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Orange juice and hesperidin increase flavanone exposure without detectable short-term vascular benefits: a randomized crossover trial

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Orange juice is a major dietary source of hesperidin, a citrus flavanone with vascular protective effects in experimental models. However, whether nutritionally realistic intake levels induce measurable benefits in humans remains unclear. We investigated the effects of orange juice and hesperidin supplementation, at realistic dietary doses, on vascular function, flavanone bioavailability, and molecular responses. Thirty-seven centrally overweight men completed a randomized, double-blind, controlled, three-period crossover trial with three 6-week interventions separated by washout periods. Participants consumed daily 330 mL of 100% orange juice (OJ), an isoenergetic control beverage (CON), or a hesperidin-enriched control beverage (HESP, 210 mg day^{-1}). Fasting vascular, metabolic and anthropometric parameters were assessed before and after each intervention, with flow-mediated dilation (FMD) as the primary endpoint. Postprandial FMD, circulating flavanone metabolites and oxylipin profiles were evaluated following a standardized high-fat meal challenge, and flavanone bioavailability was assessed by 24 h urinary excretion. Whole-blood transcriptomics were performed in a subset (n = 9). Plasma exposure to phase II hesperetin metabolites ($AUC_{0-6\ h}$) and 24 h urinary excretion were comparable after OJ and HESP, indicating effective hesperidin delivery and limited matrix effects on bioavailability. Neither intervention significantly affected fasting or postprandial FMD, vascular, metabolic or anthropometric parameters, or oxylipin profiles *versus* CON. Marked interindividual variability was observed in vascular responses and flavanone bioavailability, although treatment effects were unrelated to baseline endothelial function or flavanone exposure. Exploratory transcriptomic analyses suggested modulation of pathways involved in vascular biology following OJ and HESP. Under nutritionally realistic conditions, orange juice and hesperidin induced measurable biological engagement without detectable short-term vascular benefits, highlighting the complexity of linking flavanone exposure to functional vascular outcomes in humans.

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1 Introduction

Fruit consumption has been consistently associated with a reduced risk of cardiovascular disease (CVD), as shown in large prospective cohort studies.[1] This cardioprotective effect is likely driven by the combined action of dietary fibres, essential micronutrients, and bioactive phytochemicals, including phenolic compounds.[2] Among these, flavonoids are the class most consistently linked to lower CVD incidence.[3]

Flavanones are a major flavonoid subclass predominantly found in citrus fruits, which are among the most widely consumed fruits worldwide. Orange fruit and juice constitute the principal dietary sources of flavanones, providing mainly hesperidin, and to a lesser extent narirutin, the glycoside forms of hesperetin and naringenin, respectively.[4]

Epidemiological evidence supports a beneficial role of citrus consumption in cardiovascular prevention, with higher intakes associated with reduced incidence of CVD, particularly stroke.[5] Meta-analyses further suggest that flavanone intake may be associated with a ~20% reduction in coronary heart disease risk[6] and an inverse linear association with stroke risk.[3]

Preclinical studies have shown that hesperidin supplementation can improve lipid and glucose metabolism, reduce blood pressure and adiposity, and exert anti-inflammatory and vascular protective effects.[7–9] Circulating phase II hesperetin metabolites have also been reported to display biological activity, including antioxidant, vasodilatory, and anti-inflammatory effects.[10,11] Mechanistically, citrus flavanones may enhance nitric oxide bioavailability, regulate redox-sensitive and pro-inflammatory signalling pathways, and modulate vascular-related gene expression.[12,13]

In contrast, clinical evidence remains limited and inconsistent. While vascular benefits have been demonstrated for several flavonoid-rich foods, such as cocoa, tea, and berries,[14] trials investigating citrus flavanones have yielded mixed results as recently reviewed.[15] Some studies report improvements in vascular function, particularly under postprandial conditions or in metabolically at-risk individuals, whereas others show no significant effects.

This heterogeneity likely reflects differences in study populations, intervention doses and duration, the hesperidin-embedding food matrix, co-consumed foods, timing of vascular assessments, and the extent of flavanone bioavailability achieved. The current variability in trial findings precludes firm conclusions regarding the clinical efficacy of flavanones, particularly under conditions reflecting habitual dietary intake. To our knowledge, no controlled intervention study has simultaneously assessed fasting and postprandial vascular function alongside quantitative measures of systemic flavanone exposure. Moreover, the influence of the food matrix on flavanone absorption, metabolism, and vascular effects remains insufficiently characterized in controlled intervention studies.

In addition, emerging evidence suggests that flavonoid-rich interventions may positively influence oxylipin biosynthesis.[16,17] Oxylipins constitute a large family of bioactive lipid mediators that play critical roles in the regulation of cardiometabolic processes such as inflammation, vascular tone, endothelial permeability, and blood clotting.[18] However, the effects of flavanones on this pathway have not been investigated in clinical trials.

To address these gaps, we conducted a randomized, double-blind, controlled, three-period crossover trial in centrally overweight but otherwise healthy men, a population likely to exhibit early vascular dysfunction despite the absence of overt clinical disease. The study compared orange juice with two isocaloric control beverages—with and without added hesperidin—over three 6-week intervention periods. The primary endpoint was endothelial function assessed by flow-mediated dilation (FMD) under fasting and postprandial conditions. Secondary outcomes included blood pressure, arterial stiffness, skin microvascular endothelial reactivity, systemic endothelial biomarkers, and metabolic and anthropometric parameters. We also assessed flavanone bioavailability and explored potential effects on oxylipin metabolism and gene expression. This integrated design aimed to clarify the vascular effects of citrus flavanones, the role of the juice matrix, and the relationship between systemic exposure and functional outcomes under nutritionally relevant conditions.

2 Methods

2.1 Study design

This study was part of the HESPER-HEALTH trial, a randomized, double-blind, controlled, three-period crossover dietary intervention. The full protocol has been published elsewhere.[19] The trial was conducted at the Clinical Investigation Center of the University Hospital of Clermont-Ferrand, France. Ethical approval was obtained from the CPP Sud-Est III ethics committee (ID-RCB: 2020-A01985-34), and the trial was registered at ClinicalTrials.gov (NCT04731987, Registration date: 2021-01-22).

2.2 Subjects

Eligible participants were men or postmenopausal women, centrally overweight but otherwise healthy (40–65 years; BMI $\leq$30 kg m^{-2}; waist circumference >94 cm for men and >80 cm for women). Exclusion criteria included smoking, gastrointestinal disorders, anti-inflammatory or lipid-lowering medication use, and recent antibiotic or supplement intake known to affect vascular or inflammatory status. All participants provided written informed consent. Participants and the public were not involved in the design, conduct, reporting, or dissemination plans of this study.

2.3 Intervention

Participants were randomly assigned to one of six intervention sequences by an independent clinical assistant using a computer-generated randomization scheme, as previously described.[19] Allocation concealment and blinding were ensured by the Central Pharmacy of the University Hospital of Clermont-Ferrand. Participants, clinical center staff, outcome assessors, and analysts were blinded after assignment to the intervention conditions.

Each participant consumed 330 mL per day of the following three isoenergetic test beverages (122 kcal) across three 6-week intervention periods, separated by 4–6-week washout phases: (a) 100% orange juice naturally rich in hesperidin (OJ), (b) a sugar-matched control beverage without hesperidin (CON), and (c) the same control beverage supplemented with hesperidin (HESP) at the same level present in the juice. Before the start of each experimental period, the clinical center staff distributed the required bottles to the volunteers. Beverages were consumed at home in two equal portions (morning and noon). To preserve blinding, the control drinks mimicked flavor, color, and sensory properties of OJ, and all beverages were pro-

Table 1 Composition of study beverages

	CON	HESP	OJ[a]
Total sugars (g L^{-1}) [b]	92	93	92
Citric acid (g L^{-1})	6.4	6.3	7.8
Potassium (g L^{-1})	n. d.	n. d.	2.02
Total fibre (g L^{-1})	n. d.	n. d.	7.3
Total vitamin C (mg L^{-1}) [c]	n. d.	n. d.	587 ± 9
Total carotenoids (mg L^{-1}) [d]	n. d.	n. d.	3.7 ± 1.5
Total phenolic compounds (mg L^{-1}) [e]	n. d.	641 ± 2	960 ± 4
Hydroxycinnamic acids (mg L^{-1}) [e]	n. d.	n. d.	72 ± 1
Hesperidin (mg L^{-1}) [f,g]	n. d.	617 ± 2	647 ± 4
Narirutin (mg L^{-1}) [e]	n. d.	15 ± 0	132 ± 1
Other flavonoids (mg L^{-1}) [e,h]	n. d.	10 ± 0	109 ± 0
Natural orange aroma (mL L^{-1}) [i]	0.15	0.15	0.15
Colorant E110 (mg L^{-1}) [i]	2	2	—
Safflower extract (mg L^{-1}) [i]	400	400	—
Clouding agent (g L^{-1}) [i]	4.60	0.60	—

CON, control beverage; HESP, control beverage supplemented with hesperidin; OJ, orange juice. Values of sugars, citric acid and potassium represent a single measurement of a representative sample at production date. Values of vitamin C, carotenoids and phenolic compounds are means ± SEM (n = 2) at the production date. n. d, not determined. [a] Orange juice was obtained from concentrate (66.1 °Brix, 182 g concentrate per L) provided by Sucocítrico Cutrale (Araraquara, São Paulo, Brazil). [b] Include sucrose (50%), glucose (25%), and fructose (25%). [c] Include ascorbic acid and dehydroascorbic acid. [d] Include epoxy xanthophylls carrying three and four oxygens ($[M + H]^+$ at m/z 585.4 and 601.3–601.5, resp.), lutein, zeaxanthin, zeinoxanthin, β-cryptoxanthin, phytoene, α-carotene, and β-carotene analyzed according to Lux *et al.*[59] [e] Analyzed by HPLC-DAD according to Gilcher *et al.*[60] [f] Analyzed according to IFU methods of analysis no. 58.[61] [g] Provided by Ferrer Health Tech (Murcia, Spain). [h] Other phenolic compounds include polymethoxyflavones. [i] Alcohol-based natural orange aroma, food-grade colors E110, safflower concentrate and cloudifier were provided by Döhler (Darmstadt, Germany) in agreement with European food laws. The use of these ingredients resulted in control beverages indistinguishable to the volunteers.

vided in identical opaque brown glass bottles. Composition details are provided in Table 1. The daily hesperidin intake was 211 ± 3 mg in OJ and 206 ± 3 mg in HESP. A single production batch was used throughout the study, stored at 4 °C. Stability of hesperidin and vitamin C was monitored 11 times during the study and remained stable (data not shown).

Participants attended six clinical visits corresponding to the first and last day of each intervention period. At each visit, fasting vascular assessments and blood collections were performed, followed by postprandial measurements of FMD, at t + 3 h and t + 6 h after the assigned beverage was consumed together with a standardized high-fat meal (900 kcal; 80% fat, 13% carbohydrate, 7% protein). The t + 6 h time point was selected based on evidence indicating peak plasma concentrations of hesperetin metabolites between 4 and 6 hours following orange juice intake.[20,21] Participants also provided a 24 h urine collection corresponding to the 24 h preceding each visit. These biological samples were stored at −80 °C until analysis.

To minimize confounding, participants were asked to refrain from citrus foods throughout the trial and to limit their intake of beverages rich in phenolic compounds (coffee, tea, fruit juice, cocoa, wine) to ≤250 mL day^{-1}. During the 48 h preceding each clinical visit, they abstained from all foods rich in phenolic compounds and consumed at home a dinner devoid of phenolic compounds the evening before. Dietary intake was assessed using self-administered repeated 3-day dietary records collected at study baseline (prior to randomization) and at the midpoint of each intervention period to evaluate energy and macronutrient intake.

Compliance with the intervention was monitored using daily consumption diaries and by counting returned unconsumed bottles, and further evaluated using objective biomarkers, including urinary flavanone excretion and plasma vitamin C concentrations. Participants were asked to report any adverse events during the intervention period.

2.4 Endothelial and vascular outcomes

The primary endpoint was endothelial function assessed by flow-mediated dilation (FMD), the gold-standard non-invasive ultrasound method for evaluating vascular endothelial function in humans.[22] FMD was measured in the left brachial artery positioned longitudinally above the antecubital fossa using a high-resolution ultrasound system equipped with a 7–12 MHz linear array transducer (Vivid S5, GE Healthcare, Versailles, France). A stereotactic mechanical arm device (Vascular Imaging, Amsterdam, Netherlands) with micrometric three-dimensional adjustments ensured stable probe positioning throughout the recording. After baseline image acquisition (10 seconds), reactive hyperemia was induced by inflating a pneumatic cuff placed on the forearm to 220 mmHg for 5 minutes. Upon rapid cuff release, continuous imaging was performed for 3 minutes to capture peak arterial dilation, typically occurring between 30 and 90 seconds post-deflation. All ultrasound sequences were stored digitally for blinded analysis. Arterial diameter was quantified using automated edge-detection software (Hemodyn 3M apparatus; Dinap SRL, Buenos Aires, Argentina). FMD was expressed as the percentage change in arterial diameter between baseline and peak dilation: FMD (%) = [(Diameter_max − Diameter_baseline)/Diameter_baseline] × 100. The methodology complied with international recommendations.[22]

Secondary vascular outcomes included skin microvascular endothelial function, arterial stiffness, and blood pressure. Microvascular endothelial function was assessed on the dorsal hand using laser Doppler flowmetry (Periflux System 5000, Perimed, Sweden) combined with a post-occlusive reactive hyperemia (PORH) procedure as previously described by Roustit and Cracowski.[23] Resting flow (RF) and peak flow (PF) were extracted, and the relative change from RF to PF (RF_PF (Arbitrary Unit)) was automatically calculated and used as the primary PORH-derived index of microvascular endothelial function. Arterial stiffness was assessed using carotid–femoral pulse wave velocity (PWV) *via* applanation tonometry (SphygmoCor system, AtCor Medical Pty Ltd, Sydney, Australia). PWV was calculated automatically as distance (m) divided by pulse transit time (s), as previously described.[24] Systolic and diastolic blood pressures were measured on the right upper arm using a validated automated oscillometric

device (Dinamap V100 – GE Medical Systems), and the mean of three measurements taken one minute apart was used for analysis.

All vascular assessments were conducted after an overnight fast, following at least 5 minutes of rest in the supine position, and in a temperature-controlled room. Measurements were performed at baseline and after each 6-week intervention period. In addition to fasting assessments, postprandial FMD was assessed at t + 3 h and t + 6 h following co-ingestion of the test beverage with a standardized high-fat meal to characterize endothelial responses under metabolic challenge.

Other secondary outcomes included circulating biomarkers of endothelial activation: soluble E-selectin (sE-selectin), soluble intercellular adhesion molecule-1 (sICAM-1), and soluble vascular cell adhesion molecule-1 (sVCAM-1). Plasma concentrations were quantified using commercially available ELISA kits (Diaclone, Besançon, France) according to the manufacturer's instructions.

2.5 Metabolic and anthropometric outcomes

Fasting plasma glucose, triglycerides, total cholesterol, and HDL-cholesterol were measured using standardized laboratory procedures in samples collected at baseline and at the end of each intervention period. LDL-cholesterol was calculated using the Friedewald formula. Fasting plasma insulin was measured by ELISA. At the same time points, anthropometric parameters (waist circumference and body weight) and body composition (assessed using a multi-frequency bioelectrical impedance analyser) were measured.

2.6 Quantification of plasma and urine metabolites of phenolic compounds

Plasma and urinary metabolites of phenolic compounds, including phase II flavanone conjugates (glucuronides and sulfates) as well as their microbial catabolites, were quantified at the beginning and at the end of each 6-week intervention period. Analyses were performed using ultra-high-performance liquid chromatography coupled to diode-array detection and electrospray ionization (negative ion mode) – trapped ion mobility spectrometry-quadrupole time-of-flight high-resolution tandem mass spectrometry (UHPLC-DAD-ESI (−)-TIMS-QTOF-HR-MS/MS), as described in detail in the SI.

In urine, results were expressed as individual and total excreted amounts (μmol) of flavanone metabolites over the 24 h collection period. In plasma, these metabolites were quantified at fasting and postprandially, and exposure was calculated as the area under the concentration–time curve from 0 to 6 hours ($AUC_{0-6\ h}$) following intake of the test beverage with the standardized high-fat meal. $AUC_{0-6\ h}$ was determined using the trapezoidal rule and expressed as apparent bioavailability in μmol h L^{-1}.

2.7 Plasma oxylipins profiling

Oxylipin profiling was conducted in EDTA plasma collected at the end of the intervention periods, at baseline (t + 0 h) and postprandially (t + 6 h). A targeted and quantitative lipidomic approach based on high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS) was used as previously described.[25] Quality controls (QC, *i.e.*, intra-batch QC consisting of a plasma included in each batch of sample preparation and inter-batch QC consisting of a pool of oxylipin extracts) were prepared to control for potential bias due to sample preparation or analytical drifts. A detailed description of the different steps of oxylipins hydrolysis (to release esterified species) and extraction using solid phase extraction and LC-MS/MS analysis (electrospray ionization in negative mode and multiple reaction monitoring) can be found in Dalle *et al.*[26] This method allows the quantification of free and esterified oxylipins from all metabolic branches therefore providing a very comprehensive assessment of all oxylipins involved in the regulation of various cardiometabolic processes.

2.8 Whole blood global transcriptomics and functional analyses

RNA extraction and transcriptome profiling. Total RNA, including coding and non-coding species, was extracted from fasting whole blood samples collected at week 6 using the miRNeasy Mini Kit (Qiagen, Germantown, MD). RNA quality was assessed by agarose gel electrophoresis and NanoDrop ND-1000 spectrophotometry (Thermo Scientific, Wilmington, DE). Transcriptome profiling was performed with the Affymetrix Clariom D human array (Thermo Fisher Scientific, Santa Clara, CA) at the UC Davis Genome Center, following the manufacturer's protocol. Primary data were processed using Transcriptome Analysis Console (Thermo Fisher Scientific), and differential expression was defined as $p < 0.05$ after multiple testing correction.

Bioinformatics analyses. All the bioinformatic tools used for transcriptomic analyses are presented in the SI Table S1. Briefly, multivariate analysis was conducted by PLS-DA (MetaboAnalyst 6.0) and hierarchical clustering by Euclidean distance and Ward's linkage (ClustVis). Differentially expressed transcripts were annotated with ShinyGO to distinguish protein-coding genes from non-coding RNAs. Micro RNAs (miRNA) precursors were further analyzed with miRBase and their targets predicted using Mienturnet and miRTarBase. Long noncoding RNA (lncRNA)–RNA interactions were predicted using LncRRIsearch and targets further analyzed with ShinyGO. Pathway enrichment was assessed with Enrichr, restricted to KEGG 2021[27] and WikiPathways 2024[28] databases, excluding disease and developmental pathways. Overlaps across gene sets were explored with InteractiVenn.

Integration with public datasets. Publicly available gene expression datasets from cardiovascular patients were retrieved from GEO and analyzed with GEO2R. Correlations with intervention-induced transcriptomic profiles were tested using Pearson's correlation (SRplot). Comparative network analyses were performed with QIAGEN Ingenuity Pathway Analysis (IPA), contrasting intervention-induced profiles with IPA pre-analyzed datasets. The databases used are detailed in the SI Table S1.

2.9 Sample size and statistical analysis

The sample size calculation has been described previously.[19] Briefly, 36 participants were required to complete the study to achieve 80% statistical power to detect a minimal absolute difference of 1.6% in FMD, assuming an SD of 1.9%, a two-sided alpha level of 0.017 (adjusted for multiple comparisons), and an intraclass correlation coefficient of 0.5 in the crossover design. A total of 42 participants were planned for inclusion to ensure adequate power in case of attrition.

All statistical analyses were performed using R (version 4.5.2). Linear mixed-effects models (LMMs) were fitted using restricted maximum likelihood (REML) for continuous outcomes, with the *lmerTest* package (version 3.2-0). All models included a random intercept for subject to account for within-individual correlation inherent to the crossover design. LMMs were adjusted for period and sequence effects to account for potential residual or order-related influences.

Fasting vascular and systemic outcomes measured at baseline and after 6 weeks were analyzed using LMMs including treatment (CON, HESP, OJ), period (P1, P2, P3), and sequence as fixed effects. Period-specific baseline values were included as covariates. The false discovery rate (FDR) across multiple outcomes was controlled using the Benjamini–Hochberg procedure. All tests were two-sided, and statistical significance was defined as $p < 0.05$. *Post-hoc* pairwise comparisons between treatments were adjusted for multiple testing.

Carryover effects were not included in the primary models due to the limited sample size and the resulting risk of overparameterization and unstable estimates. This approach was supported by washout periods of 4–6 weeks, considered sufficient to minimize potential carryover effects. Potential residual effects were further explored through complementary analysis. Specifically, an interaction term between treatment and period was introduced in secondary models to evaluate whether treatment effects varied across study periods. Model fit was compared with and without this interaction using maximum likelihood estimation, likelihood ratio tests, and information criteria (AIC and BIC). No consistent improvement in model fit was observed, and no coherent pattern emerged across outcomes. In addition, potential carryover, period and sequence effects on vascular outcomes were further explored using principal component analysis (PCA) as an exploratory multivariate approach. These analyses were intended to provide descriptive insights into the data structure and should be interpreted cautiously.

Postprandial FMD responses were analyzed using LMMs including treatment, time after the challenge test (0, 3, and 6 h), period, and sequence as fixed effects, with a treatment × time interaction term to assess differential postprandial responses. Postprandial oxylipin concentrations were analyzed using LMMs including treatment, time, and their interaction as fixed effects, with subject as a random effect. Data were log-transformed prior to analysis to address skewness and heteroscedasticity.

Twenty-four-hour urinary excretion levels and postprandial plasma $AUC_{0-6\ h}$ of phenolic metabolites were analyzed using generalized linear mixed-effects models (GLMMs) fitted with a Tweedie distribution and log link function (*glmmTMB*, version 1.1.11), due to the semi-continuous distribution of the data characterized by excess zeros and right-skewed positive values. The Tweedie power parameter was estimated from the data by maximum likelihood within the glmmTMB framework. For urinary metabolite excretion, fixed effects included treatment, study phase (baseline *vs.* post-intervention at 6 weeks), their interaction, as well as period and sequence. For postprandial plasma AUC outcomes, study phase and its interaction with treatment were not included. Subject was modeled as a random effect.

Additional LMMs were performed to explore whether treatment effects on FMD at 6 weeks were modified by (1) baseline FMD values and (2) urinary excretion levels of individual or total phase II flavanone conjugates, by including appropriate interaction terms in the models.

Missing data were handled according to the type of outcome. For vascular measurements, extreme outlier values identified during predefined quality control procedures (6 baseline FMD and 2 baseline RF_PF measurements) were excluded from the dataset prior to analysis. Remaining vascular values were imputed using a *k*-nearest neighbours (KNN) approach ($k = 5$). For oxylipin analysis, analytes with more than 30% missing values within each experimental group were excluded to avoid unreliable estimates due to sparse detection. Remaining missing values were assumed to be below the limit of detection (LOD) and were imputed using random values between 10% of the LOD and the LOD itself. Prior to filtering and imputation, missing values represented 5.6% of the oxylipin dataset. For bioavailability outcomes, incomplete or missing urine collections were considered missing at random (MAR), as the missingness was not attributed to biological factors. As these outcomes were analyzed using GLMMs, which accommodate unbalanced data under this assumption, all available observations were included without imputation.

3 Results

3.1 Participant flow and baseline characteristics

Participant flow is shown in Fig. 1. Fifty participants were screened and provided written informed consent. Recruitment began in February 2021, and the last participant completed the study protocol in June 2023. Although both men and postmenopausal women were eligible according to the study protocol, only men volunteered and were enrolled. Eleven participants were excluded after the first visit, mainly due to not meeting inclusion criteria or withdrawal of consent.

The remaining 39 participants were randomized to the intervention sequences and initiated the study. Two participants discontinued after randomization, resulting in 37 participants who completed all intervention periods and were included in the analysis.

According to the prespecified sample size calculation, 36 completers were required to achieve adequate statistical power

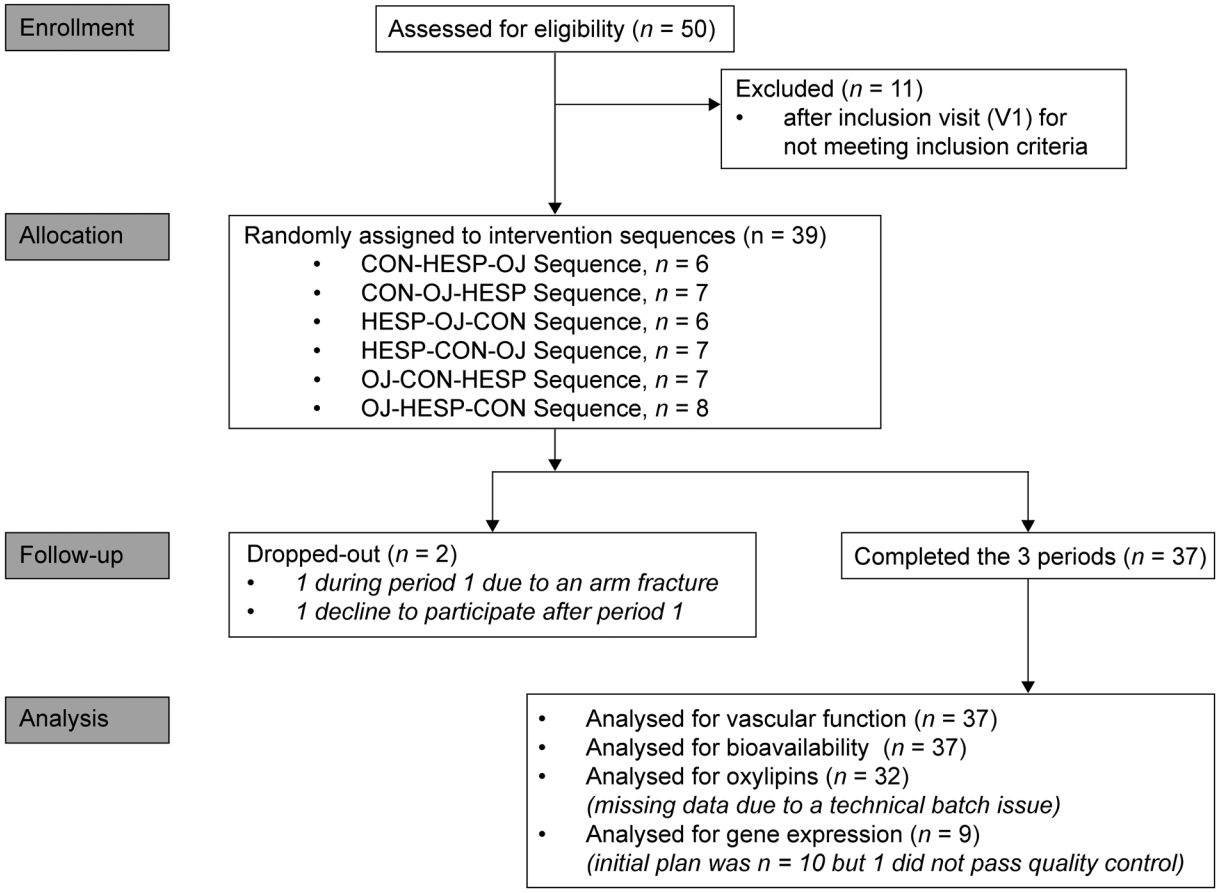

Fig. 1 Flow diagram of participant recruitment, randomization, and follow-up.

for the primary outcome (FMD). The recruitment target of 42 participants defined in the study protocol accounted for anticipated dropouts.[19] Given the high adherence observed during the trial, recruitment was stopped after 39 participants had been enrolled, resulting in 37 completers, thereby exceeding the required sample size and ensuring that statistical power was maintained.

Vascular outcomes, metabolic, and bioavailability analyses were performed in all 37 participants. Oxylipin analyses were available for 32 participants due to a technical issue affecting one analytical batch. Transcriptomic analyses were prespecified to be conducted in a subset of 10 participants with high RNA integrity; however one sample did not pass microarray quality control, resulting in a final dataset of 9 participants.

Baseline characteristics of the participants are shown in Table 2. Participants (n = 37) had a mean age of 48.8 ± 7.8 years and a BMI of 26.4 ± 1.8 kg m^{-2}, with a mean waist circumference of 100.2 ± 4.0 cm, consistent with central adiposity. Blood pressure values were within the normotensive range (128.4 ± 13.7 mm Hg systolic and 78.1 ± 8.2 mm Hg diastolic), and metabolic parameters were generally within recommended ranges. Fasting plasma glucose was 4.87 ± 0.35 mmol L^{-1}, confirming normoglycemia. Mean total cholesterol was 5.37 ± 0.91 mmol L^{-1}, with LDL-cholesterol at 3.44 ± 0.84 mmol L^{-1}, HDL-cholesterol at 1.38 ± 0.20 mmol L^{-1}, and triglycerides at 1.20 ± 0.41 mmol L^{-1}. The calculated mean 10 years Framingham risk score was 7.16 ± 5.74%, corresponding to a low to moderate cardiovascular risk. Overall, these baseline characteristics indicate that participants were generally metabolically healthy yet exhibited features compatible with

Table 2 Baseline characteristics of participants at inclusion (n = 37)

	Mean ± SD	Min–max
Age (years)	48.81 ± 7.75	40–65
Body mass index (kg m^{-2})	26.42 ± 1.79	23.9–29.8
Waist circumference (cm)	100.15 ± 4.00	94–109
Systolic blood pressure (mmHg)	128.38 ± 13.66	104–174
Diastolic blood pressure (mmHg)	78.05 ± 8.18	57–92
Glucose (mmol L^{-1})	4.87 ± 0.35	4.05–5.76
Insulin (pmol L^{-1})	52.17 ± 33.16	13.9–14.2
Triglycerides (mmol L^{-1})	1.20 ± 0.41	0.42–0.7
Total cholesterol (mmol L^{-1})	5.37 ± 0.91	3.52–7.32
HDL-cholesterol (mmol L^{-1})	1.38 ± 0.20	0.97–2.62
LDL-cholesterol (mmol L^{-1})	3.44 ± 0.84	1.87–5.00
Framingham risk score (%)[a]	7.16 ± 5.74	2–31

[a] Estimates the probability of a cardiovascular event occurring within the next 10 years in individuals without established cardiovascular disease.

early cardiometabolic alterations (age, abdominal overweight without obesity).

3.2 Dietary intake and compliance

Consumption of the study beverages provided an additional daily energy intake of 121.6 kcal, without significantly altering reported total energy or macronutrient intake, as assessed by repeated 3-day dietary records (SI Table S2), suggesting no compensatory dietary adjustments.

Compliance with the intervention was high, as indicated by bottle counts and diary records (>95% of beverages consumed), and was further supported by objective biomarkers. Substantial urinary concentrations of hesperetin phase II metabolites were observed exclusively in the HESP and OJ groups, while naringenin metabolites were detected only in the OJ group, confirming adherence to the assigned beverages (Table 3). Within-subject changes (Δ) in fasting plasma vitamin C concentrations differed significantly between treatments (Friedman test, $p < 0.001$). *Post-hoc* pairwise comparisons using Wilcoxon tests showed significantly greater increases following the OJ intervention (mean 38 ± 1 μmol L^{-1} at 6 weeks) compared with CON (25 ± 2 μmol L^{-1}) and HESP (23 ± 2 μmol L^{-1}), according to adjusted *p*-values ($p < 0.001$), whereas no significant difference was observed between CON and HESP. This result was consistent with the presence of vitamin C in the juice. No adverse events or safety concerns were reported during the study.

3.3 Exploratory PCA analysis of potential sequence and carryover effects

A principal component analysis (PCA) was performed on the vascular and endothelial dataset as an exploratory approach to examine the global variance structure and visually inspect potential period, sequence, and carryover effects. As shown in SI Fig. S1A, baseline PCA plots demonstrated substantial overlap among the different randomization sequences, indicating comparable starting profiles. The corresponding PCA performed on Δ (6 weeks – baseline) values showed a similar pattern, suggesting no clear evidence of a treatment-sequence effect on vascular outcomes. To further evaluate potential carryover effects, the same PCA score plots were examined according to the treatment received in the preceding intervention period (SI Fig. S1B). Both baseline and Δ plots showed broad overlap among treatment history groups (CON, HESP, OJ or none), with no clustering pattern suggestive of residual treatment influence. Overall, these analyses did not suggest the presence of sequence or carryover effects.

3.4 Flavanone bioavailability and phenolic microbial catabolites

Postprandial plasma exposure to phase II hesperetin metabolites, assessed by $AUC_{0-6\ h}$, reached 3.3 ± 0.6 μmol h L^{-1} following HESP supplementation and 2.3 ± 0.4 μmol h L^{-1} after OJ consumption, whereas hesperetin conjugates were undetectable after CON intake (Table 3). No significant difference was observed between HESP and OJ for plasma hesperetin metabolite exposure. Narirutin was present in OJ as naringenin rutinoside (132 mg L^{-1}, 0.23 mM), at concentrations approximately 4.9-fold lower than hesperidin (647 mg L^{-1}, 1.06 mM). However, plasma exposure to naringenin conjugates was only about 3.7-fold lower ($AUC_{0-6\ h}$: 0.62 ± 0.07 μmol h L^{-1}).

In 24 h urine, hesperetin conjugates were detected only in trace amounts at baseline and after consumption of CON, whereas total hesperetin conjugates reached 16 ± 2 μmol following HESP and 14 ± 2 μmol after OJ (Table 3). Naringenin conjugates were quantified at 6 μmol after OJ consumption, corresponding to a 2.3-fold lower excretion than hesperetin conjugates. These recoveries corresponded to 4.8% and 3.9% of the ingested hesperidin dose following HESP and OJ consumption, respectively, and to 8.2% of the ingested narirutin dose.

The main circulating and urinary phase II hesperetin metabolites were hesperetin-3′-glucuronide and hesperetin-3′-sulfate. In urine, these metabolites accounted for 73% of total hesperetin conjugates excreted after HESP intake and 68% after OJ intake, with hesperetin-3′-glucuronide being the predominant form in both conditions. The remaining metabolites mainly consisted of hesperetin-7-glucuronide (~10%), sulfoglucuronide derivatives (~12%), and, to a lesser extent, diglucuronides.

In addition to flavanone conjugates, microbial catabolites derived from phenolic compounds were analysed. The known hesperetin microbial catabolite (*R*/*S*)-3-hydroxy-3-(3′-hydroxy-4′-methoxyphenyl)propanoic acid (HMPHA) was detected at comparable levels following HESP and OJ consumption (33–43 μmol/24 h urine, Table 3). Plasma concentrations of 3-(3′-hydroxy-4′-methoxyphenyl)propanoic acid (HMPPA) were significantly higher after OJ consumption (3.79 ± 0.91 μmol h L^{-1} plasma) than after HESP consumption (1.54 ± 0.52 μmol h L^{-1}), possibly reflecting the contribution of additional phenolic compounds present in orange juice.

As a relatively non-specific microbial catabolite, (*R*/*S*)-3-hydroxy-3-(3′-hydroxyphenyl)propanoic acid (HPHPA) was the most abundant and second most abundant microbial catabolite detected in plasma (52–54 μmol h L^{-1}) and 24 h urine (121–126 μmol), respectively. The level of 4′-hydroxyhippuric acid was significantly higher after OJ consumption than after HESP consumption, possibly due to naringenin derivatives (*e.g.* narirutin, see Table 1) exclusively present in orange juice providing a 4-hydroxy substituted phenyl ring. Additionally, plasma hippuric acid was significantly higher following OJ consumption (44 ± 3 μmol h L^{-1}) than after HESP consumption (36 ± 2 μmol h L^{-1}). The total amount of microbial catabolites was significantly higher in plasma after OJ consumption (106 ± 5 μmol h L^{-1}) than after HESP consumption (95 ± 3 μmol h L^{-1}) and CON consumption (93 ± 5 μmol h L^{-1}), whereas total urinary microbial catabolite excretion was similar across CON, HESP and OJ interventions (2648–3257 μmol, Table 3).

Fig. 2 highlights the marked interindividual variability in the total 24 h urinary excretion of hesperetin conjugates within HESP and OJ groups, ranging from negligible amounts in some participants to over 40 μmol in others. When partici-

Table 3 24 h urinary excretion and postprandial plasma exposure ($AUC_{0-6\ h}$) to phenolic metabolites after 6-week consumption the study beverages

	Urine					Plasma			
	Baseline	CON	HESP	OJ	Treatment × study phase	CON	HESP	OJ	Treatment
Sum hesperetin conjugates	0.583 ± 0.271^{A}	0.176 ± 0.096^{A,a}	16.243 ± 2.486^{B,b}	13.619 ± 1.983^{B,b}	***	0.008 ± 0.008^{a}	3.281 ± 0.577^{b}	2.306 ± 0.437^{b}	***
Hes-GCA-SO_3H (1)^{a}	0.089 ± 0.042^{A}	0.035 ± 0.017^{A,a}	2.015 ± 0.313^{B,b}	1.854 ± 0.288^{B,b}	***	n. d.	0.003 ± 0.002	0.005 ± 0.005	
Hes-di-GCA (1)^{a}	0.022 ± 0.013	n. d.	0.130 ± 0.051	0.384 ± 0.073	NE	n. d.	0.025 ± 0.021	0.036 ± 0.020	
Hes-GCA-SO_3H (2)^{a}	n. d.	n. d.	n. d.	n. d.		n. d.^{a}	0.193 ± 0.058^{b}	0.063 ± 0.021^{c}	***
Hes-di-GCA (2)^{a}	0.015 ± 0.008	n. d.	0.639 ± 0.128	0.501 ± 0.080	NE	n. d.	0.054 ± 0.029	0.034 ± 0.015	
Hes-7-GCA	0.055 ± 0.028^{A}	0.014 ± 0.010^{A,a}	1.652 ± 0.267^{B,b}	1.579 ± 0.281^{B,b}	***	n. d.^{a}	0.506 ± 0.095^{b}	0.256 ± 0.049^{b}	***
Hes-3′-GCA	0.320 ± 0.150^{A}	0.107 ± 0.064^{A,a}	9.591 ± 1.476^{B,b}	7.232 ± 1.030^{B,b}	***	0.002 ± 0.002^{a}	1.219 ± 0.212^{b}	0.879 ± 0.163^{b}	***
Hes-3′-SO_3H	0.081 ± 0.037^{A}	0.020 ± 0.014^{A,a}	2.215 ± 0.332^{B,b}	2.070 ± 0.326^{B,b}	***	0.006 ± 0.006^{a}	1.282 ± 0.197^{b}	1.032 ± 0.195^{b}	***
Sum naringenin conjugates	0.612 ± 0.173^{A}	0.307 ± 0.172^{A,a}	1.043 ± 0.198^{A,b}	6.141 ± 0.888^{B,c}	***	0.001 ± 0.001^{a}	0.012 ± 0.006^{a}	0.619 ± 0.070^{b}	***
Nar-di-GCA (1)^{a}	n. d.	n. d.	n. d.	0.092 ± 0.090		n. d.	n. d.	n. d.	
Nar-di-GCA (2)^{a}	n. d.	n. d.	n. d.	0.019 ± 0.010		n. d.	n. d.	n. d.	
Nar-di-GCA (3)^{a}	n. d.	n. d.	n. d.	0.015 ± 0.010		n. d.	n. d.	n. d.	
Nar-7-GCA	0.223 ± 0.063^{A}	0.123 ± 0.070^{A,a}	0.411 ± 0.086^{A,b}	2.483 ± 0.346^{B,c}	***	n. d.^{a}	0.003 ± 0.002^{a}	0.253 ± 0.036^{b}	***
Nar-4′-GCA	0.389 ± 0.122^{A}	0.184 ± 0.102^{A,a}	0.632 ± 0.115^{A,b}	3.532 ± 0.502^{B,c}	***	0.001 ± 0.001^{a}	0.008 ± 0.005^{a}	0.366 ± 0.040^{b}	***
Sum naringenin & hesperetin conjugates	1.195 ± 0.424^{A}	0.483 ± 0.256^{A,a}	17.285 ± 2.621^{B,b}	19.760 ± 2.630^{B,b}	***	0.009 ± 0.009^{a}	3.294 ± 0.581^{b}	2.925 ± 0.475^{b}	***
Sum microbial catabolites	2473.789 ± 154.305	2809.121 ± 253.887	2648.383 ± 258.892	3257.359 ± 334.164	n. s.	92.724 ± 4.508^{a}	91.414 ± 2.462^{a}	103.476 ± 4.455^{b}	***
4′-Hydroxyhippuric acid	27.843 ± 1.925^{A}	29.899 ± 3.529^{Aa}	23.297 ± 1.728^{Aa}	42.206 ± 3.127^{Bb}	***	0.199 ± 0.054^{a}	0.117 ± 0.055^{a}	1.659 ± 0.149^{b}	***
3′-Hydroxyhippuric acid	77.880 ± 6.998	91.140 ± 14.607	91.731 ± 11.090	101.943 ± 18.350	n. s.	0.973 ± 0.217	1.044 ± 0.205	1.326 ± 0.258	n. s.
Hippuric acid	2263.706 ± 143.133	2565.030 ± 232.975	2376.159 ± 245.648	2942.718 ± 304.626	n. s.	37.904 ± 3.396^{a}	35.558 ± 1.994^{a}	43.775 ± 2.736^{b}	***
HPHPA	99.653 ± 9.259	119.181 ± 21.153	121.986 ± 16.967	126.640 ± 20.173	n. s.	53.648 ± 2.277	52.604 ± 2.1134	52.170 ± 2.716	n. s.
HMPHA	4.301 ± 1.444^{A}	3.872 ± 1.227^{Aa}	32.788 ± 3.687^{Bb}	43.446 ± 5.738^{Bb}	**	n. d.	0.551 ± 0.276	0.757 ± 0.199	
HMPPA	0.408 ± 0.281	n. d.	2.421 ± 2.388	0.406 ± 0.280		n. d.^{a}	1.541 ± 0.523^{b}	3.790 ± 0.907^{c}	***
Sum conjugates & microbial catabolites	2474.984 ± 154.399	2809.603 ± 253.915	2665.668 ± 259.159	3277.119 ± 334.597	n. s.	92.733 ± 4.508^{a}	94.708 ± 2.641^{b}	106.401 ± 4.528^{c}	***

Values are means ± SEM (n = 37), expressed in μmol per 24 h (urine) and μmol h L^{-1} (plasma). CON, control beverage; HESP, control beverage supplemented with hesperidin; OJ, orange juice; Hes, hesperetin; Nar, naringenin; GCA, glucuronide; SO_3H, sulfate; HMPHA, (*R*/*S*)-3-hydroxy-3-(3′-hydroxy-4′-methoxyphenyl) propanoic acid; HMPPA, 3-(3′-hydroxy-4′-methoxyphenyl) propanoic acid; HPHPA, (*R*/*S*)-3-hydroxy-3-(3′-hydroxyphenyl) propanoic acid. n. d., not detected (values below limit of detection); n. s., not significant. NE, not estimable; **adjusted $P < 0.01$; ***adjusted $P < 0.001$ (Benjamini-Hochberg correction). GLMMs were not fitted for HMPPA in urine as well as Hes-GCA-SO_3H (isomer 1), Hes-di-GCA (isomers 1 and 2) and HMPHA in plasma due to detection in <70% of samples within each intervention group. For some metabolites indicated as NE for the interaction effect, the Tweedie GLMM failed to converge when including the treatment × study phase interaction, due to complete separation. These metabolites were detected almost exclusively after 6 weeks in the OJ and HESP groups. Study phase refers to baseline *versus* post-intervention (6 weeks). Superscript capital letters indicate differences from baseline within each intervention, and lowercase letters indicate differences between interventions, based on *post-hoc* comparisons following GLMMs. [a] Tentatively identified compounds.

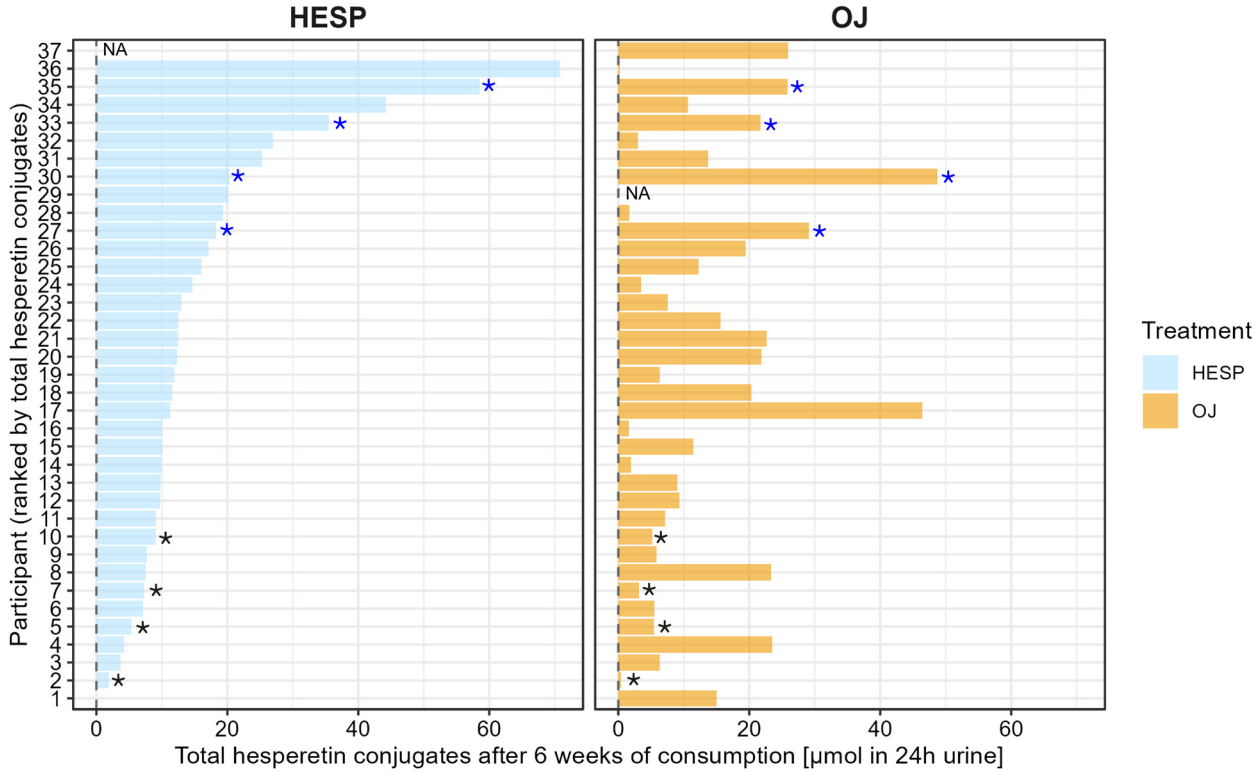

Fig. 2 Individual 24 h urinary excretion of phase II hesperetin metabolites in the HESP and OJ groups after each 6-week intervention period. Each bar represents the total amount of quantified hesperetin metabolites excreted over 24 h for an individual participant (n = 37). Hesperetin metabolites include hesperetin-7-glucuronide, hesperetin-3′-glucuronide, hesperetin-3′-sulfate, hesperetin-diglucuronide (isomers 1 and 2), and hesperetin-glucuronide-sulfate (isomers 1 and 2). NA indicates missing or incomplete 24 h urine collections. Blue asterisks (*) indicate high excretors and black asterisks (*) indicate low excretors across both interventions. HESP, control beverage supplemented with hesperidin; OJ, orange juice.

pants were ranked according to excretion levels, four individuals were consistently among the ten lowest excretors and four among the ten highest excretors across both interventions. Thus, despite the intraindividual variability, partial consistency in individual excretion rankings across the two interventions was observed.

3.5 Primary outcome: FMD in fasting and postprandial conditions

Endothelial function was assessed using flow-mediated dilation (FMD) under both fasting conditions and during a postprandial metabolic challenge. After the 6-week intervention, fasting FMD remained unchanged compared with baseline (overall mean: 5.03 ± 0.20%), and no significant differences were observed between treatments (Table 4). As shown in violin plots (Fig. 3), fasting FMD distributions were stable across intervention periods and exhibited marked interindividual variability. The distribution appeared slightly wider in the HESP arm, although this was not statistically significant.

During the postprandial challenge, plasma triglycerides increased after consumption of the high-fat meal and reached a plateau at 3 and 6 hours (SI Table S3), with $P < 0.001$ for time effect. Despite this lipemic response which was expected to transiently affect endothelial function, FMD values remained stable over time, with no decline observed at either time point. In addition, no differences between intervention arms were observed despite the presence of hesperetin conjugates in the plasma of HESP and OJ groups (Fig. 4). The mixed-effects model showed no significant effects of treatment, time, or interaction (all $P > 0.05$) (SI Table S3).

Overall, these findings indicate that neither hesperidin supplementation nor orange juice consumption modified fasting or postprandial endothelial function compared with the control beverage following 6 weeks of intervention.

3.6 Secondary vascular outcomes

Secondary vascular and endothelial parameters were also evaluated to assess potential treatment-related effects (Table 4). After 6 weeks, no significant differences between treatments were observed for skin microvascular reactivity or brachial blood pressure. A nominal treatment effect was observed for arterial stiffness in the unadjusted model ($P = 0.05$), but this effect did not remain significant after adjustment for multiple testing, and *post hoc* analysis indicated only a trend for lower PWV after OJ compared with CON ($P = 0.078$). Fasting concentrations of soluble ICAM-1 and VCAM-1 were unchanged. A nominal treatment effect was observed for E-selectin ($P = 0.03$), which was not retained after adjustment for multiple compari-

Table 4 Fasting vascular, endothelial, metabolic and anthropometric outcomes after 6-week interventions

	Means ± SEMs	Estimated marginal means ± SEMs				Treatment effect	
	Baseline	CON	CON-HESP	CON-OJ	HESP-OJ	*P* value	*P* value adjusted
Vascular function biomarkers							
FMD (%)	5.03 ± 0.20	5.20 ± 0.33	0.07 ± 0.37	0.31 ± 0.37	0.24 ± 0.37	0.67	0.84
RF_PF (A.U.)	367.49 ± 28.10	354.60 ± 53.38	−4.25 ± 75.42	−20.60 ± 75.17	−16.35 ± 75.25	0.96	0.96
SBP (mm Hg)	125.46 ± 1.08	124.96 ± 1.35	1.91 ± 1.71	1.57 ± 1.71	−0.34 ± 1.71	0.49	0.82
DBP (mm Hg)	75.23 ± 0.87	75.34 ± 1.13	0.36 ± 1.42	1.62 ± 1.42	1.26 ± 1.41	0.49	0.82
PP (mm Hg)	50.08 ± 0.86	49.16 ± 1.06	1.06 ± 1.28	−0.51 ± 1.29	−1.57 ± 1.28	0.45	0.82
PWV	6.76 ± 0.09	6.91 ± 0.17	0.02 ± 0.13	0.28 ± 0.13	0.26 ± 0.13	0.05	0.27
HR (bpm)	59.34 ± 0.72	60.19 ± 1.18	0.44 ± 1.67	−1.07 ± 1.67	−1.50 ± 1.67	0.65	0.84
Endothelial biomarkers							
sICAM (ng mL^{-1})	1108.56 ± 30.06	1179.70 ± 51.77	45.94 ± 61.85	25.89 ± 61.31	−20.05 ± 60.96	0.76	0.84
sVCAM (ng mL^{-1})	1061.57 ± 31.31	1058.79 ± 50.05	−22.90 ± 61.48	−97.34 ± 61.52	−74.43 ± 61.24	0.25	0.82
Selectin (ng mL^{-1})	129.07 ± 5.15	141.51 ± 2.78	8.51 ± 3.91	9.14 ± 3.92	0.64 ± 3.92	0.03	0.27
Metabolic parameters							
Glucose (mmol L^{-1})	5.43 ± 0.07	5.46 ± 0.13	−0.22 ± 0.19	0.02 ± 0.19	0.24 ± 0.19	0.37	0.63
Insulin (pmol L^{-1})	59.04 ± 4.59	63.37 ± 6.32	8.71 ± 4.64	5.58 ± 4.69	−3.12 ± 4.66	0.17	0.51
HOMA-IR	1.99 ± 0.17	2.15 ± 0.23	0.30 ± 0.18	0.22 ± 0.18	−0.08 ± 0.18	0.21	0.51
Triglycerides (mmol L^{-1})	1.22 ± 0.04	1.33 ± 0.07	0.02 ± 0.08	0.04 ± 0.08	0.02 ± 0.08	0.90	0.94
Total-chol (mmol L^{-1})	5.08 ± 0.08	5.06 ± 0.09	0.03 ± 0.12	−0.01 ± 0.12	−0.04 ± 0.12	0.94	0.94
LDL (mmol L^{-1})	3.22 ± 0.08	3.18 ± 0.08	0.03 ± 0.11	−0.01 ± 0.11	−0.04 ± 0.11	0.92	0.94
HDL (mmol L^{-1})	1.31 ± 0.03	1.26 ± 0.02	−0.02 ± 0.03	0.01 ± 0.03	0.03 ± 0.03	0.71	0.94
Uric Acid (μmol L^{-1})	379.85 ± 5.28	390.40 ± 5.63	12.69 ± 6.77	14.52 ± 6.80	1.83 ± 6.74	0.07	0.51
Anthropometric parameters							
Waist Circumference (cm)	99.47 ± 0.37	99.84 ± 0.37	0.91 ± 0.52	0.85 ± 0.52	−0.05 ± 0.52	0.14	0.51
Body weight (kg)	82.62 ± 0.69	82.95 ± 0.21	0.11 ± 0.29	0.08 ± 0.29	−0.03 ± 0.29	0.93	0.94
Body lean mass (%)	78.56 ± 0.33	76.72 ± 1.34	−2.72 ± 1.88	−0.45 ± 1.87	2.27 ± 1.87	0.30	0.60
Body fat mass (%)	21.41 ± 0.33	21.54 ± 0.40	0.97 ± 0.57	0.23 ± 0.57	−0.73 ± 0.57	0.21	0.51

Values are estimated marginal means ± SEMs (n = 37). Data were analyzed using linear mixed-effects models (LMMs); with treatment, period and sequence included as fixed effects, baseline measurement as a covariate, and subject included as a random effect. The treatment × period interaction was tested but not retained. *P*-Values were adjusted for multiple testing using the Benjamini-Hochberg correction. CON, control beverage; HESP, control beverage supplemented with hesperidin; OJ, orange juice. FMD, flow mediated dilation in brachial artery; RF_PF, change of flow from peak to the rest flow in skin vessels; SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; PWV, pulse wave velocity; HR, heart rates; sICAM-1, soluble intercellular adhesion molecule-1; sVCAM-1, soluble vascular cell adhesion molecule-1; E-selectin, endothelial-selectin; HOMA-IR, homeostatic model assessment of insulin resistance; chol, cholesterol; A.U., arbitrary unit.

sons. *Post hoc* analysis showed only trends for lower concentrations after OJ and HESP compared with CON (P = 0.057 and P = 0.081, respectively). Overall, neither hesperidin supplementation nor orange juice induced measurable changes in secondary vascular or endothelial biomarkers under fasting conditions.

3.7 Secondary metabolic and anthropometric outcomes

After 6 weeks of intervention, fasting glucose, insulin concentrations, and homeostatic model assessment of insulin resistance (HOMA-IR) did not differ between treatments and remained unchanged from baseline (Table 4). Similarly, no treatment effects were observed for plasma triglycerides, total cholesterol, HDL-cholesterol, and LDL-cholesterol, uric acid, and no significant changes from baseline were detected. Waist circumference, body weight, and body composition (fat and lean mass) also remained unchanged across intervention periods, with no differences between treatments or relative to baseline (Table 4).

3.8 Postprandial oxylipin response

To evaluate whether beverage consumption modulated the postprandial oxylipin response, a comprehensive oxylipin profile comprising 62 quantifiable oxylipins was assessed at baseline (t + 0 h) and t + 6 h after a high-fat challenge meal. The high-fat challenge significantly altered circulating levels of 13 oxylipins; however, these postprandial changes were similar across all study beverages, indicating no beverage-specific effect over the 6-week intervention (Fig. 5 and SI Table S4). Of these, the levels of 11 oxylipins increased by 3–23% following the challenge, whereas two linoleic acid-derived oxylipins showed a slight decrease (−2% for 9-HODE and −9% for 15-HODE) (Fig. 5D). Oxylipins that increased after the high-fat challenge were predominantly derived from arachidonic acid, including 12-HETE, 14,15-DiHETrE, 16-HETE, 5,6-DiHETrE, 5-HETE, and 8,9-DiHETrE (Fig. 5A). In addition, two eicosapentaenoic acid-derived oxylipins (5-HEPE and 8,9-DiHETE), two dihomo-γ-linolenic acid-derived oxylipins (12-HETrE and 5-HETrE), and one docosahexaenoic acid-derived oxylipin (14-HDHA) were increased (Fig. 5B, C, and E, respectively). Overall, the postprandial oxylipin profile was characterized by changes in metabolites associated with inflammatory, platelet-related, and vascular pathways classically involved in the response to a high-fat challenge, including products of the 5-lipoxygenase (5-HETE, 5-HETrE, and 5-HEPE), 12-lipoxygenase (12-HETE and 12-HETrE), and cytochrome P450/soluble epoxide hydrolase pathways (DiHETrE).

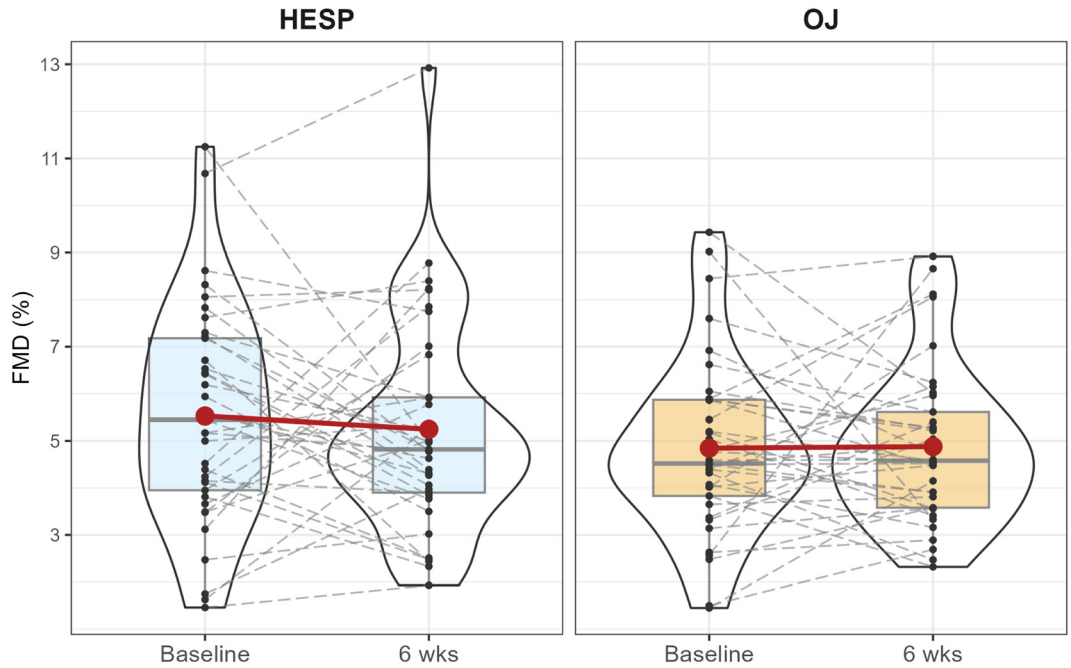

Fig. 3 Individual changes in fasting flow-mediated dilation (FMD) values and individual responses after each 6-week of intervention period across study arms. Violin plots showing the distribution of FMD (%) values at baseline and after 6 weeks in the three intervention arms. Each violin represents the distribution of the data, with embedded boxplots indicating the median and interquartile range. Dashed lines connect paired individual values, and large red circles indicate mean ± SEM (n = 37). FMD, flow-mediated dilation; HESP, control beverage supplemented with hesperidin; OJ, orange juice.

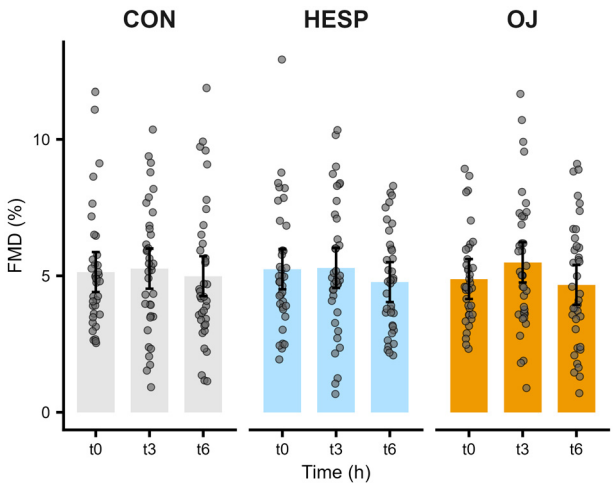

Fig. 4 Postprandial changes in flow-mediated dilation (FMD) over 6 h following consumption of a high-fat meal with the study beverages after each 6-week intervention period. FMD was assessed in the fasting state (t + 0 h) and postprandially (t + 3 h and t + 6 h). Data are presented as estimated marginal means derived from a linear mixed-effects model (LMM), with individual values shown (n = 37). No significant effects of treatment, time, or treatment × time interaction were observed (P > 0.05). FMD, flow-mediated dilation; CON, control beverage; HESP, control beverage supplemented with hesperidin; OJ, orange juice.

3.9 Exploratory interaction analysis on fasting FMD responses

The relationships between FMD responses and (1) baseline FMD values and (2) 24 h urinary excretion levels of flavanone conjugates were assessed using linear mixed models with continuous variables (SI Table S5). No significant treatment-by-baseline FMD interaction was observed (P > 0.05). Similarly, no significant interaction effects were detected between FMD responses and urinary excretion levels of total flavanone phase II metabolites, total hesperetin metabolites, or individual hesperetin metabolites (P > 0.05). Comparable non-significant results were observed when models were applied using changes in urinary metabolite excretion over the 6-week intervention periods (Δ values). Overall, these exploratory analyses provided no evidence for treatment effect modification by baseline FMD or flavanone metabolite exposure.

3.10 Exploratory transcriptomic analysis

In the subset of nine participants, multivariate analyses (PLS-DA and hierarchical clustering) revealed distinct global transcriptional signatures following OJ and HESP intake compared with CON (Fig. 6A and SI Fig. S2), indicating differential global gene expression profiles between interventions. Following statistical analysis, both interventions were associated with differential expression of numerous coding and non-coding RNAs, with only partial overlap between OJ and HESP, including 26 mRNAs, 5 miRNAs, and 6 lncRNAs commonly regulated (Fig. 6B and SI Table S6).

Functional enrichment analyses of differentially expressed genes, together with predicted miRNA and lncRNA targets, highlighted pathways related to cardiovascular function and metabolism (Fig. 6C). OJ primarily influenced pathways linked to vascular physiology and endothelial function, including

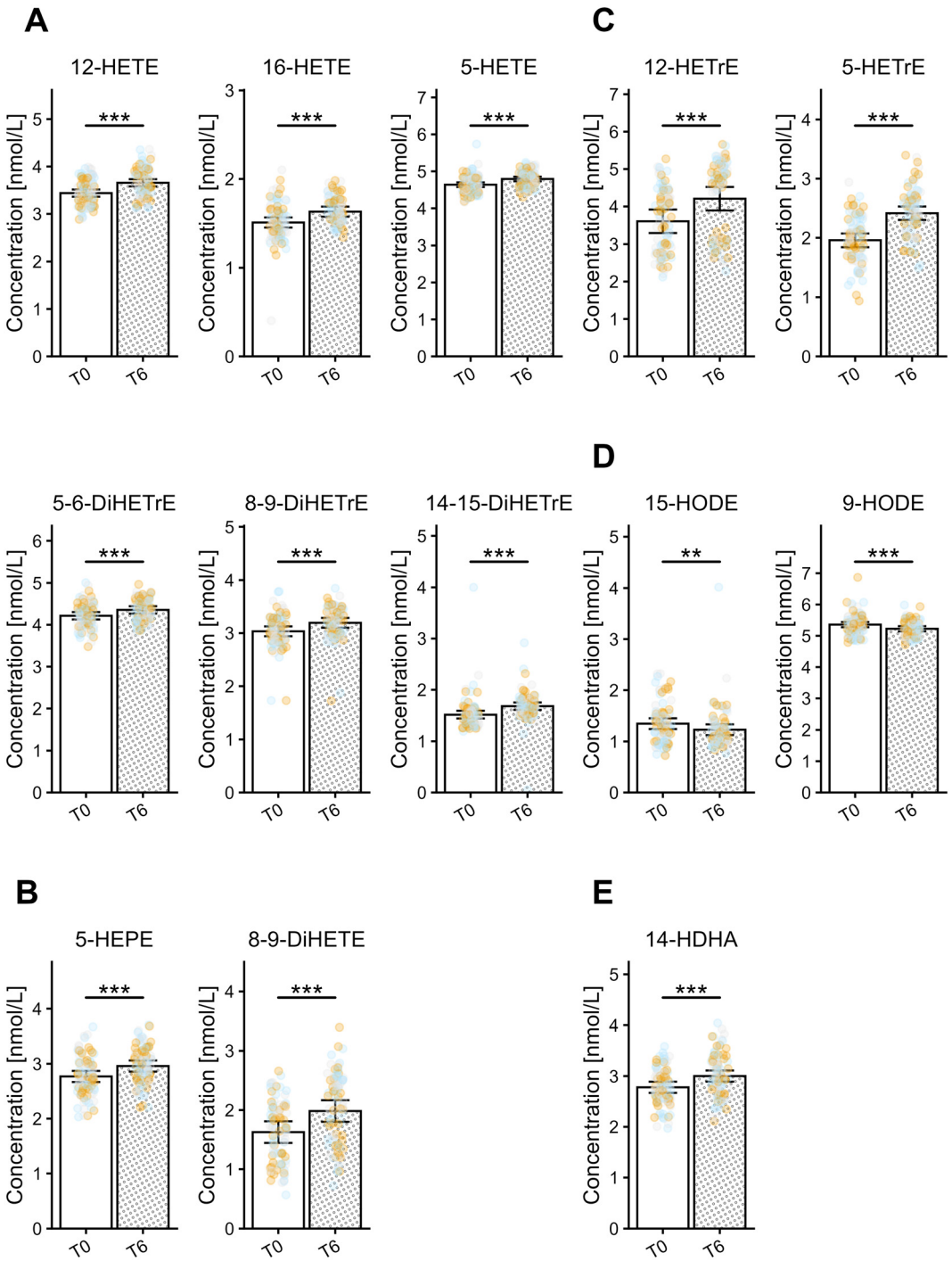

Fig. 5 Postprandial changes in plasma oxylipins over 6 h following a high-fat meal consumed with the study beverages after each 6-week intervention period. Estimated marginal means at baseline (t + 0 h) and 6 h after the high-fat meal (t + 6 h) are shown for the 13 oxylipins significantly modulated by time, independent of intervention group during the 6-week intervention periods (linear mixed-effects models; adjusted $P < 0.05$, Benjamini-Hochberg correction). The treatment × time interaction was not significant. *Post-hoc* pairwise comparisons between t + 0 h and t + 6 h were performed using Tukey's adjustment for multiple testing. Statistical significance is indicated as follows: $^{**}P < 0.01$ and $^{***}P < 0.001$. Individual values are shown according to intervention groups: CON (grey dots), HESP (blue dots), and OJ (orange dots). Panels represent oxylipins grouped according to their parent fatty acid: (A) arachidonic acid (C20:4n6), (B) eicosapentaenoic acid (C20:5n3), (C) dihomo-γ-linolenic acid (C20:3n6), (D) linoleic acid (18:2n6), and (E) docosahexaenoic acid (C22:6n3). HDHA, hydroxydocosahexaenoic acid; HEPE, hydroxyeicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid; HETrE, hydroxyeicosatrienoic acid; HODE, hydroxyoctadecadienoic acid.

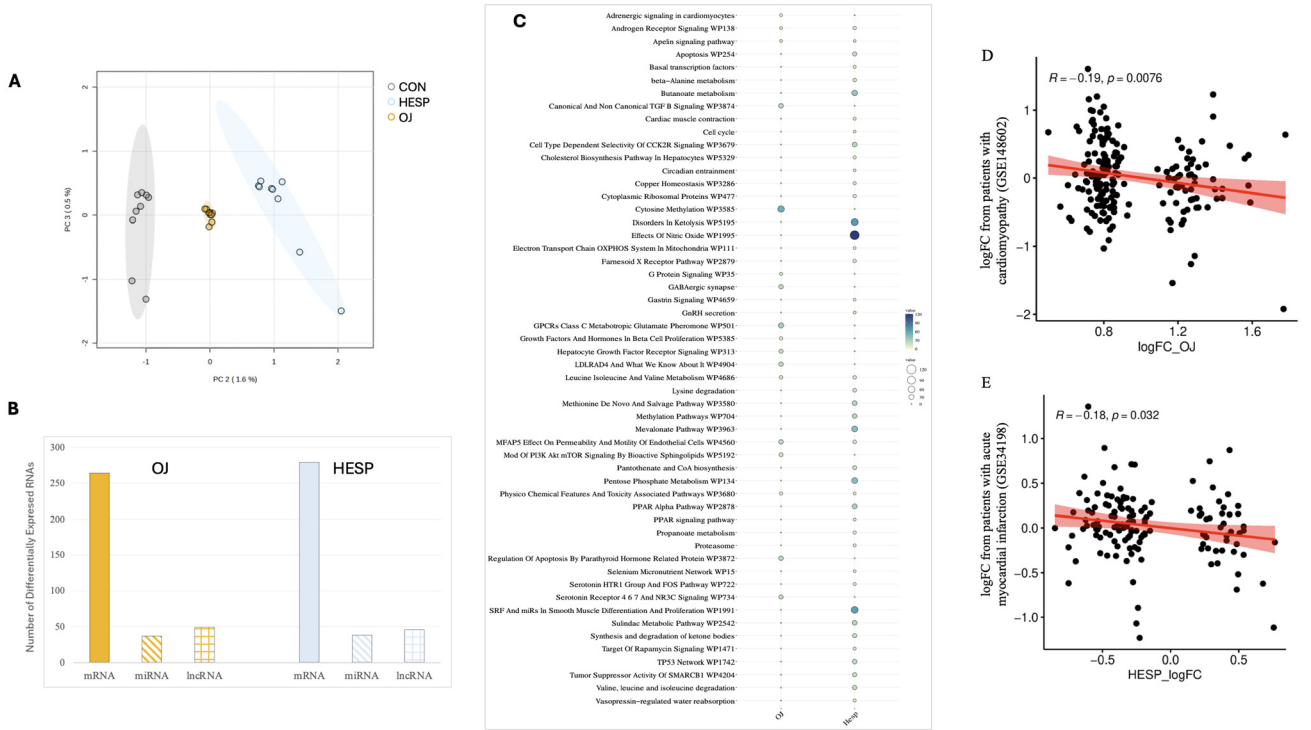

Fig. 6 Global genomic responses following orange juice and hesperidin consumption. (A) Partial Least Squares Discriminant Analysis (PLS-DA) showing separation of samples based on global gene expression profiles across intervention groups. (B) Number of differentially expressed genes (mRNAs, miRNAs, lncRNAs) identified following consumption of orange juice (left) and hesperidin (right). (C) Pathway enrichment analysis showing significantly enriched cellular pathways, with circle size indicating the number of genes contributing to each pathway. (D) Pearson correlation between gene expression profiles from participants after orange juice consumption OJ and those from patients with cardiomyopathy. (E) Pearson correlation between gene expression profiles from participants after hesperidin consumption and those from patients with acute myocardial infarction. CON, control beverage; HESP, control beverage supplemented with hesperidin; OJ, orange juice.

adrenergic and apelin signalling and canonical/non-canonical TGF-β signalling. In contrast, HESP modulated a broader spectrum of pathways, encompassing cardiovascular function (*e.g.*, nitric oxide signalling, cardiac muscle contraction, cholesterol biosynthesis) and metabolic regulation (*e.g.*, PPAR signalling, branched-chain amino acid metabolism, mTOR signalling) (Fig. 6C). Several pathways, including apelin and androgen receptor signalling, were modulated by both interventions.

To explore potential clinical relevance, intervention-induced profiles were compared with transcriptomic datasets from patients with cardiometabolic diseases (Fig. 6D, E and SI Fig. S3). OJ-induced signatures showed weak but significant inverse correlations with those of hypertension ($R = -0.18$, $P = 0.0017$), cardiomyopathy ($R = -0.19$, $P = 0.0076$), and myocardial infarction ($R = -0.17$, $P = 0.039$), while HESP-induced signatures correlated inversely with myocardial infarction ($R = -0.18$, $P = 0.032$), and thoracic aortic aneurysm ($R = -0.14$, $P = 0.03$). Comparative network analyses using IPA further identified patterns opposing disease-associated transcriptomic alterations in cardiovascular disorders and diabetes for OJ, and in diabetes for HESP (SI Fig. S4).

Taken together, these results provide preliminary mechanistic insights into the molecular effects of the interventions and indicate that both interventions were associated with transcriptomic patterns inversely related to cardiometabolic disease signatures.

4 Discussion

In this randomized, controlled, three-period crossover trial conducted in centrally overweight but otherwise healthy men, neither 330 mL day^{-1} of 100% orange juice (211 mg hesperidin) nor an isocaloric hesperidin-supplemented control beverage (206 mg hesperidin) induced measurable changes in fasting or postprandial endothelial function assessed by flow-mediated dilation (FMD). Consistently, no significant treatment effects were observed for secondary vascular outcomes including skin microvascular reactivity, blood pressure, arterial stiffness and circulating endothelial biomarkers. Nominal trends toward lower PWV after orange juice consumption and lower E-selectin concentrations after both orange juice and hesperidin supplementation were observed, but these isolated findings were small and not supported by concordant changes across vascular endpoints. Under the present conditions, moderate chronic hesperidin intake, whether consumed within orange juice or as an isolated compound, did not translate into detectable short-term vascular improvements. These find-

ings should be interpreted within a pragmatic design intended to test biologically plausible, nutritionally realistic exposure rather than pharmacological dosing.[29]

Despite providing an additional daily energy intake, neither intervention induced evidence of compensatory dietary adjustments or altered glucose homeostasis, lipid profile, or anthropometric parameters. Thus regular consumption of orange juice at the tested volume (330 mL day^{-1}), corresponding to approximately twice the recommended daily intake for fruit juice, was not associated with adverse metabolic effects over the intervention period. Overall, these findings support a neutral cardiometabolic response under the present conditions.

4.1 Comparison with previous flavanone trials and dose considerations

Evidence from previous studies helps contextualize our findings. An umbrella review of 144 meta-analyses reported neutral associations between habitual intake of 100% fruit juice (~50–240 mL day^{-1}) and major cardiometabolic outcomes, while suggesting potential improvements in vascular markers in studies using higher phenolic exposure or in metabolically impaired populations.[30] Studies investigating orange juice have yielded heterogeneous findings, with vascular benefits more consistently observed at higher intakes (400–600 mL day^{-1}, providing ~200–750 mg day^{-1} flavanones) or in individuals with metabolic impairment.[31–37] Similarly, trials using purified hesperidin (300–500 mg day^{-1} for 3–6 weeks) have reported mixed effects on endothelial function.[31,38,39] Collectively, these data suggest that vascular responsiveness to citrus flavanones is influenced by dose, delivery matrix, and baseline metabolic status.

Importantly, the hesperidin dose tested here (~210 mg day^{-1}) reflects a realistic dietary exposure achievable through moderate orange juice consumption. Although lower than doses used in several positive intervention trials,[31,38] it remains within the range of habitual nutritional intake. The absence of measurable vascular effects is therefore unlikely to be explained by dose alone, but rather by the combined influence of exposure level, participant characteristics, and other determinants of responsiveness.

4.2 Flavanone bioavailability and matrix effects

To further interpret the absence of measurable vascular effects, flavanone bioavailability must be considered. Metabolite profiling including flavanone conjugates (glucuronides and sulfates), as well as phenolic microbial catabolites, confirmed participant compliance and demonstrated systemic exposure to flavanone-derived metabolites. Approximately 4–5% of the ingested hesperidin dose was recovered as urinary hesperetin conjugates, consistent with a similar overall bioavailability between the two matrices. Excretion rates and metabolite profiles were in agreement with previous pharmacokinetic studies using orange juice or pure hesperidin.[40–43] Comparable metabolite recovery further suggests limited matrix effects on overall hesperidin bioavailability, although potential effects on absorption kinetics, tissue distribution or downstream signalling pathways cannot be excluded. Interestingly, while circulating and urinary phase II flavanone metabolite profiles were highly comparable between OJ and HESP interventions, some differences were observed for selected microbial catabolites, suggesting that the effect of the food matrix may be more pronounced at the level of downstream colonic metabolism than during intestinal absorption and phase II metabolism.

Systemic plasma exposure to hesperetin conjugates was within the expected range, although lower than that reported in studies using higher doses or extended sampling windows of up to 24 hours.[20,41] These concentrations are therefore unlikely to reflect insufficient absorption *per se*, but may have been too low to elicit measurable vascular effects. Consistent with previous studies,[21] the predominant circulating phase II metabolites were hesperetin-3′-glucuronide, hesperetin-3′-sulfate, and hesperetin-7-glucuronide, each previously associated with complementary biological activities related to endothelial function.[10,11,44,45] The diversity of circulating phase II metabolites raises the question of which metabolites, alone or in combination, contribute to flavanone bioactivity. Nevertheless, despite confirmed systemic exposure and the presence of potentially bioactive metabolites, these mechanisms did not translate into measurable vascular improvements *in vivo*. Marked interindividual variability in flavanone metabolism was also observed, consistent with previous reports,[41,46] and may contribute to heterogeneous biological responses.

4.3 Postprandial response

The postprandial setting represents an additional opportunity to detect acute vascular effects. Although the high-fat meal induced marked lipemia and altered oxylipin profiles, reflecting activation of inflammatory and vascular lipid mediator pathways, it did not impair endothelial function in our study population, despite being designed according to current recommendations.[47] While such challenge meals are commonly used to induce transient endothelial dysfunction,[48] responses vary according to study design and participant characteristics, and similar neutral findings have been reported previously.[49] Notably, the only trial reporting postprandial vascular protection by orange juice used a sequential double-meal challenge, likely inducing greater physiological stress.[50] The absence of endothelial impairment in the present study may therefore have reduced the sensitivity of the model to detect protective effects. In addition, the moderate systemic exposure to flavanone metabolites achieved may have further limited the detection of acute vascular responses, despite measurable increases in circulating metabolites, consistent with studies reporting postprandial improvements at higher hesperidin doses.[31] Collectively, these observations suggest that both the nature of the postprandial challenge and the achieved systemic exposure constrained the detection of acute vascular effects, rather than indicating an absence of biological activity.

4.4 Interindividual variability in flavanone responsiveness

Interindividual variability in response to flavonoid interventions is increasingly recognized.[51] Such variability may arise

from differences in flavonoid release from the food matrix, colonic catabolism, absorption and human metabolism, baseline physiological status, or sensitivity of molecular targets. Baseline blood pressure, for example, has been identified as a determinant of vascular responsiveness to flavanols.[52] In the present study, substantial interindividual variability was observed in both fasting FMD and flavanone exposure, yet no clear treatment-response relationship according to baseline endothelial function or flavanone metabolite levels was identified. Although these analyses were exploratory and the study was not powered to detect interaction effects, the findings do not support a distinct responder/non-responder pattern under the present conditions. Further insights into interindividual variability would require, in our view, a better understanding of the concomitantly present, pronounced intraindividual variability in flavanone excretion. Taken together, these observations suggest that interindividual variability alone is unlikely to explain the absence of measurable vascular effects.

4.5 Transcriptomic insights

Transcriptomic analysis provided additional mechanistic insight. Both interventions induced changes in gene expression compared with control, consistent with prior evidence of genomic modulation by citrus flavanones.[53,54] Expression patterns were inversely associated with cardiovascular and cardiometabolic disease signatures, suggesting that flavanone intake is linked to a transcriptional profile aligned with protective molecular patterns. Modulation of pathways including apelin signalling and MFAP5-related endothelial regulation may reflect early molecular adaptations that, if sustained over time, could contribute to vascular health.[55,56] Although exploratory, these findings suggest that citrus flavanone exposure modulates pathways involved in vascular biology despite the absence of measurable short-term functional changes. This supports the possibility that molecular adaptations may precede detectable physiological responses, particularly under moderate exposure conditions.

4.6 Overall interpretation

Overall, the present findings indicate that citrus flavanones can induce measurable biological engagement under nutritionally realistic exposure conditions, as evidenced by confirmed bioavailability and modulation of vascular-related transcriptomic pathways, without detectable short-term improvements in vascular phenotypes. These observations illustrate the complexity of translating food bioactive exposure into measurable functional outcomes in humans. Responsiveness to citrus flavanones is likely influenced by multiple interacting determinants, including exposure level, metabolic phenotype, food matrix, microbial metabolism, and interindividual variability. Accordingly, the absence of detectable vascular effects should not be interpreted as a lack of mechanistic relevance. Rather, the findings support the view that physiological responses to dietary polyphenols are subtle, context-dependent, and may emerge preferentially in individuals with metabolic dysregulation or distinct physiological response profiles. This interpretation aligns with current precision-nutrition frameworks, where variability in responsiveness to dietary bioactives is expected rather than viewed as noise.[57] More broadly, these results highlight the challenges of detecting measurable functional effects under realistic nutritional conditions, despite clear evidence of systemic exposure and molecular responses.

4.7 Strengths and limitations

This study has several strengths, including its randomized, double-blind, crossover design; the use of energy- and sensory-matched control beverages enabling the assessment of potential matrix effects; and the comprehensive evaluation of vascular, metabolic, oxylipin and molecular outcomes under both fasting and postprandial conditions. Flavanone bioavailability was extensively characterized through detailed metabolite profiling, providing important mechanistic context beyond clinical endpoints.

Limitations should also be acknowledged. Although the study was open to both sexes, only men volunteered, limiting generalizability. The intervention duration and dose were selected to reflect realistic dietary exposure, but this pragmatic design may have limited the ability to detect measurable effects, particularly in a population with relatively preserved endothelial function.[58] In addition, habitual dietary polyphenol intake was not specifically quantified, precluding assessment of whether background polyphenol exposure may have influenced individual responsiveness to the interventions. The study was not powered to detect responder subgroups, and transcriptomic analyses were exploratory and conducted in a subset of participants. Finally, the postprandial challenge did not induce endothelial dysfunction, thereby limiting the sensitivity of the model to detect protective effects.

5 Conclusion

In conclusion, moderate orange juice consumption and matched hesperidin supplementation increased systemic flavanone exposure and induced molecular signatures consistent with biological activity, but did not translate into detectable short-term vascular benefits in centrally overweight men. These findings help delineate the boundary between biological engagement and measurable functional vascular responses under realistic dietary exposure conditions. Collectively, the present results support the need for multidimensional approaches, including precision nutrition frameworks, to better characterize responsiveness to dietary bioactives in humans.

Author contributions

C. Gilcher: conceptualization, methodology, investigation, data curation, formal analysis, project administration, writing – original draft, writing – review & editing. D. Milenkovic: methodology, investigation, formal analysis, writing – original draft, writing – review & editing. M. Taillandier: formal analysis, data

curation, validation, writing – original draft, writing – review & editing. T. Ruskovska: investigation, formal analysis, writing – review & editing. C. B. Steingass: methodology, investigation, supervision, writing – original draft, writing – review & editing. N. Macian: investigation, data curation. M. A. Verny: investigation, data curation. R. Evrard: investigation, data curation. B. Pereira: supervision, validation. C. Gladine: methodology, investigation, formal analysis, writing – original draft, writing – review & editing. R. Schweiggert: conceptualization, methodology, funding acquisition, supervision, resources, writing – review & editing. G. Pickering: conceptualization, methodology, supervision, project administration, writing – review & editing. C. Morand: conceptualization, methodology, funding acquisition, supervision, project administration, writing – original draft, writing – review & editing.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Abbreviations

AU	Arbitrary unit
AUC	Area under the concentration-time curve
BP	Blood pressure
CON	Control beverage
CVD	Cardiovascular diseases
FMD	Flow-mediated dilation
GLMM	Generalized linear mixed model
HDHA	Hydroxydocosahexaenoic acid
HEPE	Hydroxyeicosapentaenoic acid
HESP	Control beverage supplemented with hesperidin
HETE	Hydroxyeicosatetraenoic acid
HETrE	Hydroxyeicosatrienoic acid
HODE	Hydroxyoctadecadienoic acid
HMPHA	(*R*/*S*)-3-Hydroxy-3-(3′-hydroxy-4′-methoxyphenyl)propanoic acid
HMPPA	3-(3′-Hydroxy-4′-methoxyphenyl)propanoic acid
HPHPA	(*R*/*S*)-3-Hydroxy-3-(3′-hydroxyphenyl)propanoic acid
IPA	Ingenuity pathway analysis (Qiagen)
LMM	Linear mixed model
lncRNA	Long noncoding RNA
miRNA	microRNA
OJ	Orange juice
PLS-DA	Partial least squares discriminant analysis
PWV	Pulse wave velocity
RCT	Randomized controlled trial.

Data availability

Deidentified data described in the manuscript, code book, and analytic code will be made available upon request pending application to and approval of the corresponding author.

Supplementary information (SI) is available. This Supplementary file provides additional methodological details and extended results to complement the main manuscript. See DOI: https://doi.org/10.1039/d6fo02778d.

Ref. 62 and 63 are cited in the SI.

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