)	87.80	(5.79)	89.30	(5.80)	1.50	-0.68 , 3.68	0.165	87.65	(6.43)	86.75	(8.32)	-0.90	-4.92 , 3.12	0.645	0.145	0.191
Insulin (µUI/ml)	7.20	(3.72)	8.69	(4.92)	1.49	0.12 , 2.85	*0.034*	7.53	(4.86)	7.30	(3.79)	-0.24	-1.96 , 1.49	0.779	0.071	0.088
HOMA index	1.57	(0.85)	1.94	(1.19)	0.37	0.03 , 0.71	*0.034*	1.65	(1.13)	1.58	(0.87)	-0.08	-0.47 , 0.32	0.691	*0.037*	0.063
Fe (µg/dl)*	80.40	(53.68)	111.80	(37.20)	31.40	-20.91 , 83.71	0.208	76.70	(46.38)	80.30	(45.85)	3.60	-20.39 , 27.59	0.742	0.289	-
Ferritin (ng/ml)*	65.43	(67.74)	69.34	(72.41)	3.91	-8.00 , 15.82	0.477	24.06	(30.22)	19.33	(21.72)	-4.73	-22.35 , 12.89	0.559	0.370	-

Data are expressed as mean and standard deviation (SD). [a] Paired t-test on the post-intervention minus pre-intervention difference. [b] Paired t-test assessing the between juices comparison of the post-intervention minus pre-intervention difference. [c] Effect of the factor "Product" in the mixed model analysis. * Analysis of data from the first arm of the study (n=10). PR, polyphenol rich apple juice; VCR, vitamin C rich apple juice; TG, triglycerides; TC, total cholesterol; HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol; HOMA, homeostasis model assessment.

Table 5. Effect of the two intervention juices on inflammation related parameters.

	Juice PR							Juice VCR							P^b	P^c
	Pre-intervention		Post-intervention		Difference	95% CI	P^a	Pre-intervention		Post-intervention		Difference	95% CI	P^a		
	Mean	SD	Mean	SD				Mean	SD	Mean	SD					
CRP (mg/dl)	0.11	(0.22)	0.09	(0.17)	-0.02	-0.16 , 0.12	0.756	0,06	(0.15)	0,06	(0.08)	-0.01	-0.07 , 0.05	0.775	0.860	1.000
C3 (mg/dl)	107.14	(16.93)	108.25	(18.42)	1.11	-5.86 , 8.09	0.742	105.72	(14.08)	107.50	(16.60)	0.73	-4.09 , 5.54	0.755	0.861	0.819
C4 (mg/dl)	24.63	(7.92)	24.12	(6.99)	-0.51	-3.17 , 2.15	0.693	22.43	(5.85)	23.59	(6.23)	0.92	-0.43 , 2.27	0.170	0.326	1.000
ICAM-1 (ng/ml)	119.61	(28.96)	127.82	(23.01)	8.20	-7.83 , 24.24	0.298	131.97	(24.27)	119.94	(19.08)	-12.03	-24.63 , 0.58	0.060	0.*033*	0.119
VCAM-1 (ng/ml)	741.34	(238.35)	794.34	(201.67)	53.01	-32.00 , 138.01	0.207	816.12	(229.47)	753.92	(139.55)	-62.20	-149.5 , 25.07	0.152	0.*021*	0.078
e-SEL (ng/ml)	27.59	(8.98)	29.15	(8.62)	1.56	-1.08 , 4.20	0.231	30.41	(11.18)	30.31	(11.08)	-0.10	-2.74 , 2.53	0.936	0.361	0.412
PAI-1 (ng/ml)	27.41	(23.47)	25.87	(17.12)	-1.54	-6.50 , 3.41	0.523	28.87	(18.86)	28.32	(17.75)	-0.54	-5.05 , 3.96	0.803	0.743	0.558
IL-8 (pg/mL)	10.88	(13.05)	8.35	(7.43)	-2.53	-6.49 , 1.43	0.196	8.98	(9.17)	8.72	(8.08)	-0.26	-1.68 , 1.16	0.702	0.294	0.216
MCP-1 (pg/mL)	138.58	(51.27)	148.15	(55.68)	9.58	-6.81 , 25.97	0.236	140.71	(50.49)	154.68	(48.93)	13.97	-0.85 , 28.79	0.063	0.662	0.483
IL-6 (pg/mL)	5.34	(3.42)	6.60	(6.39)	1.25	-1.24 , 3.74	0.306	4.83	(2.86)	5.31	(2.95)	0.48	-1.21 , 2.17	0.561	0.544	0.396
IL-10 (pg/mL)	8.36	(9.09)	11.53	(16.04)	3.17	-4.85 , 11.19	0.418	7.04	(6.24)	7.25	(5.62)	0.21	-1.39 , 1.82	0.783	0.430	0.364

Data are expressed as mean and standard deviation (SD). PR, polyphenol rich apple juice; VCR, vitamin C rich apple juice. [a] Paired t-test on the post-intervention minus pre-intervention difference. [b] Paired t-test assessing the between juices comparison of the post-intervention minus pre-intervention difference. [c] Effect of the factor "Product" in the mixed model analysis. CRP, C-reactive protein; C3, complement factor 3; C4, complement factor 4; ICAM-1, intercellular adhesion molecule; VCAM-1, vascular cell adhesion molecule; e-Sel, e-selectin; PAI-1, Plasminogen activator inhibitor-1; IL, interleukin; MCP-1, Monocyte chemoattractant protein-1