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Hesperidin in orange juice improves human endothelial function in subjects with elevated blood pressure and stage 1 hypertension: A randomized, controlled trial (Citrus study)

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ABSTRACT

In a randomized, parallel, double-blind, placebo-controlled trial, participants with elevated blood pressure and stage 1 hypertension (n = 159) received 500 mL/day of control drink, orange juice (OJ), or hesperidin-enriched OJ (EOJ) for 12-weeks, and their ischaemic reactive hyperemia (IRH) was assessed at baseline and after 4, 8, and 12-weeks. Two dose–response studies were nested within the sustained-consumption study: at baseline and after 12-weeks, a single dose of 500 mL was administered. All treatments increased postpandrial IRH, and a higher increase was obtained with EOJ. Moreover, hs-CRP and IL6 increased but not after EOJ. After 12 weeks of sustained consumption: IRH values after EOJ increased versus control group; EOJ treatment increased *DSP* and decreased *IEX-1* gene expression in PBMCs; and IRH directly correlated to NO and inversely to MPO and *IEX1*. Thus, hesperidin in OJ improves human endothelial function, lower inflammatory status at systemic level and changes at transcriptomic level might account for the increased IRH observed.

1. Introduction

The term endothelial dysfunction most commonly refers to an impairment of endothelium-dependent vasodilation with concomitant abnormalities in endothelial integrity and homeostasis (Quyyumi, 2003). Specifically, endothelial dysfunction, an important contributor to the pathobiology of atherosclerotic cardiovascular disease (Gimbrone & García-Cardeña, 2016), is the result of a balance between atherogenic and atheroprotective factors, including nontraditional and unknown factors. Endothelial dysfunction reportedly improves the prediction of cardiovascular events compared with that obtained with the traditional risk factors (Matsuzawa et al., 2015; Matsuzawa & Lerman, 2014).

Hesperidin, which is a flavonoid present in *Citrus*, has been shown to exhibit vasodilator, antihypertensive, antithrombotic, anti-

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inflammatory, antilipemic, and antioxidant activities in experimental models (Dobiaš et al., 2016; Ohtsuki et al., 2002; Sun et al., 2017; Yamamoto et al., 2008, Yamamoto et al., 2013). Hesperidin is hydrolysed into hesperetin in the gastrointestinal tract and is conjugated during absorption (Yamamoto et al., 2013). The bioactive properties of hesperidin are particularly mediated by the hesperetin-7-O-β-d-glucuronide conjugate in animal models (Yamamoto et al., 2013). Hozawever, the data on the impact of *Citrus* hesperidin consumption on vascular function in humans are controversial. A single dose of water-dispersible hesperetin (150 mg) is effective for peripheral vasodilatation in women with cold sensitivity (Takumi et al., 2012), and oral hesperidin administration (500 mg/day, 3 weeks) improves endothelial function in patients with metabolic syndrome (Rizza et al., 2011). Additionally, the daily consumption of 500 mL of orange juice (OJ) for 1 week significantly improves endothelial function in subjects with an increased cardiovascular risk (Buscemi et al., 2012). However, no effect on endothelial function was observed in elderly men with mild hypercholesterolaemia after the consumption of hesperidin (212 mg/day) in OJ for 4 weeks (Constans et al., 2015), in healthy volunteers with overweight or mild obesity after the intake of hesperidin (500 mg/day) for 6 weeks (Salden et al., 2016), or in healthy middle-aged participants after the acute ingestion of 320 mg of hesperidin in OJ or as a supplement (Schar et al., 2015).

Thus, as we previously stated (Pla-Pagà et al., 2019), the effect of hesperidin consumption on endothelium-dependent vasodilation in humans remains to be elucidated. Therefore, we aimed to assess both the acute and sustained effects, as well as the influence of sustained consumption on acute effects, of real-life doses of OJ and hesperidin-enriched OJ (EOJ) on endothelial function in individuals with elevated blood pressure and stage 1 hypertension. We hypothesized that hesperidin would benefit endothelial function both in the postprandial state and after sustained consumption.

2. Methods

2.1. Subjects

Participants were recruited via newspaper advertisements and tableaux in civic centres from the general population at the Hospital Universitari Sant Joan (HUSJ)-Eurecat, Reus, Spain, between January 2016 and June 2017. A total of 311 subjects were assessed for eligibility, and 159 (53 female and 106 male) individuals with pre- or stage 1 hypertension were recruited from this group of subjects according to current guidelines (Chobanian et al., 2003; Whelton et al., 2018). The inclusion criteria were as follows: systolic blood pressure (SBP) $\geq$ 120 mmHg, aged 18–65 years, no family history of cardiovascular disease or evidence of chronic disease, and willingness to provide informed consent before the initial screening visit. The exclusion criteria were as follows: SBP $<$ 120 mmHg or $\geq$160 mmHg and diastolic blood pressure (DBP) $>$ 100 mmHg or taking antihypertensive medications; fasting glucose $>$ 125 mg/dL; hyperlipemia or anti-lipemic medication; body mass index (BMI) $\geq$ 35 kg/m^2; smoking habit; pregnant or intending to become pregnant; use of medications, antioxidants, or vitamin supplements; following a vegetarian diet; chronic alcoholism; physical activity $>$5 h/week; intestinal disorders; anaemia (haemoglobin $\leq$ 13 mg/dL in men and $\leq$12 mg/dL in women); consumption of a research product in the 30 days prior to inclusion in the study; and failure to follow the protocol guidelines. Prior to their participation in the study, the participants signed an informed consent form. The protocol was approved by the Clinical Research Ethical Committee of the HUSJ (14–12-18/12aclaassN1), Reus, Spain. Both the protocol and trial were conducted in accordance with the Helsinki Declaration and Good Clinical Practice Guidelines of the International Conference of Harmonization (ICH GCP) and reported as CONSORT criteria. The trial was registered in ClinicalTrials.gov: NCT02479568.

2.2. Intervention products

The intervention products (supplied by the Department of Citrus of the Florida State Government, USA) were OJ containing 690 mg/L hesperidin, EOJ containing 1200 mg/L hesperidin, and a control drink (CD) without hesperidin. The hesperidin, narirutin, and vitamin C contents of the beverages were measured by LC-MS/MS. The daily doses of CD, OJ, and EOJ 500 mL provided 0 mg/day, 345 mg/day, and 600 mg/day hesperidin and 0 mg/day, 64 mg/day, and 77.5 mg/day narirutin, respectively. The drinks were dispensed in frozen, sealed, opaque cans and sequentially numbered by the codes “000”, “111” and “222” according to the randomization schedule, which was assigned by an independent researcher who was not related to the study, to guarantee blinding. Both the participants and researchers were blinded to the allocation until the end of the intervention. The composition of the intervention products is depicted in Supplementary Table 1.

2.3. Study design

In this randomized, parallel, double-blind, placebo-controlled clinical trial, the participants were randomly assigned to one of the three intervention groups: CD, OJ, and EOJ groups. The participants in each group consumed a 500 mL of the corresponding intervention product every day for 12 weeks. At the beginning of the study and after 12 weeks of the intervention, two dose–response studies were nested within the sustained-consumption study: at these time points, a single dose of 500 mL was administered, and the changes in the outcomes at the postprandial state were recorded. The random allocation of the participants to the three intervention groups was performed using a computerized random-number generator. A block-randomization method for sequence generation was prepared by an independent statistician. The SAS 9.2 (Cary, NC, USA: 83 SAS Institute Inc.) statistical software PROC PLAN with a 1:1:1 allocation using random block sizes of 2, 4, and 6 was used. After enrolment, the participants were subjected to a 1-week run-in period in which they ate a control diet based on their normal dietary habits and nutritionist recommendations and maintained their lifestyle. Throughout the study period, the participants were instructed to maintain their own dietary habits, to completely refrain from consuming citrus-containing foods and to limit their total intake of flavonoid-rich beverages (tea, coffee, cocoa, wine, and citrus juices). During the sustained-consumption study, the participants visited the HUSJ-Eurecat, where the clinical trial was performed, 7 times (every 2 weeks). Blood and urine samples were collected at the fasting state during visits (V) 1, 3, 5 and 7. The dose–response postprandial studies, which were performed at V1 and V7, lasted from 08:00 am to 02:00 pm, and the participants received a light meal before leaving. Blood samples were collected under fasting conditions at baseline (0 h) and 2 h, 4 h, and 6 h after the consumption of a single dose of 500 mL. Three-day food records, which were submitted at V1, V3, V5, and V7, were used to monitor the adherence of the volunteers to their dietary habits throughout the study. In all the visits, the subjects underwent a physical examination by a general practitioner and completed the Physical Activity Questionnaire Class AF (Vallbona, Roure, Violan, & Alegre, 2007). After 15 min of rest, the blood pressure was measured three times at 1-minute intervals using an automatic sphygmomanometer (OMRON HEM-907; Peroxfarma, Barcelona, Spain). The blood was centrifuged at 1500*g* and 4 °C for 20 min, and the obtained serum and plasma samples were stored at −80 °C in the central laboratory’s Biobanc of HUSJ (biobanc.reus@iispv.cat) until required for batch analyses.

2.4. Measures of compliance

The level of plasma hesperetin-7-O-β-d-glucuronide, one of the compliance biomarkers determined in the *Citrus* study, in the plasma samples was measured by LC-MS/MS (Valls et al., 2020). Briefly, 20 μL of internal standard (hesperetin d4) was mixed with 125 μL of plasma

and 750 μL of methanol. The mixture was vortexed and centrifuged at 4700 rpm and 4 °C, and 900 μL was evaporated in a SpeedVac at room temperature. The residues were reconstituted in 25 μL of MeOH and 75 μL of H_2O (1% HFor) and injected into the LC-MS/MS instrument. The extraction was performed using a semi-automated process with the Agilent Bravo Automated Liquid Handling Platform. An Agilent 1200 series UHPLC instrument coupled to a 6490 Triple Quad LC-MS mass spectrometer was used, and electrospray source ionization (ESI) was performed in the negative mode.

2.5. Anthropometric measurements

Anthropometric data were obtained with participants wearing lightweight clothing and no shoes. Waist circumference (WC) was measured at theumbilicus using a 150-cm anthropometric steel measuring tape. Body-weight and body composition were obtained by a calibrated scale (Tanita SC 330-S; Tanita Corp., Barcelona, Spain). Height was measured using a wall-mounted stadiometer (Tanita Leicester Portable; Tanita Corp., Barcelona, Spain). Body mass index (BMI) was calculated as the ratio between measured weight (kg)/and the square of height (m).

2.6. Endothelial function assessment

Endothelial-dependent vasomotor function was measured as ischaemic reactive hyperemia (IRH) using a Laser-Doppler linear flowmeter (PeriFlux 5000, Perimed AB, Järfälla, Stockholm, Sweden). The measurements were performed with the patient lying in the supine position in a room with a stable temperature (20–22 °C). The patients were at rest for 15–20 min before the test. The blood pressure cuff (Big Ben floor design, Riester GmbH, Jungingen, Germany) was placed above the elbow of the dominant arm, and the laser probe was attached to the palmar surface of the second finger. After a 5-min resting period, the basal capillary flow was measured for 1 min (t_0). Thereafter, 4-min distal ischaemia was induced by inflating the cuff to supra-systolic pressure (220 mmHg). Subsequently, the cuff was deflated, and after 30 s, the flow was recorded for 1 min (t_d). The data were recorded and stored using PeriSoft 2.5 software for Windows. The system monitor showed how the perfusion units (PU) decreased regularly to reach comportment equal or similar to the basal conditions. The results are expressed as arbitrary units (AUs), and the calculations were performed using the following formula: $IRH=((PUt_d- PUt_0)/PUt_0) \times 100)$. The IRH value of the area under the curve (AUC) was calculated using Microsoft Excel for pharmacokinetic functions. The AUC, not the peak itself, was the critical determinant of the peak flow-mediated dilatation (FMD) response because this measure constitutes the best method of quantifying reactive hyperemia shear stimulus for IRH normalization (Pyke & Tschakovsky, 2007). The intra- and inter-assay variabilities in the IRH values were 8.5% and 9.05%, respectively. The measurements were performed at baseline and after 4, 8, and 12 weeks of the intervention in the sustained-consumption study and 2 h, 4 h, and 6 h after ingestion of the single dose of the corresponding intervention product in both dose–response studies, which were performed at the beginning and the end of the 12-week period.

2.7. Systemic biomarkers

Serum glucose and insulin concentrations were measuerd by standardized methods in a Cobas Mira Plus autoanalyzer (Roche Diagnostics Systems, Madrid, Spain).

The levels of nitrites and nitrates, as measures of nitric oxide (NO), in the plasma samples were determined using a colorimetric assay kit (Cayman Chemical Company MI, USA). High-sensitivity C-reactive protein (hsCRP) was determined using standardized methods with an autoanalyser (Beckman Coulter-Synchron, Galway, Ireland). The level of interleukin (IL) 6 (IL6) was measured by ELISA (Abcam, Cambridge, UK), and serum myeloperoxidase (MPO) and IL18 were determined with a BIO-PLEX 200 Multiplex Reader (Bio-Rad, CA, USA). Serum endothelin 1 was determined with an ELISA kit (Invitrogen-ThermoFisher Scientific, Waltham, MA, USA). All the markers were measured within the framework of the sustained intervention. In both dose–response studies, hsCRP, NO, and endothelin 1 were also measured 2 h, 4 h, and 6 h after the single dose, and IL6 was measured 6 h after the single dose.

2.8. Transcriptomic analyses

Gene expression in peripheral blood mononuclear cells (PBMCs) collected from a subsample (n = 37) of participants (11, 15, and 11 in the CD, OJ, and EOJ groups, respectively) under fasting conditions at baseline (V1) and after 12 weeks (V7) was assessed using an Agilent Microarray Platform (Agilent Technologies, Santa Clara, CA, USA). Ficoll gradient separation (GE Healthcare Bio Sciences, Barcelona, Spain) was used for the isolation of PBMC RNA. The RNA yield (Nanodrop UV–VIS Spectrophotometer) and integrity (Agilent 2100 Bioanalyzer using the Total RNA Nano kit and the Eukaryote Total RNA Nano; Agilent Technologies, Santa Clara, CA, USA) were assessed. Total RNA from PBMCs was labelled with one color (Cy3) and hybridized using a Gene Expression Hybridization Kit (Agilent Technologies, Santa Clara, CA, USA). Image scanning was performed using an Agilent Microarray Scanner System with SureScan High-Resolution Technology (Agilent Technologies, Santa Clara, CA, USA).

Functional and biochemical pathway analyses using Gene Ontology and the KEGG (Kyoto Encyclopedia of Genes and Genomes (www.genome.jp/kegg) and PANTHER (Protein Annotation Through Evolutionary Relationship classification system) (http://www.pantherdb.org/) biochemical pathway databases, respectively, were performed to assess the role of the differentially expressed genes. The analyses were performed using GeneCodis (http://genecodis.dacya.ucm.es) (Nogales-Cadenas et al., 2009) software.

Selected genes related to endothelial function were validated by PCR. Briefly, cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4 Barcelona, Spain) and MyGene Series Peltier Thermal Cycler (LongGene Scientific, Zhejiang, China) and used for reverse transcription. The cDNA was subjected to quantitative reverse transcription-polymerase chain reaction amplification using LightCycler 480 SYBR Green I Master (Roche Diagnostic, Sant Cugat del Vallès, Barcelona, Spain) and a LightCycler 480 II system (Roche Diagnostic, Sant Cugat del Vallès, Barcelona, Spain).

2.9. Sample size and power analyses

A sample size of 159 individuals allows at least 80% power to detect a statistically significant difference among groups of 8 units of IRH assuming a drop rate of 20% and a type I error of 0.05 (2-sided). The common standard deviation of the method is 11 units (Ruano et al., 2005).

2.10. Statistical analyses

The normality of the variables was assessed with the Kolmogorov-Smirnov test. Nonparametric variables were log transformed. Differences in baseline characteristics were assessed by one-factor analysis of variance (ANOVA). The analyses were made based on intention-to-treat. A general linear model with Bonferroni correction and age and sex as covariables was fitted for intra-treatment comparisons. An ANCOVA model adjusted for age and sex was used for inter-treatment comparisons. For the transcriptomic analyses, the statistical comparisons were performed with Student's *t*-test or Welch's *t*-test as appropriate. The Benjamini-Hochberg false discovery rate (FDR) was used for multiple testing correction. Probes were assumed to be differentially expressed if they presented a P-value < 0.05 and a fold change $<=-0.58$ or $>=0.58$ on the log2 scale (corresponding to a 1.5-fold difference on the natural

scale). The calculations were performed using the R statistical language. The comparisons of both systemic and transcriptomic variables among the treatments were conducted with an ANCOVA model adjusted for age and sex and the baseline values. A P value ≤ 0.050 from a two-sided test was defined as indicating statistical significance. We performed the analyses using SPSS for Windows (version 21, IBM corp., Armonk, NY, USA).

3. Results

3.1. Characteristics of the study participants

Among the 311 subjects who were assessed for eligibility, only 159 met the inclusion criteria and were randomly allocated to the CD, OJ, and EOJ groups (n = 53 in each group). A total of 129 participants completed the study (43 in the CD group, 46 in the OJ group, and 40 in the EOJ group). In addition, three and six of the 52 allocated participants discontinued the intervention in the first and second dose–response studies, respectively. Moreover, 16 participants in the CD group, 21 in the OJ group, and 13 in the EOJ group were available for the first dose–response study, and 13 in the CD group, 18 in the OJ group, and 11 in the EOJ group were available for the second dose–response study. The flow chart of the study is depicted in Supplementary Fig. 1.

The baseline characteristics of the total study population divided by intervention group are shown in Table 1. No adverse effects were reported during the study. No differences were observed among the groups. The baseline characteristics of the participants in the dose–response study were similar to those of the whole sample. No differences in the level of physical activity or dietary habits were observed from the beginning to the end of the study in any of the groups with the exception of the protein (% energy) intake, which was greater in the OJ group than in the EOJ group at 12 weeks (P = 0.031) (Supplementary Table 2).

Table 1
Baseline characteristics of participants by intervention group.

Variable	Control (n = 53)	OJ (n = 53)	Enriched OJ (n = 53)	P
Age, *y*	45.4 ± 13.0	43.3 ± 12.0	43.6 ± 11.8	0.629
Females, *%*	34.0	32.1	34.0	0.981
SBP, *mm Hg*	132 ± 9.94	132 ± 9.11	134 ± 9.82	0.687
DPB, *mm Hg*	79 ± 8.14	80 ± 8.42	79 ± 10.2	0.868
Pulse pressure, *mm Hg*	53 ± 9.09	52 ± 8.05	54 ± 6.74	0.261
Weight, *kg*	77.3 ± 15.4	78.8 ± 12.2	75.9 ± 11.6	0.523
BMI, kg/m^2	26.1 ± 3.8	26.4 ± 3.6	26.1 ± 3.3	0.858
Waist circumference, *cm*	93.0 ± 11.0	91.7 ± 10.9	91.4 ± 10.7	0.766
Waist/height, *cm*	0.54 ± 0.06	0.53 ± 0.07	0.54 ± 0.07	0.790
Conicity index	1.50 ± 0.76	1.30 ± 0.35	1.39 ± 0.62	0.269
Glucose, *mg/dL*	91.6 ± 9.2	93.6 ± 11.6	93.6 ± 9.6	0.517
Cholesterol, *mg/dL*				
Total	196 ± 30.1	198 ± 32.7	196 ± 31.6	0.937
LDL	124 ± 26.4	125 ± 31.5	127 ± 25.1	0.900
HDL	50.9 ± 13.4	51.0 ± 14.7	49.8 ± 13.0	0.889
Triglycerides*, *mg/dL*	82 (67–118)	85 (65–121)	81 (63–116)	0.624
Physical activity, *AU*	3.08 ± 0.06	3.12 ± 1.38	3.12 ± 1.26	0.986

Data expressed as mean ± standard deviation, or percentages. OJ, orange juice; SBP, systolic blood pressure; DBP, diastolic blood pressure; Pulse pressure = SBP-DBP; BMI, body mass index; LDL, low density lipoproteins; HDL, high density lipoproteins * median (25th – 75th percentiles). AU, arbitrary units: 0, inactive; 1, very low activity; 2, low activity; 3, moderately active; 4,very active. *P* for ANOVA with logarithmic transformation for triglycerides.

3.2. Compliance biomarkers

Dose-response studies: At the beginning of the study, the level of plasma hesperitin-7-β-D-glucuronide measured 4 and 6 h after the consumption of OJ and EOJ was higher than that in the CD group ($P < 0.05$) (Fig. 1A). A similar pattern was observed at the end of the study.

Sustained-consumption study: After 12 weeks of intervention (Fig. 1B), the administration of hesperidin increased the level of plasma hesperitin-7-β-D-glucuronide in a dose-dependent manner (P < 0.001 for the linear trend). The level of hesperitin-7-β-D-glucuronide was increased by the OJ and EOJ treatments ($P < 0.001$ versus changes in CD), and the increase observed with EOJ was significantly higher than that obtained with OJ ($P < 0.05$).

3.3. Anthropometric measurements

Changes in anthropometric parameters (body weight, BMI and WC) are shown in Supplementary Tables 3, 4 and 5. Compared with baseline, the OJ group had a significantly increased body weight maximum by 0.87 (0.01; 1.72) kg ($P < 0.05$) and BMI maximum by 0.292 (0.01; 0.57) kg/m^2 ($P < 0.05$). WC was maintained throughout the study with the consumption of all three intervention products.

3.4. Endothelial function

Dose-response studies: Fig. 2 shows the changes in IRH after the interventions. At the beginning of the study (Panel A), the IRH values increased after all the treatments (P < 0.05); however, the increases obtained with EOJ were higher than those obtained with the other treatments, and the EOJ-induced increase at 6 h postprandial was significantly higher than those obtained with the CD and OJ treatments (P > 0.05). After 12 weeks of treatment (Panel B), an increase in the IRH value versus the baseline was observed at 6 h postprandial with all the treatments (P < 0.05), and no intertreatment differences were detected.

Sustained-consumption study: The changes in IRH after 4, 8, and 12 weeks of intervention are shown in Fig. 3. No significant changes were observed after 4 and 8 weeks. After 12 weeks of sustained consumption, the changes in IRH in the EOJ group were higher than those found in the CD group (P = 0.043). In addition, the increases in IRH at the end of the intervention period was directly correlated with the increases in the hesperetin-7-B-glucuronide values (R = 0.233, $P = 0.009$).

3.5. Systemic biomarkers

Dose-response studies: No hyperglycemia or hyperinsulinemia had been observed in the postprandial state (Supplementary Table 6). No changes in the endothelin values were observed in either dose–response study. A decrease in NO at the postprandial state was obtained after 6 h with all the treatments (no differences among the treatments) in the dose–response study conducted at the beginning of the study (P < 0.05) and with the OJ treatment in the dose–response study conducted at the end of the study (P < 0.05) (Supplementary Table 7). After 12 weeks of sustained treatment, the 4-h postprandial IRH values were directly related to the corresponding NO levels (R = 0.396, $P = 0.013$) (Supplementary Fig. 2). In the dose–response study conducted at the beginning of the study, the postprandial hsCRP was increased after 4 h and 6 h after the dose of CD (P < 0.05) (Supplementary Table 8). The postprandial IL6 level was increased 6 h after the consumption of CD and OJ (P < 0.05) but not after the consumption of EOJ, and this finding was obtained in both dose–response studies (Fig. 4).

Sustained-consumption study: Serum glycemia, insulin concentrations and HOMA-IR remained unchanged (Supplementary Table 9). Although neither intra- nor inter-treatment differences in hsCRP, IL6, MPO, or

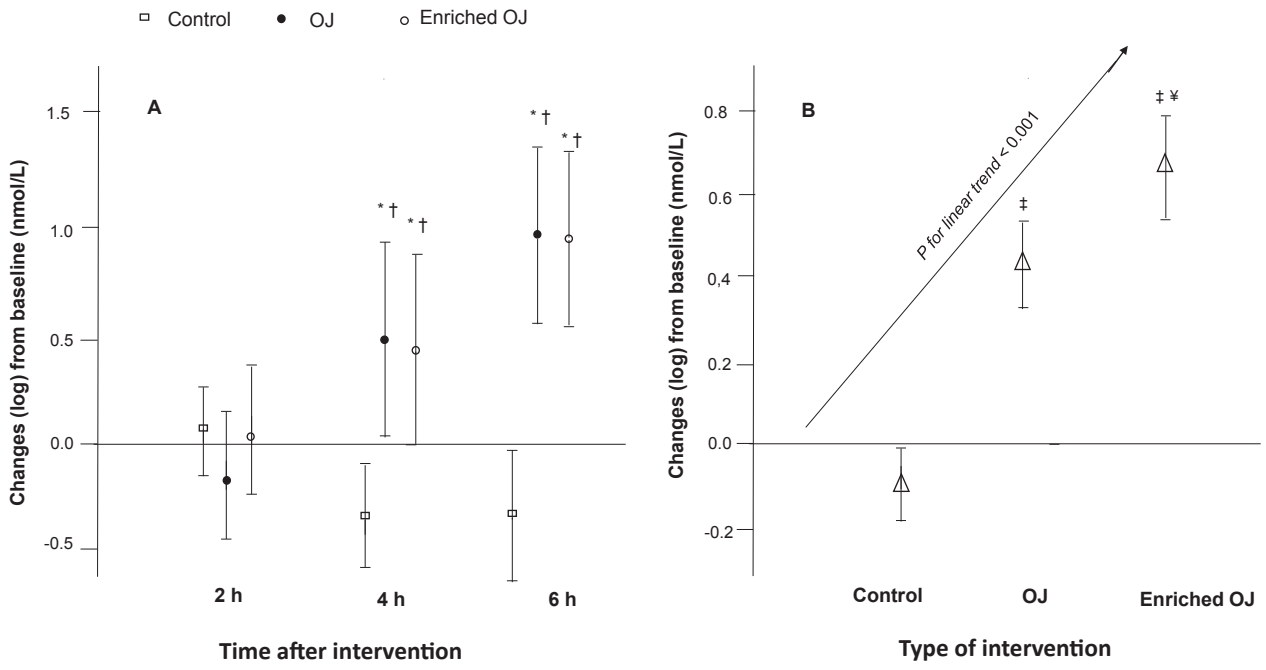

Fig 1. Changes in plasma hesperitin-7-β-D-glucuronide after ingestion of the control drink (CD), orange juice (OJ), and enriched OJ. Panel A, at the beginning of the study after a single dose of 500 mL. Panel B, after sustained consumption for 12 weeks (500 mL/day). *$P < 0.05$ versus the baseline; †$P < 0.05$ versus the control group; ‡$P < 0.001$ versus the control group; ¥$P < 0.05$ versus the OJ group.

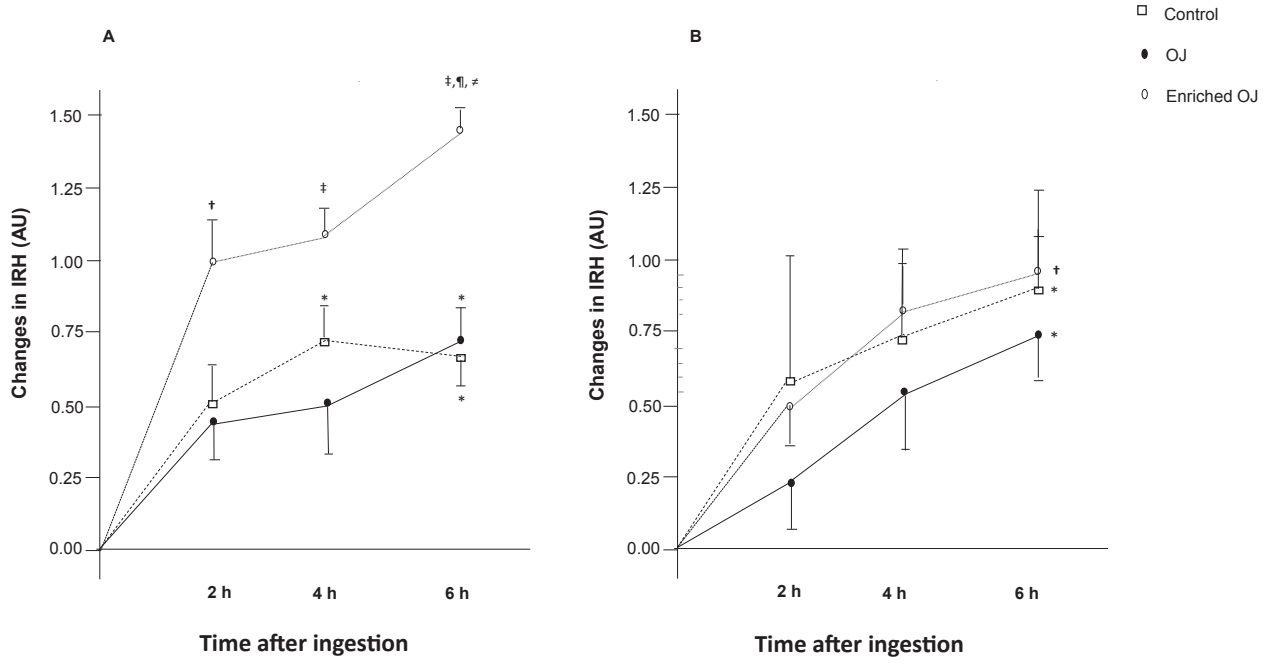

Fig 2. Changes in ischaemic reactive hyperemia (IRH) after a single dose of 500 mL of the control drink (CD), orange juice (OJ), and enriched OJ at the beginning (Panel A) and end of the study (Panel B). AUs, arbitrary units (log). *$P < 0.05$, †$P < 0.01$, and ‡$P < 0.001$ versus the baseline; ¶ $P < 0.05$ versus the 2-h time point; ≠$P < 0.05$ versus the control and OJ groups.

IL18 were observed in all the evaluated weeks, the IRH values after 12 weeks of sustained consumption were directly related to the NO levels (R = 0.175, $P = 0.049$) (Supplementary Fig. 3), and the changes in IRH were inversely related to changes in MPO (R = −0.203, $P = 0.023$).

3.6. Transcriptomic analyses

Four genes directly related to endothelium integrity were differentially expressed after 12 weeks of treatment: desmoplakin (DSP), early growth response 3 (ERG3), inhibitor of DNA binding 1 (ID1), and immediate early response 3 (IEX-1).

EOJ intervention increased the expression of *DSP* and significantly decreased that of *IEX-1* compared with that obtained in the CD group ($P < 0.05$). No changes were observed after the CD and OJ interventions. Fig. 5 shows the comparisons among the interventions, and the dot axis at $P < 0.05$ indicates significance. At week 12, the expression of *IEX-1*

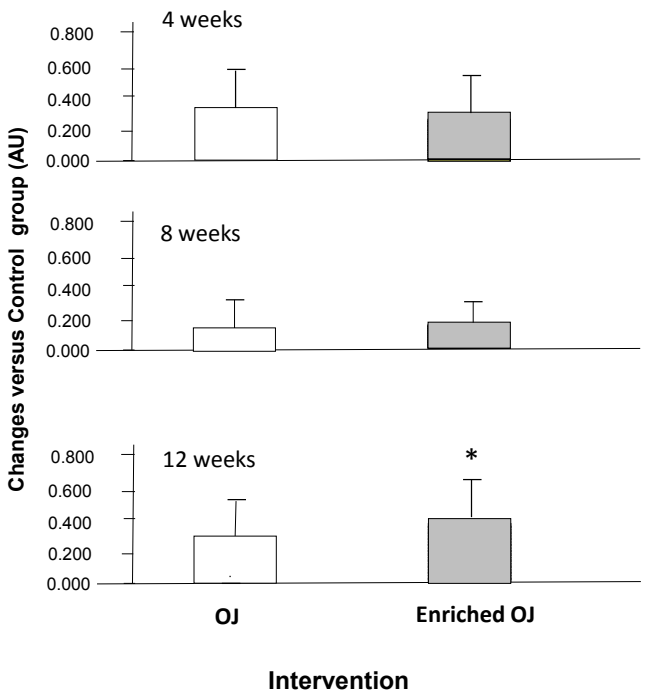

Fig 3. Changes in ischaemic reactive hyperemia after 4, 8, and 12 weeks of treatment. OJ, orange juice; EOJ, enriched OJ; AUs, arbitrary units (log). *$P <$ 0.05 versus the control group.

was inversely related to the IRH values with borderline significance (R = -0.310, $P = 0.062$). Additionally, at this time point, the expression of *IEX-1* was directly related to the levels of *EGR3* (R = 0.654, $P < 0.001$) and *ID1* (R = 0.599, $P < 0.001$), and the expression of *EGR3* was related to that of *ID1* (R = 0.799, $P < 0.001$).

4. Discussion

In the present work, we examined the effect of the hesperidin content in OJ on endothelial function and associated markers. The 6-h postprandial increase in IRH after the consumption of EOJ was higher than that observed after the CD and OJ treatments. Concomitant with this increase in IRH, postpandrial significant increases versus its baseline observed in hsCRP after the CD treatment and in IL-6 after the CD and OJ treatments were not observed with the EOJ intervention. After 12 weeks of sustained consumption, the changes in the IRH values in the EOJ group were higher than those found in the CD group. Additionally, sustained consumption of EOJ increased the expression of the *DSP* gene and decreased that of the *IEX-1* gene in PBMCs. The IRH values after 12 weeks of sustained consumption were directly correlated with the NO values and inversely correlated with the MPO and *IEX1* levels.

In the present study, we used IRH, which was determined using the laser-Doppler technique and is considered a noninvasive indicator of endothelial dysfunction (Vuilleumier et al., 2002). IRH reduction is a reflex of impaired flow-mediated vasodilation, which is considered an early indicator of atherosclerosis and is associated with organ damage, including increased carotid intima media thickness and left ventricular hypertrophy (Bruno et al., 2014). A low FMD is also associated with an increased risk of incident arterial stiffness (AF), which is an independent predictor of cardiovascular and cerebrovascular diseases and is considered a consequence of endothelial function impairment (Tomiyama et al., 2018). Both AF and FMD are related to new-onset atrial fibrillation (Shaikh et al., 2016). In our study, increases in IRH after EOJ intervention were observed both after a single dose and after sustained consumption. The mean increase in IRH after 12 weeks of EOJ treatment was 1.5-fold and 2.6-fold higher than those observed after the consumption of OJ and CD, respectively. NO enhances the FMD response in the brachial artery (Bruno et al., 2014), and oxidative stress impairs this response (Gimbrone & García-Cardeña, 2016). Accordingly, after 12 weeks of EOJ consumption, the IRH values were related directly to the NO levels and inversely correlated with the MPO values.

After sustained consumption of EOJ, but not CD and OJ, we observed changes in the expression of *DSP* and *IEX-1*, which are two genes related to endothelial function. Desmoplakin (DSP), a cytoplasmic desmosomal protein that attaches intermediate filaments to the plasma membrane, is assembled with VE-cadherin into dermal microvascular endothelial intercellular junctions to maintain the monolayer integrity (Kowalczyk et al., 1998). Endothelial cell–cell junctions provide a restrictive barrier that is tightly regulated to allow dynamic responses to permeability-inducing angiogenic factors as well as to inflammatory agents and adherent leukocytes (Timmerman et al., 2015). In our study, *DSP* gene expression increased after EOJ intervention and exerted a positive effect on the microvascular endothelial membrane. *IEX-1*, also known as *IER-3*, is a response gene that has been shown to modulate cell growth and has been implicated in venous neointimal hyperplasia (Brahmbhatt et al., 2014; Im et al., 2002). We observed decreased expression of *IEX-1* after EOJ intervention. An increase in the lumen vessel area accompanied by a decrease in the neointima area is associated with venous stenosis in *IEX-1*$^{-/-}$ mice. The inhibition of *IEX-1* results in decreased proliferation, increased apoptosis and higher positive vascular remodelling

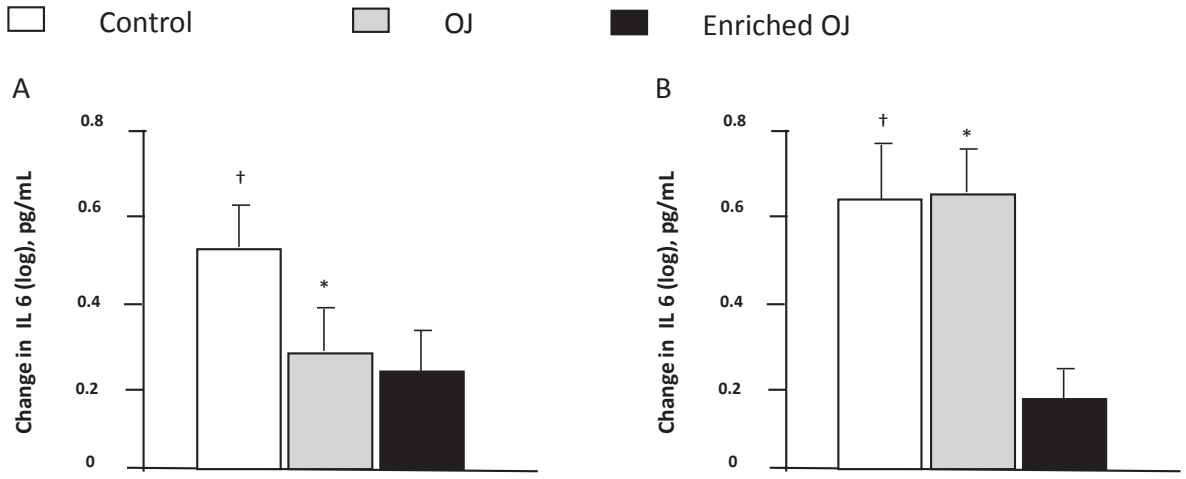

Fig 4. Changes in the postprandial interleukin 6 (IL6) level at 6 h after a single dose of 500 mL of the control drink (CD), orange juice (OJ), and enriched OJ. Panel A, at the beginning of the study. Panel B, after sustained consumption for 12 weeks (500 mL/day). *$P <$ 0.05 and † $P < 0.001$ versus the baseline.

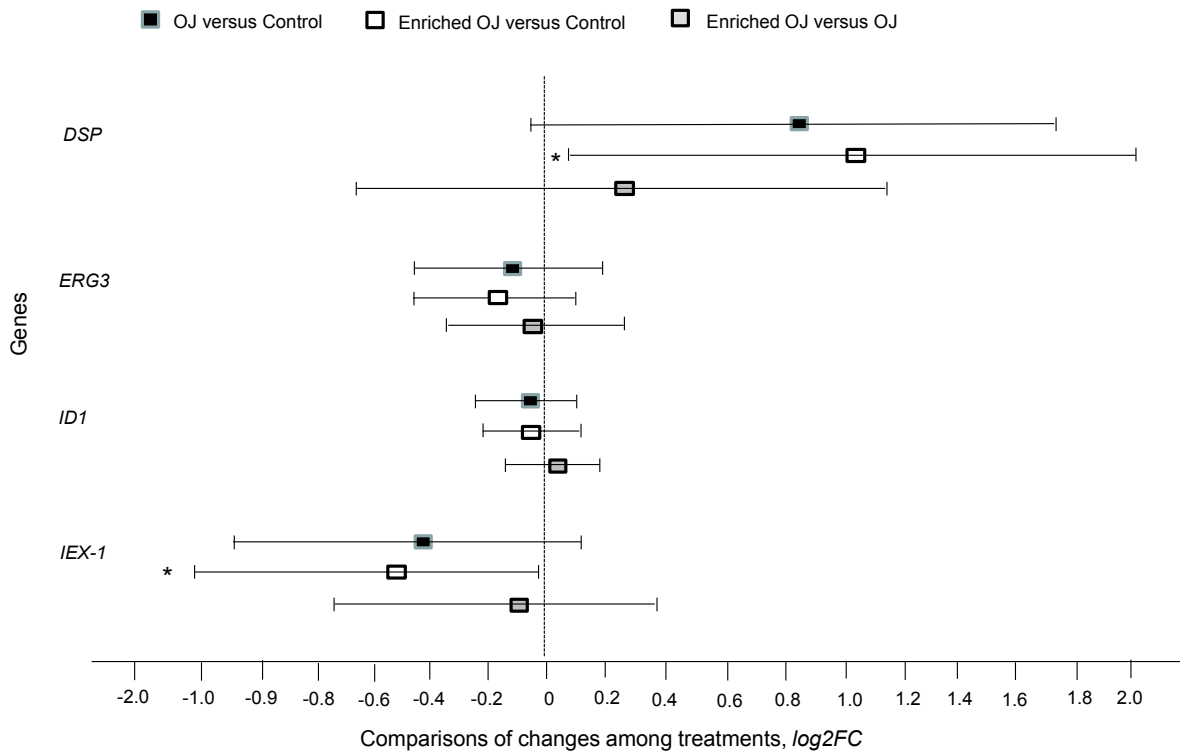

Fig 5. Mean (95% CI) of changes in gene expression after the interventions. The dotted axis indicates significance at the $P < 0.05$ level. *DSP*, desmoplakin; *ERG3*, early growth response 3; *ID1*, inhibitor of DNA binding 1; *IEX-1*, immediate early response.

(Brahmbhatt et al., 2014). Accordingly, in our study, the observed decrease in *IEX-1* was inversely related to the IRH value.

Individuals in Western populations spend most of their time in a non-fasting state. The postprandial state, an active field of research in cardiovascular disease, influences cardiovascular risk due to an impairment of endothelial-dependent vasodilatation (Ghiadoni et al., 2012). Moreover, the postprandial inflammatory state (Alipour et al., 2007) triggers microvascular responses such as vasomotor dysfunction, leukocyte recruitment, increased vascular permeability, angiogenesis and thrombosis (Nettleton et al., 2006). In our study, a high hesperidin dose in OJ promoted the best postprandial IRH response. Accordingly, in contrast to the observations after CD or OJ treatment, no increases in the inflammatory state, i.e., in either hsCRP or IL6, were observed after EOJ treatment. Our results agree with those reported by Morand et al. (2011), who observed an improvement in postprandial microvascular endothelial reactivity in healthy elderly men after a single dose of 292 mg of hesperidin in OJ. However, Morand et al. (2011) found that sustained consumption of 292 mg of hesperidin for 4 weeks did not exert any significant effect (Morand et al., 2011). Additionally, flavanone-rich citrus beverages have been shown to counteract the transient decline in postprandial endothelial function in healthy men (Rendeiro et al., 2017).

Rich-polyphenol dietary interventions have been reported to provide benefits on endothelial function in humans. We (Valls et al., 2015, Valls et al., 2017) and other researchers (Ruano et al., 2005) have previously described the benefits of polyphenol-rich virgin olive oils on endothelial function in patients with hypertension or hyperlipemia both after sustained consumption or at the postprandial state. Additionally, other flavonoid-rich foods, such as cocoa, black tea, and red wine, have been shown to provide benefits on endothelial function in individuals with hypertension (Ghiadoni et al., 2012). In July 2011, the European Food Safety Authority (EFSA) released a claim concerning the benefits of the daily ingestion of at least 200 mg of cocoa flavanols in the maintenance of normal endothelium-dependent vasodilation (EFSA Panel on Dietetic Products, 2014). The present study provides the first demonstration that citrus flavanones can provide dose-dependent effects on endothelium-dependent vasodilation in individuals with elevated blood pressure and stage 1 hypertension. Additionally, our data are reinforced by our previous data concerning the beneficial effect of a hesperidin-enriched beverage on blood and pulse pressure in a population with hypertension (Valls et al., 2020).

Another relevant result is that the intake of the hesperidin-enriched OJ managed to improve endothelial function, maintaining glucose and insulin concentrations and subjects' body weight stable. The increase in body weight observed by the group consuming OJ was considered to be non-clinically relevant as the increase was less than 1 kg of the body.

The study has strengths and limitations. As a strength, the vitamin C content, a key possible confounder, was similar across the intervention groups. Other strengths of the present study are the assessment of hesperitin-7-β-D-glucuronide as a compliance marker and the integration of multiple omic approaches, such as targeted metabolomics and transcriptomics, which provided a comprehensive overview of citrus biomarker metabolites and of the expression profile of key genes related to endothelial function. One limitation of this study was the inability to assess potential interactions between the interventions and other dietary components. Although no clinical trials have reported a benefit of naringenin on endothelium-dependent vasodilation, the contribution of the narirutin present in the treatments cannot be discarded. An improvement in arterial stiffness, without any FMD changes, has been observed in menopausal women after the daily consumption of grapefruit juice containing 210 mg of naringenin for 6 months (Habauzit et al., 2015). In addition, the participants in this study were individuals with pre- and stage 1 hypertension, which limits the extrapolation of our results to the general population. Whether additional or different effects of hesperidin would have been observed over longer time periods is unknown, but longer intervention periods could have affected the compliance of the individuals.

5. Conclusion

In summary, our results show that the intake of EOJ improves endothelial function both postprandially and after sustained consumption, which to the best of our knowledge, has not been previously reported. This improvement in endothelial function was found to be directly related to the hesperidin content of the beverage administered. A better inflammatory status at the systemic level and changes at the transcriptomic level could account for the increased endothelial function observed after the consumption of EOJ.

Therefore, EOJ might be a useful co-adjuvant tool for the management of elevated blood pressure and stage 1 hypertension by improving endothelial function and maintaining blood glucose and insulin concentrations and body weight stable.

6. Ethics statement

Prior to their participation in the study, the participants signed an informed consent form. The protocol was approved by the Clinical Research Ethical Committee of the HUSJ (14-12-18/12aclaassN1), Reus, Spain. Both the protocol and trial were conducted in accordance with the Helsinki Declaration and Good Clinical Practice Guidelines of the International Conference of Harmonization (ICH GCP) and reported as CONSORT criteria. The trial was registered in Clinical-Trials.gov: NCT02479568.

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CRediT authorship contribution statement

Rosa M. Valls: Conceptualization, Investigation, Methodology, Data curation, Formal analysis, Writing - original draft, Writing - review & editing. **Anna Pedret:** Investigation, Methodology, Data curation, Formal analysis, Writing - original draft, Writing - review & editing. **Lorena Calderón-Pérez:** Investigation, Methodology. **Elisabet Llauradó:** Investigation, Methodology. **Laura Pla-Pagà:** Investigation, Methodology. **Judit Companys:** Investigation, Methodology. **Ana Moragas:** Investigation, Methodology. **Francisco Martín-Luján:** Investigation, Methodology. **Yolanda Ortega:** Investigation, Methodology. **Montse Giralt:** Investigation, Methodology. **Laura Rubió:** Investigation, Methodology. **Núria Canela:** . **Francesc Puiggrós:** Conceptualization, Data curation, Formal analysis, Writing - review & editing. **Antoni Caimari:** Conceptualization, Investigation, Methodology, Data curation, Formal analysis, Writing - review & editing. **Josep M. Del Bas:** Conceptualization, Data curation, Formal analysis, Writing - review & editing. **Lluís Arola:** Conceptualization, Writing - review & editing. **Rosa Solà:** Conceptualization, Investigation, Methodology, Data curation, Formal analysis, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2021.104646.

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