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ORIGINAL CONTRIBUTION

Four-week ingestion of blood orange juice results in measurable anthocyanin urinary levels but does not affect cellular markers related to cardiovascular risk: a randomized cross-over study in healthy volunteers

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Abstract
Purpose Blood orange juice (OJ) is an important source of anthocyanins (ACN). The latter molecules are endowed with antioxidant activity and might thus modulate different cell function. Our aim was to investigate ACN absorption following a 1-month daily supplementation of blood OJ and their potential effects on cell markers of platelet and leukocyte activation and interaction.
Methods Eighteen healthy subjects (10 men and 8 women) were supplemented for 4 weeks with 1 L/day of either blood OJ or blond OJ (that contains no ACN), following a cross-over design. Blood samples were obtained from fasting participants both at baseline and after each week of treatment to measure plasma ACN concentration. At the same time-intervals, 24-h urinary excretion of these molecules was also measured. At the beginning and the end of each 4-week intervention period, platelet and leukocyte markers and mixed cell conjugates were assessed both in basal condition and upon in vitro collagen/ADP activation.
Results After 1 week supplementation with blood OJ, 24-h urinary excretion of ACN reached average levels of 11.47 ± 5.63 nmol that significantly differed from baseline and remained substantially unchanged until the end of treatment. No plasma accumulation of ACN following blood OJ supplementation was observed. Cellular markers were not significantly affected by either OJ after 4-week supplementation.
Conclusions Following supplementation of healthy volunteers with 1 L/day of blood OJ for 4 weeks, the ACN plasma levels reached were insufficient to significantly modify cell markers of platelet and leukocyte activation and interaction.

Keywords Blood orange juice · Anthocyanins · Bioavailability · Platelets · Leukocytes

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Introduction

Anthocyanins (ACN) are the largest group of water-soluble pigments in the plant kingdom and are responsible for the blue, red and purple colors of many fruits, vegetables, grains, flowers and leaves. In human, the intake of these compounds was estimated within the range of 3–200 mg/day [1–3]. ACN were reported to modify biomarkers of heart diseases or cancer in vitro. They inhibit release of reactive oxygen species from activated human granulocytes [4], suppress free-radical-mediated lipid peroxidation and cell death in cultured aortic endothelial cells [5, 6] and reduce P-selectin expression in resting platelets [7]. However, these results cannot be applied to the in vivo condition, where nutritional exposure to ACN is limited to relatively low concentrations, also depending on bioavailability and metabolism. Nonetheless, some cohort studies reported a reduced risk of coronary heart disease, cardiovascular disease (CVD) and total mortality with increased ACN consumption [8].

Increasing evidence that platelets and their interaction with inflammatory cells are essentially involved in the

initiation and progression of atherosclerosis has been provided in recent years. Mixed platelet–leukocyte conjugate formation could take place within the circulation in relation to some clinical inflammatory conditions or acute thrombotic events, as well as in subjects with cardiovascular risk factors. Mixed cell conjugate levels in the circulation and their formation upon in vitro activation could, therefore, be useful markers of atherosclerosis and cardiovascular risk [9]. Ex vivo platelet aggregation seems to be inhibited in human by a single-dose administration of both red wine and purple grape juice that are rich in ACN [10, 11], but whether these ACN-rich juices were able to affect platelet–leukocytes activation and interaction in vivo remains to be investigated.

Blood oranges (*Citrus sinensis* variety: Moro, Tarocco and Sanguinello), one of the most common orange cultivar in the Italian region of Sicily, are also a rich source of ACN, in particular of cyanidin-3-glucoside (C3G), delphinidin-3-glucoside (D3G) and cyanidin-3-(6″malonyl)-glucoside (CMG) [12–14]. Consumption of blood oranges in Southern Europe is quantitatively relevant and is also increasing in other European regions as well as in the US, mainly due to the availability of pasteurized juice. The high antioxidant capacity of these orange juices (OJ) was demonstrated to be strictly correlated to their ACN rather than vitamin C concentration [15, 16]. A kinetic study, conducted after human oral ingestion of blood OJ in a single dose, revealed that ACN were rapidly but poorly absorbed as intact molecules, subsequently undergoing a rapid metabolism [17]. Because of the potential role of ACN in promoting health and the prevention of CVD, it is important to understand whether a regular dietary intake of blood OJ could increase human ACN absorption to levels consistent with significant changes of cellular biomarkers of CVD, in particular on platelet and leukocyte activation and interaction.

Starting from these considerations, the aim of our study was to investigate the ACN bioavailability and the potential effect on biomarkers correlated with platelet and leukocyte reactivity and platelet–leukocyte interaction after a prolonged intake of blood OJ in healthy volunteers, according to a modality closer to a nutritional one, rather than a "nutraceutic" supplementation. In order to exclude the effects deriving from other antioxidant components of OJ, the study followed a cross-over design in which blood OJ was administered in comparison with a blond OJ that contains no ACN but has the same antioxidants and flavonoids composition. A preliminary study of ACN bioavailability was performed after administration of a single, acute dose of blood OJ.

Materials and methods

Study subjects

Recruitment of subjects was divided in two phases: in the first one, eight healthy volunteers (4 male and 4 female) aged 23–44 years, with BMI between 18 and 27 kg/m^2, were selected for a preliminary kinetic study of ACN following acute administration of blood OJ in a single dose; in the second one, eighteen healthy subjects (10 men and 8 women) aged 25–47 years, with BMI between 19 and 26 kg/m^2, were recruited for the cross-over supplementation with OJ. All subjects were recruited among the personnel of Catholic University and their relatives. All of them denied taking medication or vitamin and mineral supplements. Exclusion criteria were: pregnancy/lactation, alcohol abuse, gastrointestinal disorders, CVD, hypertension (Systolic Blood Pressure >140 mmHg and/or Diastolic Blood Pressure >90 mmHg), type 2 diabetes (blood glucose >690 mmol/L), dyslipidemia (cholesterol >6.2 mmol/L and/or triglycerides >2.3 mmol/L), metabolic and endocrine diseases, vegetarian or other restrictive dietary requirements. Each subject underwent a clinical screening (body weight and height, blood pressure/pulse, lipids, liver and renal function, medical anamnesis and dietary questionnaire) before the study. All the participants to the cross-over were recommended to maintain their regular lifestyles and usual extent of physical activities throughout the study.

The study protocol was approved by the Ethical Committee of the Catholic University, and all volunteers gave their informed written consent before starting the experiment.

Orange juices

Blood OJ was obtained from Moro, Tarocco and Sanguinello varieties, while blond OJ derived from Valencia, Navel and Belladonna varieties, properly selected by Istituto Sperimentale per l'Agrumicoltura (Acireale, Italy) and supplied by Ortogel (Caltagirone, Italy). For the first kinetic study, blood OJ was previously twofold concentrated by using an evaporator and stored at −20 °C until use. Pasteurized blood and blond OJ stored at 4 °C were used for cross-over supplementation. Blood OJ and blond OJ were both characterized by the same nutritional facts and phenolic composition (reported in Tables 1 and 2, respectively) with the exception of ACN that were absent in blond OJ. The analysis of both phenolic and ACN content of OJ was performed by using previously published method [15, 18].

Table 1 Nutritional facts of orange juice

	Blond OJ	Blood OJ
Energy (kJ)	180	183
Carbohydrate (g)	12.5	10.5
Sugars (g)	8.5	9.5
Proteins (g)	0.9	0.8
Fats (g)	0.1	0.2
Fiber (g)	1.2	0.5
Vitamin C (mg)	59.1	57.3
Sodium (mg)	1.0	2.0

The values are referred to 100 mL of product

Table 2 Phenolic composition of orange juice

	Blond OJ	Blood OJ
Total flavonoids (mg/L)	225.02 ± 8.51	217.38 ± 8.70
Total hydroxycinnamic acids (mg/L)	94.71 ± 3.00	100.66 ± 2.70
Total anthocyanins (mg/L)	ND	53.09 ± 5.31
Delphinidin-3-glucoside (mg/L)	ND	3.96 ± 0.20
Cyanidin-3-glucoside (mg/L)	ND	25.79 ± 1.17
Cyanidin-3-(6-malonylglucoside) (mg/L)	ND	17.88 ± 0.95

The values are expressed as means ± SD ($n = 10$)

Study design

Study design was made up of two phases: in the first one, the absorption and excretion of ACN was examined after a single, acute ingestion of blood OJ; the second one was a controlled, randomized, cross-over dietary intervention trial in which 2 treatment groups were supplemented for 4 weeks with blood OJ and blond OJ.

Participants to the first phase were instructed to consume a polyphenol-free diet for 2 days before the study and during the experimental day, excluding fruits, fruit juices, vegetables, coffee, tea, wine and related products. Milk, pasta, rice, chicken, tuna and white bread were allowed as food. This controlled diet was reported in a food diary. On the morning of the experimental day, after overnight fast, the subjects consumed a single dose of 500 mL of twofold concentrated blood OJ within 5 min at the study site. The volunteers were allowed to eat for the first time 4 h after the ingestion of the juice. Blood was collected into 15 mL evacuated tubes containing 3.8% (1:10, v:v) sodium citrate from a catheter inserted into the brachial vein at different time points: before and 0.3–0.7–1–2–4–6–8 h after juice drinking. Plasma for ACN analysis was obtained after blood centrifugation at 1,880*g* for 20 min at 4 °C, acidified (pH ≈ 2) with HCl, divided in aliquots and stored at −80 °C until analysis. Baseline (T_0) urine was collected into plastic bottles before OJ administration; 24-h urine samples were collected after OJ in five different fractions (0–2, 2–4, 4–6, 6–12, 12–24 h). These fractions were acidified with HCl, and after volume measurement, aliquots were stored at −80 °C until analysis.

Subjects enrolled in cross-over study, after 1 week run-in period, were randomly assigned to two periods of 4 weeks dietary treatment with daily consumption of (A) one liter of blood OJ, (B) one liter of blond OJ, respectively. The subjects were instructed to divide the juice in three doses: at breakfast, at lunchtime and in the evening. There was a 4-week washout period between each treatment. During the study, volunteers were invited to follow their usual dietary habits, avoiding further orange consumption; they were asked to complete a simple form throughout the study in order to report the daily consumption of food and drinks. During each phase of cross-over study, volunteers made five visits to the research laboratories at baseline (T_0) and at the end of each week of supplementation (T_1, T_2, T_3 and T_4), during which they received the amount of juice to consume during the week and delivered a 24-h urine collection for ACN measurements. Further, at the same time points, the subjects, after a blood pressure/pulse evaluation, underwent a fasted blood collection in EDTA-K tubes for a complete blood-count and in sodium citrate tubes for plasma ACN assessment. Plasma for ACN analysis was obtained and stored as described previously. Twenty-four hour urine sample for ACN analysis was collected into plastic bottles containing ascorbic acid. After volume measurements, aliquots were acidified with HCl and stored at −80 °C until analysis. For the evaluation of platelet function and of cellular activation markers, blood samples were drawn from fasting subjects in tubes containing sodium citrate at baseline (T_0) and at the end of each period of supplementation (T_4). The technicians performing the laboratory tests were blind.

Extraction and quantification of ACN in plasma and urine

ACN molecules (D3G, C3G and CMG) were extracted from plasma and urine samples and analyzed by liquid chromatography tandem mass spectrometry (LC–MS/MS) using a previously published method [19]. Briefly, a solid phase extraction using C_{18} cartridges (Sep-Pak, 500 mg, 6 mL, Waters) was performed, eluting ACN with 5% formic acid in methanol. Eluants were evaporated to dryness under a stream of nitrogen at 37 °C. The dried residue was reconstituted with 200 μL of water–methanol–formic acid (76:20:4, v:v) and analyzed by LC–MS/MS. HPLC separations were obtained using a Perkin-Elmer 200 micro LC pump system (Norwalk, CT, USA) and were performed using a reversed phase Luna column (150 × 3.00 mm;

C_{18}, 5 μm, 100 Å) (Phenomenex, Torrance, CA) using acidified water with 5% formic acid and methanol (Sigma-Aldrich, Steinheim, Germany) as mobile phase. The HPLC system was coupled directly to an API 4000 triple-quadrupole mass spectrometer (AB Sciex, Toronto, Canada) equipped with a TurboIon Spray source. The positive ion mode and multiple reaction monitoring (MRM) for tandem MS were used. The quantification of our analytes was performed using calibration curves obtained from blank plasma and urine spiked with pure standards (Extrasynthese, Genay, France). Quantification of CMG was obtained using C3G calibration curves. Limit of detection (LOD) was 0.05 and 0.10 ng/mL for plasma and urine, respectively. Limit of quantification (LOQ) was 0.2 ng/mL for both plasma and urine.

Platelet function and cellular biomarkers

Citrated whole blood samples were used to measure shear stress-induced platelet aggregation by the Platelet Function Analyzer (PFA-100, Dade, Siemens, Milan, Italy); the test reports the time the platelets aggregating under shear forces need to occlude a collagen/epinephrine (C/EPI) coated or a collagen/ADP (C/ADP) coated cartridge [20]. Markers of platelet (P-selectin and PAC-1, the fibrinogen-activated receptor) and leukocyte (CD11b and Mac-1) activation and mixed platelet–leukocyte conjugates were measured in whole blood by flow cytometry, both in basal condition and upon stimulation with collagen (2 μg/mL) and ADP (5 μmol/L), stirring at 1,000 rpm in an aggregometer for 10 min, as described [21]. Briefly, 100 μL of blood, unstimulated or stimulated by C/ADP, was fixed by addition of Thrombofix® (Instrumentation Laboratory, IL, Milan, Italy), as specified by the manufacturer; 1 h after fixation, samples were labeled with the appropriate monoclonal antibody to detect antigen expression for 20 min in the dark, at room temperature. Then, red cells were lysed by adding the IOTest 3 lysing solution (Instrumentation Laboratory, IL, Milan, Italy), vortexed and incubated for 10 min. The supernatant was discarded and samples resuspended in 1 mL of PBS buffer and acquired at flow cytometer.

Flow cytometric analyses were performed with a Coulter EPICS XL flow cytometer (Beckman Coulter, IL) that was daily checked by the acquisition of Flow-Check™ Fluorospheres (Beckman Coulter, IL). For each measurement, 10,000 events were analyzed. Platelets were defined by morphological characteristics, using forward light scatter (FS) versus side scatter (SS) intensity dot-plot representation and by CD42b positivity. PMN and monocyte populations were defined on the basis of the SS characteristics within the CD45-positive population; monocyte population was detected also as CD14-positive events. Data have been reported as percentage of fluorescence-positive events in platelet, PMN or monocyte populations.

Statistical analysis

The pharmacokinetic indexes were calculated by a non-compartmental model. The peak plasma concentration (C_{max}) and the time to reach it (T_{max}) were taken directly from the data. The elimination half-life ($T_{½}$) was calculated from the equation $T_{½} = \ln 2/k$, using the terminal mono-exponential log-linear slope of the time vs. concentration curve of each subject for the estimation of k by the least-square method. The area under the plasma concentration–time curve ($AUC_{0–8h}$) was calculated using the linear trapezoidal rule. AUC was extrapolated to infinity ($AUC_{0–\infty}$) by adding the last quantifiable concentration divided by the elimination rate constant.

A paired-samples t was performed to analyze data obtained by the cross-over design before (T_0) and after (T_4) juice supplementation as well as to determine possible significant differences in ACN levels in plasma and urine between each time point (T_0, T_1, T_2, T_3 and T_4) of OJ supplementation. A paired-samples t test between values at the baseline (T_0) before blood OJ supplementation and those at T_0 before blond OJ was used to establish the correct performance of the washout. Results yielding P-level lower than 0.05 were considered statistically significant. All statistical analyses were performed using SPSS software package for Windows (version 15.0; 2006).

Results

Kinetic study

C3G plasma levels were quantifiable up to 8 h after the ingestion of an acute dose of 500 mL of twofold concentrated blood OJ in each subject participating to the preliminary study (Fig. 1a); pharmacokinetic indexes were reported in Table 3. D3G plasma levels were detected, but not quantifiable (<LOQ) in six subjects out of eight subjects and not detected (<LOD) in two of them. CMG levels were quantifiable only in plasma samples collected at 0.3, 0.7 and 1 h after OJ ingestion, with C_{max} ranging between 0.37 and 0.93 nmol/L. C3G, D3G and CMG reached quantifiable levels in each urine fraction measured for all the subjects (Fig. 1b). The maximum concentration was reached in the 0–2 h fraction with a percentage of total excretion of 46, 43 and 53% for D3G, C3G and CMG, respectively. Urinary excretion reached a plateau 6 h after OJ ingestion for all the analytes.

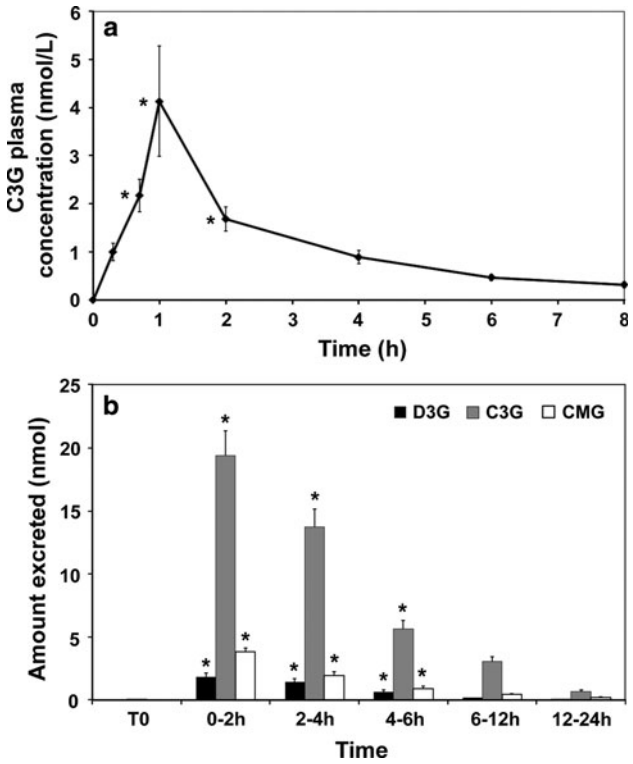

Fig. 1 Plasma concentration time curves of cyanidin-3-glucoside (**a**) and urinary excretion of anthocyanins (**b**) in healthy volunteers ($n = 8$) after a single administration of 500 mL of twofold concentrated blood OJ. Values are means ± SD. *Different from baseline, $P < 0.05$

Cross-over study

ACN levels in plasma and urine

Baseline plasma concentrations of ACN (C3G, D3G and CMG) were under LOQ in all the enrolled subjects. The 4-week supplementation with blood OJ did not affect these levels that remained unquantifiable for all the subjects. C3G, CMG and D3G urinary excretions showed a statistically significant increase ($P < 0.05$) between baseline (T_0) and all the weekly end points of blood OJ supplementation ($T_0 \neq T_1$–T_2–T_3–T_4) (Fig. 2). Blond OJ supplementation resulted in no quantifiable plasma and urine ACN concentrations.

Statistical analysis of ACN urinary excretion before each OJ supplementation confirmed an effective washout between treatments.

Platelet function and cellular biomarkers

Table 4 reports the parameters of platelet function, of markers of platelet and leukocyte activation and of platelet–leukocyte mixed conjugates. Platelet function detected by the closure time of C/EPI or C/ADP PFA-100 cartridges was not affected by either 4-week OJ supplementation. As expected, platelet activation, measured by the expression of P-selectin and of PAC-1, markers of alfa-granule release and of the active fibrinogen binding receptor, respectively, was significantly increased following stimulation. A reduction in these parameters was observed after either OJ supplementation, but not significantly in either case ($P = 0.1$). The leukocyte activation markers CD11b and Mac-1, measured on polymorphonuclear (PMN) leukocyte and monocyte surface, were also significantly increased upon activation, but unaffected by OJ intake. OJ supplementation did not affect also mixed conjugates of platelets with PMN or monocytes, either in basal or in stimulated conditions.

Discussion

The aim of this study was to investigate ACN bioavailability following blood OJ supplementation and to establish whether a regular and prolonged intake of blood OJ at nutritional amounts could beneficially affect platelet function and/or other cellular biomarkers in healthy subjects. We tested the hypothesis that a prolonged, daily intake of blood OJ at nutritional amounts could increase ACN levels in plasma of fasting healthy subjects and, consequently, induces measurable urinary excretion of these molecules during the 4 weeks of supplementation. A kinetic approach represents our starting point, as we collected data about absorption and elimination of ACN after acute administration of the same blood OJ used in the chronic study. Our findings about the parent molecules C3G, D3G and CMG measured in human plasma and urine are consistent with those previously reported [17]. Previous studies [22] in healthy subjects showed ACN absorption and maximum plasma levels from 1 to 120 nmol/L, after consumption of different food sources containing doses of ACN from 0.7 to 10.9 mg/kg$_{bw}$. In our study, C3G reached a maximum plasma level of 0.63 nmol/L with an

Table 3 Cyanidin-3-glucoside kinetic indexes in human plasma after acute blood OJ administration

C_{max} (nmol/L)	C_{max}/dose ($\times 10^{-3}$) (L^{-1})	t_{max} (h)	$T_{1/2}$ (h)	AUC_{0-8} (nmol h/L)	AUC_{0-8}/dose ($*10^{-3}$) (h/L)	$AUC_{0-\infty}$ (nmol h/L)
0.63 ± 1.06	0.07 ± 0.02	0.96 ± 0.04	2.58 ± 0.31	8.99 ± 1.57	0.16 ± 0.03	10.30 ± 1.63

Values are reported as mean ± SD ($n = 8$). For further details, see "Materials and methods"

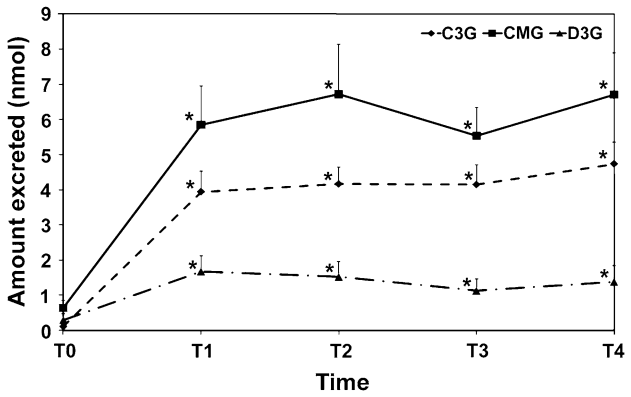

Fig. 2 Anthocyanins cumulative urinary excretion in healthy volunteers ($n = 18$) following blood OJ supplementation for 4 week. Values are means ± SD. *Different from baseline, $P < 0.05$

administered average dose of 0.43 mg/kg$_{bw}$. D3G and CMG content in blood OJ was lower than that of C3G, as 500 mL of twofold concentrated juice provided average doses of 0.07 and 0.29 mg/kg$_{bw}$ for D3G and CMG, respectively. These doses explain the unquantifiable levels of D3G as well as the partial quantification of CMG in plasma samples in our kinetic study. Previous in vivo studies dealing with the metabolism of ACN reported that glucuronidated compounds were the most abundant ACN metabolites found in urine [23–26]. In particular, Vitaglione et al. [17] found that cyanidin-glucuronides accounted for 83% of total C3G urinary excretion after an acute ingestion of blood OJ in human volunteers. A complete investigation about ACN metabolism was out of the purposes of the present work, as our aim was to establish the fate of ACN-glucosides as such, representing the parent molecules ingested from the food matrix. In the attempt to elucidate the biological effects deriving from ACN assumption, we focused only on parent molecules which are endowed with biological activity.

Our cross-over study established that detectable but hardly quantifiable levels of ACN in fasted plasma samples were reached with a prolonged blood OJ supplementation for 4 weeks; this result was consistent with our kinetic data about ACN elimination from plasma, as C3G was rapidly eliminated from plasma and its concentration decreased to values close to baseline 6 h after acute administration of blood OJ. During the cross-over study, blood samples were taken about 10 h after the last intake of juice and ACN could not have been detected as they were already cleared from the circulation. Twenty-four hour urinary ACN excretion supports the effective absorption as well as the presence of these molecules in the bloodstream. The significant increment in urinary ACN in the first week of

Table 4 Effect of blood and blond OJ 4-wk supplementation on different parameters of platelet and leukocyte function and mixed cell conjugate formation

Parameters	Blood OJ		Blond OJ	
	T_0	T_4	T_0	T_4
Platelet function test (PFA-100)[a]				
Closure time (C/EPI, s)	127 ± 21	126 ± 20	129 ± 26	125 ± 24
Closure time (C/ADP, s)	80 ± 13	80 ± 10	80 ± 15	77 ± 10
Markers of platelet activation[b]				
P-selectin (basal)	1.0 ± 0.5	1.4 ± 1.1	1.2 ± 0.8	0.9 ± 0.6
P-selectin (C/ADP)	30.0 ± 8.6	28.4 ± 9.1	27.1 ± 7.0	23.2 ± 5.2
PAC-1 (basal)	10.6 ± 5.9	9.6 ± 5.6	7.8 ± 5.4	7.1 ± 4.2
PAC-1 (C/ADP)	43.5 ± 9.6	38.6 ± 8.3	38.3 ± 9.6	35.6 ± 9.5
Markers of leukocyte activation				
PMN CD11b (basal)	49.2 ± 11.8	49.8 ± 12.1	49.0 ± 9.9	48.9 ± 8.6
PMN CD11b (C/ADP)	90.0 ± 4.4	89.5 ± 4.7	88.7 ± 9.3	90.0 ± 4.5
Monocyte CD11b (basal)	36.2 ± 18.3	38.2 ± 19.5	37.5 ± 20.8	42.5 ± 19.6
Monocyte CD11b (C/ADP)	70.4 ± 20.8	76.4 ± 18.3	71.8 ± 21.6	77.6 ± 10.8
PMN Mac-1 (basal)	12.5 ± 5.9	11.4 ± 3.9	11.9 ± 7.6	14.2 ± 7.5
PMN Mac-1 (C/ADP)	76.9 ± 16.8	76.0 ± 15.7	77.7 ± 18.3	77.1 ± 12.8
Monocyte Mac-1 (basal)	12.0 ± 6.8	12.1 ± 4.6	10.0 ± 3.8	12.8 ± 4.7
monocyte Mac-1 (C/ADP)	60.9 ± 23.8	68.5 ± 18.0	62.3 ± 26.9	66.5 ± 13.8
Mixed conjugates				
Platelet-PMN (basal)	4.7 ± 1.5	5.0 ± 2.2	4.5 ± 1.7	4.7 ± 1.9
Platelet-PMN (C/ADP)	10.9 ± 2.7	11.2 ± 3.7	11.4 ± 4.5	9.1 ± 2.4
Platelet-monocyte (basal)	5.0 ± 3.0	6.1 ± 6.1	4.5 ± 1.7	5.6 ± 3.7
Platelet-monocyte (C/ADP)	7.6 ± 3.0	8.0 ± 1.9	7.3 ± 2.4	8.1 ± 2.7

Values are expressed as means ($n = 18$) ± SD

[a] PFA-100 test was performed with collagen/epinephrine (C/EPI) and collagen/ADP (C/ADP) cartridges

[b] Basal and C/ADP represent, respectively, blood samples unstimulated and stimulated with collagen and ADP for 10 min at 37 °C under stirring. For further details, see "Materials and methods"

supplementation, followed by a plateau until the end of treatment, suggests a rapid and efficient clearance of these molecules, without accumulation.

Only few studies focused on the bioavailability of ACN, following a prolonged administration of different fruit juices or extracts. Bub et al. [27] did not find any increase in plasma and urine polyphenols after 2-week ingestion of a juice containing 210 mg of C3G, a dose about 10 times greater than that employed in our study. Duthie et al. [28] supplemented healthy volunteers for 2 weeks with 750 mL/day of cranberry juice (that provided 2.1 mL/day of malvidin-3-glucoside equivalents) and measured no difference in plasma levels of ACN before and after the treatment. Recently, Curtis et al. [29] failed to observe any ACN accumulation in plasma of postmenopausal women, after supplementation with elderberry extract for 12 weeks, despite the high dosage of ACN (500 mg/day) administered. Conversely, Riso et al. [30] measured an increase in fasted plasmatic levels of C3G from 0.6 to 8 nmol/L after 3 weeks of treatment with blood OJ, while CMG was not detectable. Considering that they employed a daily dose of 600 mL of blood OJ (i.e., about 21 mg of C3G), their results seem to be in contrast with present and previous findings. However, in Riso et al. [30] study, the quantification of ACN levels in 24-h urine, that could confirm data obtained from plasma, was not considered. In our study, the determination of urinary levels of ACN was fundamental in assessing the transition of these molecules through the bloodstream.

The potential effect of a regular intake of ACN-rich blood OJ on platelet function and on markers of platelet and leukocyte activation and interaction, apparently investigated for the first time in the present study, did not result in any significant change. These results were consistent with those obtained by Curtis et al. [29] that observed no change in platelet reactivity. Keevil et al. [11] compared the ex vivo platelet aggregation in human volunteers before and after drinking ~500 mL/day of purple grape juice, OJ or grape fruit juice for 7–10 days and found that only purple grape juice was able to prevent platelet activation. Different classes of flavonoid compounds are contained in the three juices tested: purple grape juice was rich in flavonols (quercetin) and ACN, while OJ and grape fruit juice contained flavanones (hesperidin and naringenin) and flavones. Blood OJ used in our study was rich in flavanones hesperidin and naringenin and ACN, but not in quercetin. However, a strict comparison between our study and Keevil's cannot be made, as they assessed platelet aggregation in blood collected 2 h after juice consumption; moreover, the exact concentration of ACN administered was not provided [11]. Another aspect to be considered is that the food matrix may alter both bioavailability and functional effects of ACN: these molecules may act synergistically with other components to exert biological effects. Moreover, the dietary habits may influence ACN absorption and affect the bioavailability and biological efficacy. It is, therefore, quite important to evaluate bioavailability to support functional studies on food supplementation, and in particular on polyphenols.

The healthy status of the subject population may have also masked the protective antioxidant efficacy of ACN that may be evidenced instead in studies conducted on subjects with medium–high level of CVD risk. Furthermore, the lack of effect on cellular parameters related to CVD risk of our interventional cross-over study could be the result of a too limited period of time of the intervention; indeed, epidemiological studies demonstrated a long-term risk reduction due to the regular presence in the diet of ACN, together with other healthy food components, as in the Mediterranean diet.

In conclusion, our study shows that following blood OJ 4-week supplementation at nutritional amounts, ACN are absorbed but undergo rapid and efficient clearance and no accumulation, thus being ineffective in reducing cellular markers of CVD risk. Future investigations should select populations with moderate-level risk factors or inflammatory status conditions that may benefit from a regular ACN intake.

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Conflict of interest The authors declare that they have no conflict of interest.

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