BMJ Open

Study protocol for a randomized controlled trial evaluating the role of Orange juice, HESPERidin in vascular HEALTH benefits: The HESPER-HEALTH Study

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2021-053321
Article Type:	Protocol
Date Submitted by the Author:	10-May-2021
Complete List of Authors:	Verny, Marie-Anne; INRAE, UNH Milenkovic, Dragan; INRAE, UNH MACIAN, Nicolas; University Hospital Centre Clermont-Ferrand Pereira, Bruno; University Hospital Centre Clermont-Ferrand Evrard, Rémy; University Hospital Centre Clermont-Ferrand Gilcher, Caroline; Geisenheim University Steingass, Christof B.; Geisenheim University Mosoni, Pascale; INRAE, MEDIS Gladine, Cécile; UNH Monfoulet, Laurent-Emmanuel; UNH Schweiggert, Ralf; Geisenheim University PICKERING, Gisèle; University Hospital Centre Clermont-Ferrand MORAND, Christine; INRAE, UNH
Keywords:	MOLECULAR BIOLOGY, NUTRITION & DIETETICS, VASCULAR MEDICINE

I, the Submitting Author has the right to grant and does grant on behalf of all authors of the Work (as defined in the below author licence), an exclusive licence and/or a non-exclusive licence for contributions from authors who are: i) UK Crown employees; ii) where BMJ has agreed a CC-BY licence shall apply, and/or iii) in accordance with the terms applicable for US Federal Government officers or employees acting as part of their official duties; on a worldwide, perpetual, irrevocable, royalty-free basis to BMJ Publishing Group Ltd ("BMJ") its licensees and where the relevant Journal is co-owned by BMJ to the co-owners of the Journal, to publish the Work in this journal and any other BMJ products and to exploit all rights, as set out in our licence.

The Submitting Author accepts and understands that any supply made under these terms is made by BMJ to the Submitting Author unless you are acting as an employee on behalf of your employer or a postgraduate student of an affiliated institution which is paying any applicable article publishing charge ("APC") for Open Access articles. Where the Submitting Author wishes to make the Work available on an Open Access basis (and intends to pay the relevant APC), the terms of reuse of such Open Access shall be governed by a Creative Commons licence – details of these licences and which Creative Commons licence will apply to this Work are set out in our licence referred to above.

Other than as permitted in any relevant BMJ Author's Self Archiving Policies, I confirm this Work has not been accepted for publication elsewhere, is not being considered for publication elsewhere and does not duplicate material already published. I confirm all authors consent to publication of this Work and authorise the granting of this licence.

Study protocol for a randomized controlled trial evaluating the role of Orange juice, HESPERidin in vascular HEALTH benefits: The HESPER-HEALTH Study

Marie-Anne Verny[1], Dragan Milenkovic[1], Nicolas Macian[2], Bruno Pereira[3] Rémy Evrard, [2] Caroline Gilcher[4], Christof B. Steingass[4], Pascale Mosoni[5], Cécile Gladine[1], Laurent-Emmanuel Monfoulet[1], Ralf Schweiggert[4], Gisele Pickering[2,], Christine Morand[1]

[1] Université Clermont Auvergne, INRAE, UNH, F-63000 Clermont-Ferrand, France

[2] Plateforme d'Investigation Clinique/CIC Inserm 1405, University Hospital CHU, 63000 Clermont-Ferrand, France.

[3] Biostatistics Unit (DRCI), Gabriel-Montpied University Hospital, Clermont-Ferrand, France

[4] Chair of Analysis and Technology of Plant-based Foods, Department of Beverage Research, Hochschule Geisenheim University, Geisenheim, Germany.

[5] Université Clermont Auvergne, INRAE, MEDIS, F-63000 Clermont-Ferrand, France

Author emails :

Marie Anne Verny : marie-anne.verny@inrae.fr

Dragan Milenkovic: dragan.milenkovic@inrae.fr

Macian Nicolas: nmacian@chu-clermontferrand.fr

Bruno Pereira : Bpereira@Chu-Clermontferrand.Fr

RemyEvrard: revrard@chu-clermontferrand.fr

Caroline Gilcher : Caroline.gilcher@hs-gm.de

Christof Steingaß: Christof.Steingass@hs-gm.de

Pascale Mosoni : pascale.mosoni@inrae.fr

Cécile Gladine : cecile.gladine@inrae.fr

Laurent Emmanuel Monfoulet : laurent-emmanuel.monfoulet@inrae.fr

Ralf Schweiggert: Ralf.Schweiggert@hs-gm.de

Gisele Pickering: gisele.pickering@uca.fr

Christine Morand: Christine.morand@inrae.fr

Corresponding author :

Christine MORAND

INRAE- Unité de Nutrition Humaine – UMR 1019

Site de Theix INRAE - Clermont-Ferrand – Theix- 63122 Saint-Genès-Champanelle

France

Email : christine.morand@inrae.fr

Tel : +(33) 4 73 62 40 84

Key words :

Randomized controlled trial ; vascular function ; orange juice ; hesperidin ; flavanones bioavailability and metabolism

ABSTRACT

Introduction: Although epidemiological studies associate the consumption of sugary beverages with adverse health effects, human experimental studies have demonstrated substantially different metabolic responses when 100% fruit juices are compared with artificial beverages. Fruit juices do not just provide sugars and associated calories, but they are also rich in bioactive compounds. Flavanones are bioactives specifically and abundantly found in citrus foods, with hesperidin as the major representative in sweet oranges. Flavanone intake has been associated with a lower incidence of mortality from cardiovascular disease (CVD). However, cinical evifdence are too scarce to confirm the vasculo-protective effects of 100% orange juice (OJ) presumably mediated by flavanones, and thereby do not allow firm conclusions to be drawn about their efficacy.

Methods and analysis: The HESPER-HEALTH study aims to assess the efficacy of OJ in improving vascular function and the contribution of hesperidin to these effects. This double-blind, randomised, controlled, crossover study will be carried out in 42 volunteers predisposed to CVD, based on age and waist circumference. It will include three 6-week periods of consumption of 330 mL/d of OJ versus control drinks with and without hesperidin at a dose in agreement with a daily OJ serving (approx. 200-215 mg). The primary outcome is endothelial function, assessed by flow mediated dilation (FMD), with measurements performed at fasting and postprandially in response to a challenge meal. The secondary outcomes include bioavailability and metabolism of flavanones, changes in other markers of vascular function, systemic biomarkers of cardiovascular risk, endothelial dysfunction, and inflammation, vitamin C and carotenoids status, anthropometry and body composition, gut microbiota composition, nutrigenomic response, and in oxylipin profiling.

Ethics and dissemination: The study was approved in February 2021 by an independent ethics committee (n°20.10.15.60521, CPP Sud-Est III, Bron, France) and registered on ClinicalTrials.gov (NCT04731987). The results will be disseminated in peer-reviewed journals.

ARTICLE SUMMARY

Strengths and Limitations of this study

- HESPER-HEALTH is a controlled randomised dietary intervention carried out in men and women (40-65 yrs, postmenopausal, overweight) in which the effects of OJ consumption on vascular function will be studied together with the capacity of hesperidin to contribute to these effects.
- This trial includes an analysis of hesperidin bioavailability and metabolism in biofluids to enable further correlation with the vascular response of individuals.
- This trial will also substantiate the vascular effects by providing insights on the in-vivo underlying molecular mechanisms using innovative approaches (i.e., oxylipin profiling and nutrigenomics).
- This study will clarify interractions between OJ, hesperidin and the gut microbiota.
- While subjects and clinic staff will be unable to differentiate between the drinks rich in, or free from, hesperidin due to their matched color, turbidity, taste and flavor, they may sensorially differentiate these two artificial control drinks from the fully natural OJ due to its unique sensory properties.

INTRODUCTION

Recent epidemiological studies have associated the frequent consumption of sugar-sweetened beverages with some adverse health effects, such as early death, weight gain and cancer [1; 2] Findings of such studies are often extrapolated to fruit juice consumption, including 100% fruit

juices. However, these observational, epidemiological results contrast with experimental studies which have demonstrated clear differences in the metabolic response of the human body to fruit juices as compared with that seen for sugar-sweetened beverages[3; 4].

One characteristic of citrus foods is that they are a rich and exclusive source of dietary flavanones, a category of (poly)phenol compounds, mainly present as hesperidin in orange. Flavanone intake has been repeatedly associated with a lower incidence of mortality from CVD [5; 6]. Results from preclinical studies using different models of atherosclerosis also provide evidence for a role of citrus flavanones in cardiovascular protection, with a slowdown in atherosclerosis development [7].These atheroprotective effects have been related to the capacity of flavanones to modulate the expression of genes involved in cellular processes responsible for vascular dysfunction[8]. Evidence of the vascular protective effects of citrus flavanones have been reported in few randomised controlled clinical trials [4; 9; 10; 11]. However, published trial with orange flavanones do not yet allow firm conclusions to be drawn about their efficacy to modulate vascular function, mainly due to the high degree of discrepancies between the study designs.

The ability of citrus flavanones to exert beneficial effects depends on their bioavailability. The gut microbiota plays a key role in flavanone absorption, because naturally-occuring flavanones are present as molecules with glycosyl moieties, e.g. a rutinosyl, i.e. a α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoosyl moiety in hesperidin. The sugar moiety of hesperidin must be hydrolysed by bacterial glycosidases to yield a format which the human body can absorb i.e. the aglycone, hesperetin. Upon release, hesperetin can be absorbed by intestinal cells or may be further catabolized into diverse phenolic compounds by microbial action in the colon. After absorption of hesperetin and its microbial catabolites, they enter the blood circulation and are subject to further human metabolism. For instance, hepatic metabolism includes several conjugation reactions, e.g., sulphatation or glucuronidation[12; 13]. Hence, the extent of hesperetin released in the colon, the

formation of colonic catabolites and their subsequent absorption could largely depend on the gut microbial composition, which has been shown to vary between subjects [14]. In agreement with this hypothesis, previous studies have reported a large interindividual variability in the urinary excretion level of flavanones [15; 16] that could reflect differences in gut microbiota composition, but this remains to be demonstrated. A recent clinical study found that regular consumption of OJ can modulate the gut microbiota profile and these changes were associated with positive shifts in some metabolic outcomes[17].

Dietary (poly)phenols and their circulating metabolites encompass a huge diversity of compounds [18] whose health effects seem increasingly linked to their capacity to exert complex genomic modifications, such as changes to the expression of genes [19]. In preclinical studies, flavanones have been shown to induce changes in the expression of a number of genes in aorta and endothelial cells that relate to inflammation, and endothelial cell function [7; 20; 21; 22], revealing potential molecular mechanisms of action to explain their health properties. However, such mechanisms of action are still largely unexplored in humans [20]. Based on a huge body of experimental results demonstrating the anti-inflammatory effects of polyphenols and related mechanisms [23], these compounds are suggested as key players in the protective effects of their food sources for chronic inflammatory diseases. However, in humans, consumption of polyphenol-rich foods induces subtle changes in inflammatory and oxidative status that can only typically be measured usingsensitive techniques, such as the lipidomic profiling of oxylipins [24]. Oxylipins are a superclass of lipid mediators comprising hundreds of metabolites which regulate a diversity of biological processes including inflammation, cell adhesion, migration and proliferation, blood clotting and vessel permeability [25].

Based on this state of the art, the present human randomised, controlled, double-blind, cross over intervention conducted on subjects predisposed to CVD aims to establish a cause-and-effect relationship between hesperidin intake and the vascular protective effects of drinking OJ naturally rich in hesperidin. The study will also provide insights into the mechanisms responsible for the observed effects.

STUDY OBJECTIVES

Primary Objective

The primary objective of this trial is to assess the effect of a subchronic consumption (6 weeks) of a naturally flavanone-rich OJ or a control drinks supplemented with orange flavanones on endothelial function, by assessing flow-mediated dilation (FMD) in subjects with a predisposition to CVD, based on age, body mass index and waist circumference.

Secondary Objectives

The secondary objectives of the trial are to:

- assess the effects of the intervention: (1) on other markers of vascular function using a range of well-established measurements in the macrocirculation (blood pressure, arterial stiffness) and in the microvasculature at skin level (microvascular reactivity by Flow Laser Doppler, FLD); (2) on the postprandial endothelial response in response to a challenge meal; (3) on biomarkers of cardiovascular risk, endothelial dysfunction and inflammation; (4) on anthropometric parameters and body composition; (5) on the vitamin C and carotenoids status; (6) on the gut microbiota composition (microbial communities profiling)
- measure the bioavailability of hesperidin and its metabolites in biofluids (plasma, urine)

- ascertain the underpinning molecular mechanisms of the vascular responsiveness (nutrigenomic analysis, oxylipins profiling).

METHOD AND ANALYSIS

Study Design

HESPER-HEALTH is a human dietary intervention study designed as a randomized, double-blind, controlled, cross-over trial with three arms that will be conducted at the Clinical Investigation Center, Inserm 1405 (PIC/CIC) of the University Hospital of Clermont-Ferrand, France. This trial will be carried out on subjects predisposed to CVD, based on age (40-65 years old) and overweight (waist circumference ≥ 80 cm for woman, ≥ 94 cm for man). Forty-two participants will be recruited and will receive the three treatments in a random order: (A) a commercially-available OJ naturally rich in hesperidin (ca. 600-650 mg/L), (B) a control beverage with a total sugar concentration identical to (A), and (C) a control beverage identical to (B) but supplemented with hesperidin at the level present in the natural OJ (A).

For each volunteer, the study is divided in three identical experimental periods of 45 days. These periods include a three day run in period during which time specific dietary recommendations will be followed, samplings and measurements will be performed at home. This run in period will be followed by a 6-weeks treatment period of consumption of one of the three beverages. A four to six week wash-out is planned between each of the three experimental periods. The protocol includes seven visits to PIC/CIC, including one visit (V1) at inclusion, and will last in total for 28 to 33 weeks (**Figure 1**). Over the last 24h prior to each visit, volunteers will be asked to collect

stool samples and 24h urine samples to assess gut microbiota composition and flavanone bioavailability and metabolism, respectively.

At the beginning and end of each experimental period, overnight-fasted volunteers will be invited to attend the PIC/CIC (visits V2 to V7) for vascular function tests, blood sampling, anthropometric measurements and body composition analysis. Blood will be sampled for further assessment of plasma flavanones including metabolites, carotenoids and vitamin C, oxylipin profiling, systemic biomarkers of endothelial activation and inflammation, metabolic parameters, and for the analysis of the nutrigenomic response.

At each visit (V2 to V7), after measurements and blood collection in the fasting state, a challenge meal together with the respective study drink will be administered to all subjects before further exploration. This will enable evaluatation of the acute postprandial effects (at T+3h, T+6h) of the study products on endothelial function (V2,V4,V6), to also be repeated after the 6 weeks intervention period (V3, V5, V7). The challenge meal will consist of fresh cream, sucrose and milk proteins, providing 900 kcal – a pro-oxidant and pro-inflammatory meal that is known to induce a transient endothelial dysfunction during the post prandial period [26].

During each experimental period, volunteers will be asked not to eat citrus foods and to consume no more than 250 mL/d of polyphenol-rich beverages (coffee, tea, fruit juices, wine, cocoa). To check that volunteers do not change their eating habits during the study, for each experimental period they will be asked to complete food questionnaires over three defined days. The first questionnaire will be completed prior to visit V2 to PIC/CIC and two others at mid-term of each intervention period. On the two days preceeding each visit at the PIC/CIC, volunteers will be asked

not to consume polyphenol-rich foods and beverages and they will have to consume exactly the same dinner without polyphenols at 8 pm the day before each visit at the PIC/CIC.

Inclusion and Exclusion criteria

These criteria are listed in the Table 1.

Study products

Three study beverages will be used in cross-over and double-blinding conditions. These include (A) an OJ from a commercially-available OJ concentrate (Sucocítrico Cutrale, Araraquara, São Paulo, Brazil) containing approx. 600-650 mg hesperidin per L, (B) a control drink with a total sugar concentration identical to that of (A), and (C) a control drink identical to (B) but supplemented with hesperidin at the level found in the OJ (Figure 2). The daily administered dose will be 330 mL to be distributed over two intakes (2 × 165 mL) - one in the morning during breakfast and the second during lunch. This will correspond to ca. 200-215 mg hesperidin per day (drinks A and C, resp.).

The control beverages (B) and (C) will be made isocaloric to (A), all containing an identical total sugar concentration (ca. 9.0 g/100 mL), comprising glucose, fructose, and sucrose. Thus, the amount of total sugar provided by 330 mL of each drink will amount to ca. 30 g. All drinks will contain citric acid at similar levels. To match the visual appearance of the OJ, food colorants as well as a clouding agent will be added to the control drinks. Flavour will be matched by adding a natural orange aroma to all beverages (A-C), including the OJ from concentrate to which orange aroma addition is required by European law. All the beverages will be pasteurized according to commercial practice and filled into 330 mL brown glass bottles with no visual distinction possible

between bottles of different drinks. Despite careful colour and flavor matching, both the hesperidin-free (B, placebo) and the hesperidin-rich (C) control drink may be differentiated sensorially from the OJ by attentive subjects. In contrast, subjects and clinic staff will be unable to identify which of the artificial control drinks (B) and (C) is the one rich in or free from hesperidin.

Finally, bottles will be labelled and packaged for each cross-over period in a blinded fashion on the basis of the randomisation schedule. They will be stored at +4°C until distribution to the participants, who will be asked to store the beverages in a dark place at room temperature or cooler. Hesperidin levels in drinks (A) and (C) as well as levels of ascorbic and dehydroascorbic acid (vitamin C) in the OJ (A) will be monitored over the entire study period. Colony forming units of yeasts, total viable counts, lactic acid bacteria and moulds will also be assessed to ensure the microbiological safety of the products.

Assignment of intervention

Randomisation and Allocation : Subjects will be randomly assigned to treatment groups according to a pre-established list of randomisation designed by a PIC/CIC clinical research supervisor, independently from the investigators and the sponsor. The treatment number will be allocated by order of entrance in inclusion. Subjects will be randomised to receive drink A, B, or C using a Latin-square random design. Subject allocation to treatments will take place after inclusion and will be based on a computer-generated randomisation list. Access to the randomisation list will be restricted to staff performing this task. The randomisation number will be allocated by order of entrance in inclusion. Under normal circumstances, the blinding should not be broken until all subjects have completed the trial and the database will be locked. However, the blinding can be broken should a specific emergency treatment at the site require knowledge of

the treatment status of the subject. In such a case, the investigator can reveal the treatment assignment through the randomisation plan and then inform the sponsor as soon as possible (Procedure ORAGA MO-028).

Participant eligibility

Forty-two volunteers will be recruited from the existing PIC/CIC volunteer's database supplemented by announcement in local press. Each subject who meets the recruitment criteria (**Table 1**) will be pre-selected by the investigator and will be given a detailed explanation of the protocol. If the subject accepts, he or she will be asked to come to PIC/CIC for an initial study visit (V1, **Figure 1**) to give written consent to participate, and to have a medical examination, an interview with a dietitian and a blood checkup to ensure that all clinical criteria are met.

Sample size

Sample size calculation has been performed based on the FMD response (main criteria) observed in previous dietary interventions. The targeted statistical power was based on interindividual variability for FMD measurement of the operator (SD = 1.9%). Aiming for a statistical power greater than 80% and a two-tailed type I error at 0.017 (to take into account the three comparisons to be considered), the total number of subjects required to provide sufficient power to detect a minimal absolute difference of FMD equals 1.6% in a 3-sequences cross-over study (with an intra-class correlation coefficient at 0.5) is 36. Assuming unforeseen drop-outs and follow-up losses, 42 participants will be recruited.

OUTCOME MEASURES

The time table of the HESPER-HEALTH study is presented in **Table 2**.

Primary outcome

The primary outcome is endothelial function assessed at the level of of the brachial artery using the non-invasive technique FMD which constitutes the gold standard to evaluate vascular endothelial function in humans. The FMD technique measures the diameter of the brachial artery by non-invasive ultrasound before and after increasing shear stress by inducing a reactive hyperemia, with the degree of dilation reflecting mostly the arterial endothelial nitric oxide release. The procedure for the FMD measurement is full compliant with the reference method described by Coretti et al. [27]. FMD will be assessed at V2, V3, V4, V5, V6 and V7 under both fasting and postprandial conditions.

Secondary Outcomes

- ***Other vascular function measurements***

Endothelial function in the microcirculation will be assessed using Flowmetry by Laser Doppler (FLD), which is a non-invasive and validated technique for continuous measurement of the endothelial dependent microvascular reactivity. FLD will be assessed during V2, V3, V4, V5, V6 and V7 in fasting and postprandial conditions.

Arterial stiffness will be evaluated by the carotid-femoral Pulse Wave Velocity (PWV) which is calculated from measurements of pulse transit time and the distance travelled between the carotid and femoral arteries using a validated non-invasive device. PWV will be assessed after fasting at V2, V3, V4, V5, V6 and V7.

Blood pressure will be monitored by subjects at home using tensiometers which will be loaned to them for the study duration. They will be asked to perform three repeated measurements in fasting condition during the three consecutive days preceding their visits to the PIC/CPC (V2, V3, V4, V5, V6 and V7), and to record obtained values on a dedicated data sheet.

- ***Flavanone metabolism and bioavailability in biofluids***

Phase 2 metabolites and microbial-derived catabolites of flavanones will be analyzed by LC-MS in 24h urine and in plasma samples according to the method reported by Aschoff et al. [15]. Prior to analysis, the above-mentioned analytes will be extracted from plasma samples using solid phase extraction cartridges These analyses will be done for the biological fluids collected (urine) or sampled (plasma) at V2, V3, V4, V5, V6 and V7.

- ***Systemic biomarkers related to CVD risk, endothelial dysfunction and inflammation***

These include metabolic parameters (plasma glucose, insulin, TAG, Total Cholesterol, HDL-chol, uric acid that will be measured by spectrometric and enzymatic methods; total fatty acids, by gas chromatography), soluble adhesion molecules and inflammatory markers (ICAM, VCAM and e-selectin, IL-6, TNFalpha, hs-CRP, by using Elisa assays), plasma nitrites (by chemi-luminescence) and the release of endothelial extracellular vesicles (by flux cytometry). These analyzes will be performed in blood sampled at V2, V3, V4, V5, V6 and V7.

- ***Carotenoids and vitamin C status***

Carotenoids will be quantitated from human plasma after a protein-crash with ethanol and repeated extraction with hexane using the method described by Aschoff et al. [28]. Vitamin C status will be

quantified in deproteinized plasma by HPLC-fluorescence detection as previously described [29]. These measures will be performed in blood sampled at V2, V3, V4, V5, V6 and V7.

- ***Anthropometry and body composition assessment***

Body Mass Index (BMI), waist to height ratio (waist circumference/height) and the percentages of fat mass, lean mass and water (obtained using a multi-frequency Bioelectrical Impedance Analyser) will be determined. These parameters will be recorded at V2, V3, V4, V5, V6 and V7.

- ***Gut microbiota composition assessment***

The composition of the gut microbiota will be determined by 16S metabarcoding. This method involves the amplification by PCR of variable regions of the 16S rDNA gene from fecal DNA followed by the preparation of DNA libraries and high throughput next-generation sequencing (Illumina technology). Analysis will be completed by an absolute quantification by qPCR of target groups of the human gut microbiota considered as beneficial to the host, or involved in polyphenol metabolism. These analyses will be performed in faeces samples collected during the 12h preceding V2, V3, V4, V5, V6 and V7.

Mechanistic outcomes

In the HESPER-HEALTH study, we aim to explore the molecular mechanisms of action underlying vascular effects of OJ and hesperidin consumption. To this end, we propose to perform global and integrated analyses of expression of genes (nutrigenomic analysis) in the blood and to analyse the oxylipin profiling to identify changes in biological processes involved in inflammation and vascular dysfunction.

Nutrigenomic response assessment: RNA will be extracted from whole blood using PAXgene Blood RNA System. Global gene expression profile of both protein coding and protein non-coding RNAs (miRNAs, snoRNAs, lncRNAs) will be assessed using a microarray approach. For genes identified as differentially expressed, integrated multi-omic bioinformatic analyses will be performed to identify gene ontologies, gene network, cellular pathways and interactions between different types of RNAs. Potential transcription factors involved will be searched and capacity of flavanone metabolites to bind to transcription factors as well as cell signaling proteins regulating their activity will be predicted using 3D docking analysis. Analysis will be performed from blood sampled at V3, V5 and V7.

Oxylipin profiling: a comprehensive assessment of circulating oxylipins will be performed using a targeted MS-based based method (LC-MS/MS) using 22 internal standards and providing both qualitative (i.e. oxylipin signature) and quantitative information [30]. Analysis will be performed in plasma sampled at V2, V3, V4, V5, V6 and V7.

Statistical analyses

All analyses will be performed with Stata software (version 15, StataCorp, College Station, USA) before the breaking of the randomiszation code, according to International Conference on Harmonization-Good Clinical Practice guideline. Continuous variables will be presented, according to their statistical distribution, as mean and standard-deviation, or median and interquartile range. The Shapiro-Wilk test will be used to assess normality. Categorical data will be presented as exact number and percentage.

The primary outcome will be analysed using random-effects model (i.e. mixed linear model for continuous dependent variable) for a 3-treatment crossover study. The full statistical model includes treatment group, sequence, period, carry-over on FMD baseline value as fixed effects.

Subject nested in sequence will be included in the model as a random effect. A Sidak's type I error will be applied to take into account multiple comparisons. The *treatment group x period* interaction will be studied. If this interaction is not significant, the main effect of treatment will be assessed. If this interaction is significant, there will be a particular focus on the first period. The normality of residuals from random-effects model will be studied as aforementioned, with the Shapiro-Wilk statistic and visual inspection of residual plots. If appropriate, a transformation (for example logarithmic) of the primary outcome could be proposed to achieve its normality. The results will be expressed with effect-sizes and 95% confidence intervals.

The primary analysis will be completed by multivariable approach using the statistical model described above with, additionally, covariates determined according to univariate results and clinical relevance: BMI, gender, weight at enrolment and age. The other continuous outcomes will be analyzed with the same statistical analysis plan. For categorical data, generalized mixed linear modelling will carried out.

ETHICS AND DISSEMINATION

Consent: Subject will be informed in a complete and fair manner, in accessible terms, of the objectives and constraints of the study, the possible risks incurred, the necessary surveillance and security measures, their rights to refuse to participate in the study or the possibility to withdraw at any time. The patient's free, informed and written consent will be collected by the investigator. Document templates have been approved by the ethics committee and are to be used for the test concerned, to the exclusion of any other documentation. One original copy co-signed by the investigating doctor and the subject will be given to the volunteer.

Data management: Experienced and trained study coordinators will be dedicated to data acquisition, coding, security and storage under the responsibility of the investigator. The study data will be computerised in a coded manner, and in accordance with the information technology law and freedom, by the Clermont-Ferrand CIC. The data will be entered into the computer files using a double entry procedure meeting the standards set by good clinical practice. After comparing the double entry, the computer data files and any corrections made to them will be stored and retrievable on request.

Data availability statement: data not available due to legal restriction.

Dissemination: The results will be communicated in peer-review journals and presented at international conferences in the domain of Food, Nutrition & Health.

Patient and Public Involvement statement: patients and the public were not involved in the design or conduct of this protocol

DISCUSSION

The controlled randomised HESPER-HEALTH study will use reliable and sensitive clinical biomarkers of human health to provide a comprehensive picture of the effects of a flavanone-rich OJ on different components of vascular function. It will also produce mechanistic insights of relevance to support the contribution of citrus flavanones in vascular protective effects. Together, the generated knowledge should strengthen the level of evidence of the link between flavanone

intake and the effects of a moderate consumption of OJ on vascular endpoints in humans. The results will also improve understanding of the role of gut microbiota in the interindividual variability in the absorption and metabolism of citrus flavanones, and the putative prebiotic-like effect of flavanone-rich foods. Ultimately, results from HESPER-HEALTH could help health professionals communicate science-driven dietary advice about fruit juice consumption to their patients. They should also be useful for the citrus sector to encourage the selection of orange varieties naturally rich in hesperidin, and to provide health-focused guidance on how to adapt processing methods to produce fruit juice richer in flavanones.

Author contributions: MAV, DM, NM, RE, CG, CBS, PM, CG, LEM, RS, GP, CM contributed to the conceptualisation, design, and implementation of this research protocol; BP led to the development of the statistical analysis plan.

All authors read and approved the final manuscript.

Acknowledgments : We thank the medical team of the PIC/CIC for carrying the clinical investigations and Lise Bernard at the Central Pharmacy of the University Hospital Clermont-Ferrand. We thank Dr. Volker Herdegen, Dietmar Gürster, Caroline Grimm, and Tobias Schardt from Eckes Granini (Niederolm, Germany) for providing a pilot production line and helping to produce and package the drinks to be tested. We thank Peter Bach, Tim Dreifke, Paul Luka Dreis, and Anna-Maria Schmelzer (all Geisenheim University) for their technical assistance during beverage development and production. We thank Sucocítrico Cutrale (Araraquara, São Paulo, Brazil) for donating the OJ concentrate. Döhler (Darmstadt, Germany) is gratefully acknowledged for donating the colorants, clouding agent, and flavouring used in control drink development and production. HealthTech Bio Actives (Beniel, Spain) is acknowledged for providing the hesperidin formulation.

Funding : This work was supported by a financial contribution from a consortium of orange producers, juice manufacturers and packaging companies based in Europe and Brazil under the umbrella of the European Fruit Juice Association (AIJN).

Disclaimer: The views presented in this paper are those of the authors. Funding companies have played no role in the study beyond providing financial support.

Competing interest : None declared

459 **Table 1**: **Inclusion and exclusion criteria for participation in HesperHealth study**

Man or woman	Treated pre-diabetic or diabetic
40-65 years (inclusive)	Treated for hypertension
Post-menopausal woman	Use of statins or other medications for lowering cholesterol
Overweight (BMI ≤30; waist circumference ≥80 cm for women and ≥94 cm for men)	Treated with antibiotics, antifungals, probiotics or prebiotics in the 3 months before the enrolment
	Menopausal hormone therapy
Ability to give informed consent to participate in research.	Diagnosed gastrointestinal illness
Willingness to accept randomization and undergo the testing and intervention procedures and deliver stool, blood and urine samples for testing	Any serious medical condition that precludes safe participation in the study
No aversion or intolerance to citrus foods	History of eating disorders
Accept to limit their total intake of flavonoid rich beverages (tea, coffee, cocoa, wine, fruit juice) to 250 ml/day	Digestive disorders with diarrhea during the 3 months preceding the beginning of the study
	Self-declared vegetarian, vegetalian, vegan
	History of substance or alcohol abuse
	Involvement in a weight loss program within the 3 past months or who had a bariatric surgery
	Current smokers (within the last 30 days)
	Use of dietary supplements currently or in the past one month
	Strenuous exercise greater than 6 hours per week

460

Table 2: Time table of the HESPER-HEALTH study

3 PERIODS / n=42		Visit 1						Visit 2, 4, 6					Visit 3, 5, 7
		Inclusion	day -5	day -4	Day -3	Day -2	Day -1	Day 1	Day 1 to 38	Day 39	Day 40	Day 41	Day 42
CIC visit		X						X					X
Consent forms signature		X											
Medical exam		X											
Dietician interview		X											
Anthropometry		x						x					x
BP		x											
Impedancemetry/Body composition								x					x
Food Questionnaire			x	x	x				x				
Diet low in Low PPs						x	x				x	x	
Standardized meal at diner							x					x	
24h urine collection							X					X	
	PPs urinary excretion							x					x
Feces collection							x					x	
	Gut Microbiota analysis							x					x
Blood sample		x						x					x
	NF, platelets	x											
	ASAT, ALAT, Gamma GT	x											
	Glucose, Insulin	x						x					x
	TSH	x											
	TAG, Total and HDL Chol	x						x					x
	Total FA							x					x
	Creatinin	x											
	Uric acid							x					x
	hsCRP	x						x					x
	I-CAM, V-CAM, e-selectin,IL6, TNFα							x					x
	Nitrites							x					x
	Evs												X
	Vit C, Carotenoids							x					x
	Flavanones							x					x
	Oxylipins profiling							x					x
	Nutrigenomic analysis												x
Vascular function	Endothelial Function (FMD)							x					x
	Microvascular reactivity (FLD)							x					x
	Arterial Stiffnessb (PWV)							x					x
	BP self measurement (SBP+DBP)				X	X	X			X	X	X	

FIGURE LEGENDS

Figure 1: General scheme of HESPER-HEALTH study progress.

V: visits in PIC/CIC; Drink A: OJ naturally rich in hesperidin; Drink B: control beverage with sugar concentrations identical to (A); Drink C: control beverage identical to (B) but supplemented with hesperidin at the level of (A); FQ: 3 days food questionnaire.

Figure 2. HPLC-DAD chromatogram of an OJ from a commercially available OJ concentrate naturally rich in hesperidin (A), a control beverage with an identical total sugar concentration (B), and a control beverage additionally supplemented with hesperidin (C). Flavonoids were extracted according to IFU (2005) [31] and analysed by HPLC-DAD using a C18 column (250 × 4.6 mm, particle size 5.0 µm, Kinetex®, Phenomenex, Aschaffenburg, Germany) and an acetonitrile-based elution gradient.

References

1. Chazelas E, Srour B, Desmetz E *et al.* (2019) Sugary drink consumption and risk of cancer: results from NutriNet-Sante prospective cohort. *BMJ* **366**, l2408.
2. Collin LJ, Judd S, Safford M *et al.* (2019) Association of Sugary Beverage Consumption With Mortality Risk in US Adults: A Secondary Analysis of Data From the REGARDS Study. *JAMA network open* **2**, e193121.
3. Busing F, Hagele FA, Nas A *et al.* (2019) High intake of orange juice and cola differently affects metabolic risk in healthy subjects. *Clin Nutr* **38**, 812-819.
4. Morand C, Dubray C, Milenkovic D *et al.* (2011) Hesperidin contributes to the vascular protective effects of orange juice: a randomized crossover study in healthy volunteers. *The American journal of clinical nutrition* **93**, 73-80.
5. Cassidy A, Bertoia M, Chiuve S *et al.* (2016) Habitual intake of anthocyanins and flavanones and risk of cardiovascular disease in men. *The American journal of clinical nutrition* **104**, 587-594.
6. Mizrahi A, Knekt P, Montonen J *et al.* (2009) Plant foods and the risk of cerebrovascular diseases: a potential protection of fruit consumption. *The British journal of nutrition* **102**, 1075-1083.
7. Chanet A, Milenkovic D, Claude S *et al.* (2013) Flavanone metabolites decrease monocyte adhesion to TNF-alpha-activated endothelial cells by modulating expression of atherosclerosis-related genes. *The British journal of nutrition* **110**, 587-598.
8. Morand C, Barber-Chamoux N, Monfoulet L *et al.* (2019) Dietary (Poly)Phenols and Vascular Health. In *Recent Advances in Polyphenol Research*, vol. 6 [H Halbwirth, K Stich, V Cheynier and S Quideau, editors].
9. Habauzit V, Verny MA, Milenkovic D *et al.* (2015) Flavanones protect from arterial stiffness in postmenopausal women consuming grapefruit juice for 6 mo: a randomized, controlled, crossover trial. *The American journal of clinical nutrition* **102**, 66-74.
10. Li L, Lyall GK, Martinez-Blazquez JA *et al.* (2020) Blood Orange Juice Consumption Increases Flow-Mediated Dilation in Adults with Overweight and Obesity: A Randomized Controlled Trial. *The Journal of nutrition* **00**, 1–8.
11. Valls RM, Pedret A, Calderon-Perez L *et al.* (2020) Effects of hesperidin in orange juice on blood and pulse pressures in mildly hypertensive individuals: a randomized controlled trial (Citrus study). *European journal of nutrition*.
12. Cani PD, Everard A (2016) Talking microbes: When gut bacteria interact with diet and host organs. *Molecular nutrition & food research* **60**, 58-66.
13. Tomas-Barberan FA, Selma MV, Espin JC (2016) Interactions of gut microbiota with dietary polyphenols and consequences to human health. *Current opinion in clinical nutrition and metabolic care* **19**, 471-476.
14. Vallejo F, Larrosa M, Escudero E *et al.* (2010) Concentration and solubility of flavanones in orange beverages affect their bioavailability in humans. *Journal of agricultural and food chemistry* **58**, 6516-6524.
15. Aschoff JK, Riedl KM, Cooperstone JL *et al.* (2016) Urinary excretion of Citrus flavanones and their major catabolites after consumption of fresh oranges and pasteurized orange juice: A randomized cross-over study. *Molecular nutrition & food research* **60**, 2602-2610.
16. Tomas-Navarro M, Vallejo F, Sentandreu E *et al.* (2014) Volunteer stratification is more relevant than technological treatment in orange juice flavanone bioavailability. *Journal of agricultural and food chemistry* **62**, 24-27.
17. Fidelix M, Milenkovic D, Sivieri K *et al.* (2020) Microbiota modulation and effects on metabolic biomarkers by orange juice: a controlled clinical trial. *Food & Function* **11**, 1599-1610.
18. Kay CD, Clifford MN, Mena P *et al.* (2020) Recommendations for standardizing nomenclature for dietary (poly)phenol catabolites. *American Journal of Clinical Nutrition* **112**, 1051-1068.

19. Krga I, Milenkovic D, Morand C *et al.* (2016) An update on the role of nutrigenomic modulations in mediating the cardiovascular protective effect of fruit polyphenols. *Food & Function* **7**, 3656-3676.
20. Milenkovic D, Deval C, Dubray C *et al.* (2011) Hesperidin Displays Relevant Role in the Nutrigenomic Effect of Orange Juice on Blood Leukocytes in Human Volunteers: A Randomized Controlled Cross-Over Study. *Plos One* **6**.
21. Milenkovic D, Deval C, Gouranton E *et al.* (2012) Modulation of miRNA Expression by Dietary Polyphenols in apoE Deficient Mice: A New Mechanism of the Action of Polyphenols. *Plos One* **7**, 156-167.
22. Milenkovic D, Jude B, Morand C (2013) miRNA as molecular target of polyphenols underlying their biological effects. *Free Radical Biology and Medicine* **64**, 40-51.
23. Yahfoufi N, Alsadi N, Jambi M *et al.* (2018) The Immunomodulatory and Anti-Inflammatory Role of Polyphenols. *Nutrients* **10**.
24. Borkowski K, Yim SJ, Holt RR *et al.* (2019) Walnuts change lipoprotein composition suppressing TNF alpha-stimulated cytokine production by diabetic adipocyte. *Journal of Nutritional Biochemistry* **68**, 51-58.
25. Gabbs M, Leng S, Devassy JG *et al.* (2015) Advances in Our Understanding of Oxylipins Derived from Dietary PUFAs. *Advances in Nutrition* **6**, 513-540.
26. Burton-Freeman B (2010) Postprandial metabolic events and fruit-derived phenolics: a review of the science. *The British journal of nutrition* **104 Suppl 3**, S1-14.
27. Corretti MC, Anderson TJ, Benjamin EJ *et al.* (2002) Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: a report of the International Brachial Artery Reactivity Task Force. *Journal of the American College of Cardiology* **39**, 257-265.
28. Aschoff JK, Kaufmann S, Kalkan O *et al.* (2015) In vitro bioaccessibility of carotenoids, flavonoids, and vitamin C from differently processed oranges and orange juices [Citrus sinensis (L.) Osbeck]. *Journal of agricultural and food chemistry* **63**, 578-587.
29. Tessier F, BirlouezAragon I, Tjani C *et al.* (1996) Validation of a micromethod for determining oxidized and reduced vitamin C in plasma by HPLC-fluorescence. *International Journal for Vitamin and Nutrition Research* **66**, 166-170.
30. Mainka M, Dalle C, Petera M *et al.* (2020) Harmonized procedures lead to comparable quantification of total oxylipins across laboratories. *Journal of Lipid Research* **61**, 1424-1436.
31. IFU (2005) Determination of Hesperidin and Naringin HPLC In *Methods of analysis*, International Federation of Fruit Juice Producers (Ed.) ed., vol. 58 [IFoFJP (Ed.), editor]. Zug, Switzerland.

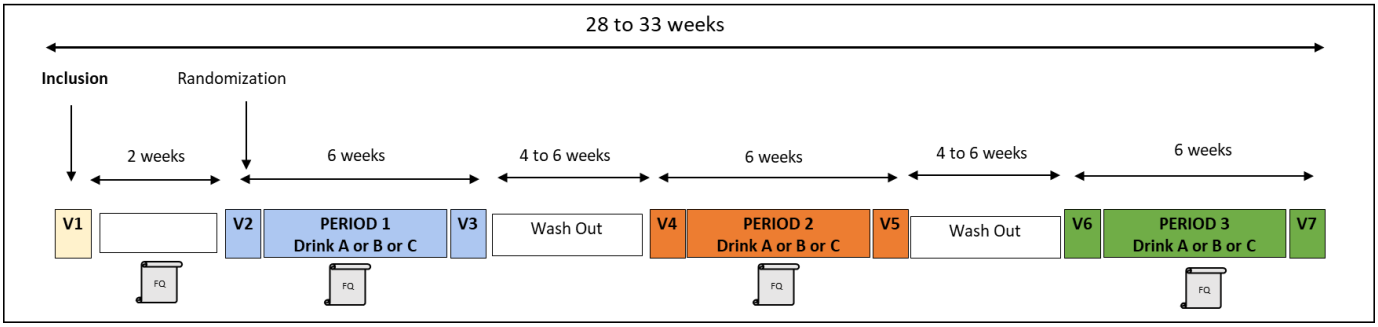

Figure 1

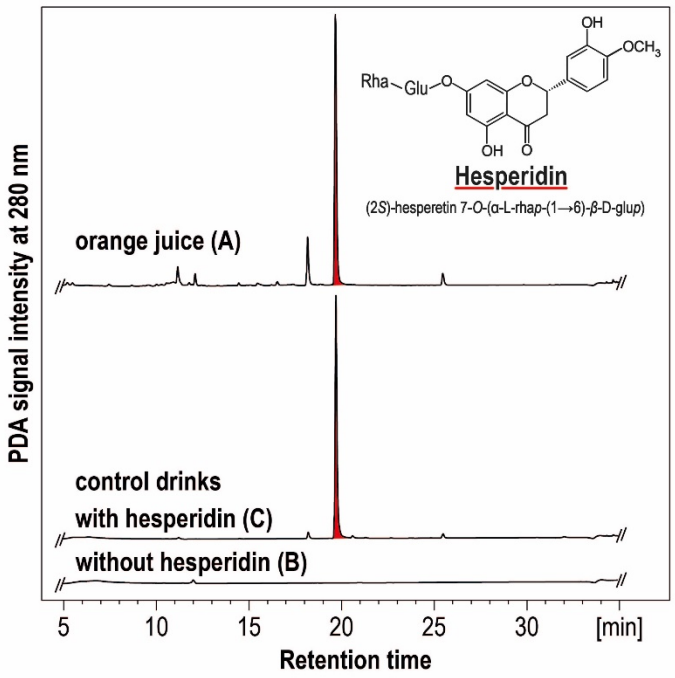

Figure 2. HPLC-DAD chromatogram of an OJ from a commercially available OJ concentrate naturally rich in hesperidin (A), a control beverage with an identical total sugar concentration (B), and a control beverage additionally supplemented with hesperidin (C). Flavonoids were extracted according to IFU (2005) [31] and analysed by HPLC-DAD using a C18 column (250 × 4.6 mm, particle size 5.0 µm, Kinetex®, Phenomenex, Aschaffenburg, Germany) and an acetonitrile-based elution gradient.

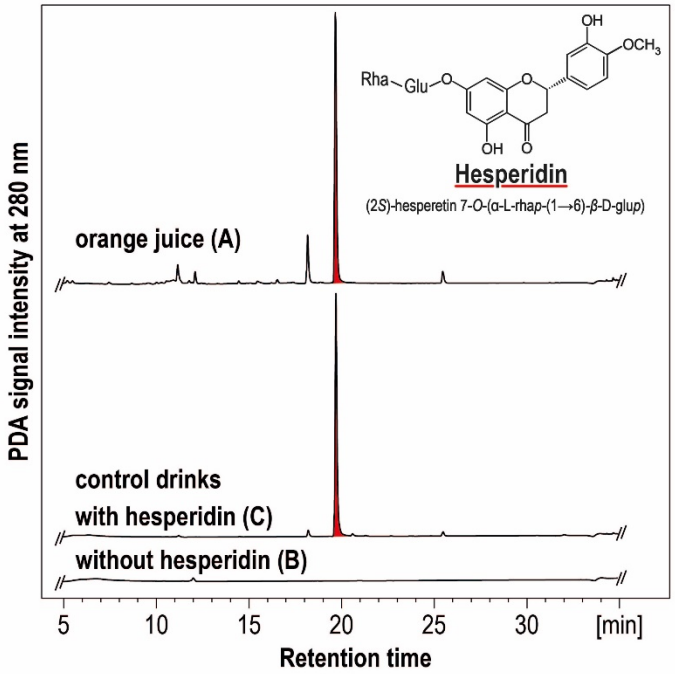

Figure 2.

BMJ Open

Study protocol for a randomized controlled trial evaluating the role of Orange juice, HESPERidin in vascular HEALTH benefits: The HESPER-HEALTH Study

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2021-053321.R1
Article Type:	Protocol
Date Submitted by the Author:	11-Oct-2021
Complete List of Authors:	Verny, Marie-Anne; INRAE, UNH Milenkovic, Dragan; INRAE, UNH MACIAN, Nicolas; University Hospital Centre Clermont-Ferrand Pereira, Bruno; University Hospital Centre Clermont-Ferrand Evrard, Rémy; University Hospital Centre Clermont-Ferrand Gilcher, Caroline; Geisenheim University Steingass, Christof B.; Geisenheim University Mosoni, Pascale; INRAE, MEDIS Gladine, Cécile; INRAE, UNH Monfoulet, Laurent-Emmanuel; INRAE, UNH Schweiggert, Ralf; Geisenheim University PICKERING, Gisèle; University Hospital Centre Clermont-Ferrand MORAND, Christine; INRAE, UNH
Primary Subject Heading:	Nutrition and metabolism
Secondary Subject Heading:	Cardiovascular medicine
Keywords:	MOLECULAR BIOLOGY, NUTRITION & DIETETICS, VASCULAR MEDICINE

I, the Submitting Author has the right to grant and does grant on behalf of all authors of the Work (as defined in the below author licence), an exclusive licence and/or a non-exclusive licence for contributions from authors who are: i) UK Crown employees; ii) where BMJ has agreed a CC-BY licence shall apply, and/or iii) in accordance with the terms applicable for US Federal Government officers or employees acting as part of their official duties; on a worldwide, perpetual, irrevocable, royalty-free basis to BMJ Publishing Group Ltd ("BMJ") its licensees and where the relevant Journal is co-owned by BMJ to the co-owners of the Journal, to publish the Work in this journal and any other BMJ products and to exploit all rights, as set out in our licence.

The Submitting Author accepts and understands that any supply made under these terms is made by BMJ to the Submitting Author unless you are acting as an employee on behalf of your employer or a postgraduate student of an affiliated institution which is paying any applicable article publishing charge ("APC") for Open Access articles. Where the Submitting Author wishes to make the Work available on an Open Access basis (and intends to pay the relevant APC), the terms of reuse of such Open Access shall be governed by a Creative Commons licence – details of these licences and which Creative Commons licence will apply to this Work are set out in our licence referred to above.

Other than as permitted in any relevant BMJ Author's Self Archiving Policies, I confirm this Work has not been accepted for publication elsewhere, is not being considered for publication elsewhere and does not duplicate material already published. I confirm all authors consent to publication of this Work and authorise the granting of this licence.

Study protocol for a randomized controlled trial evaluating the role of Orange juice, HESPERidin in vascular HEALTH benefits: The HESPER-HEALTH Study

Marie-Anne Verny[1], Dragan Milenkovic[1], Nicolas Macian[2], Bruno Pereira[3] Rémy Evrard, [2] Caroline Gilcher[4], Christof B. Steingass[4], Pascale Mosoni[5], Cécile Gladine[1], Laurent-Emmanuel Monfoulet[1], Ralf Schweiggert[4], Gisele Pickering[2,], Christine Morand[1]

[1] Université Clermont Auvergne, INRAE, UNH, F-63000 Clermont-Ferrand, France

[2] Plateforme d'Investigation Clinique/CIC Inserm 1405, University Hospital CHU, 63000 Clermont-Ferrand, France.

[3] Biostatistics Unit (DRCI), Gabriel-Montpied University Hospital, Clermont-Ferrand, France

[4] Chair of Analysis and Technology of Plant-based Foods, Department of Beverage Research, Hochschule Geisenheim University, Geisenheim, Germany.

[5] Université Clermont Auvergne, INRAE, MEDIS, F-63000 Clermont-Ferrand, France

Author emails :

Marie Anne Verny : marie-anne.verny@inrae.fr

Dragan Milenkovic: dragan.milenkovic@inrae.fr

Macian Nicolas: nmacian@chu-clermontferrand.fr

Bruno Pereira : Bpereira@Chu-Clermontferrand.Fr

RemyEvrard: revrard@chu-clermontferrand.fr

Caroline Gilcher : Caroline.gilcher@hs-gm.de

Christof Steingaß: Christof.Steingass@hs-gm.de

Pascale Mosoni : pascale.mosoni@inrae.fr

Cécile Gladine : cecile.gladine@inrae.fr

Laurent Emmanuel Monfoulet : laurent-emmanuel.monfoulet@inrae.fr

Ralf Schweiggert: ralf.schweiggert@hs-gm.de

Gisele Pickering: gisele.pickering@uca.fr

Christine Morand : Christine.morand@inrae.fr

Corresponding author :

Christine MORAND

INRAE- Unité de Nutrition Humaine – UMR 1019

Site de Theix INRAE - Clermont-Ferrand – Theix- 63122 Saint-Genès-Champanelle

France

Email: Christine.morand@inrae.fr

Tel : +(33) 4 73 62 40 84

Key words :

Randomized controlled trial ; vascular function ; orange juice ; hesperidin ; flavanones bioavailability and metabolism

ABSTRACT

Introduction: Although epidemiological studies associate the consumption of sugary beverages with adverse health effects, human experimental studies have demonstrated substantially different metabolic responses when 100% fruit juices are compared with artificial beverages. Fruit juices do not just provide sugars and associated calories, but they are also rich in bioactive compounds. Flavanones are bioactives specifically and abundantly found in citrus foods, with hesperidin as the major representative in sweet oranges. Flavanone intake has been associated with a lower incidence of mortality from cardiovascular disease (CVD). However, clinical evidence are too scarce to confirm the vasculo-protective effects of 100% orange juice (OJ) presumably mediated by flavanones, and thereby do not allow firm conclusions to be drawn about their efficacy.

Methods and analysis: The HESPER-HEALTH study aims to assess the efficacy of OJ in improving vascular function and the contribution of hesperidin to these effects. This double-blind, randomised, controlled, crossover study will be carried out in 42 volunteers predisposed to CVD, based on age and on overweight. It includes three 6-week periods of consumption of 330 mL/d of OJ versus control drinks with and without hesperidin at a dose in agreement with a daily OJ serving (approx. 200-215 mg). The primary outcome is endothelial function, assessed by flow mediated dilation (FMD), with measurements performed at fasting and postprandially in response to a challenge meal. The secondary outcomes include bioavailability and metabolism of flavanones, changes in other markers of vascular function, systemic biomarkers of cardiovascular risk, endothelial dysfunction and inflammation, vitamin C and carotenoids status, anthropometry and body composition, gut microbiota composition, nutrigenomic response and in oxylipin profiling.

Ethics and dissemination: This ongoing study was approved by the Ethics committee Sud-Est III, Bron, France on November 17, 2020. The trial is registered on ClinicalTrials.gov (NCT04731987). The results will be disseminated in peer-reviewed journals.

ARTICLE SUMMARY

Strengths and Limitations of this study

- This randomised controlled dietary intervention is carried out to assess the effects of OJ consumption on vascular function and to determine the contribution of hesperidin to these effects.
- This trial includes an analysis of hesperidin bioavailability and metabolism in biofluids to enable further correlation with the vascular response of individuals.
- The use of innovative approaches (i.e., oxylipin profiling and nutrigenomics) will provide insights on the molecular mechanisms underlying the vascular effects.
- By including an analysis of the gut microbiota, this study will clarify interactions between OJ, hesperidin and the microbiome.
- The sensoriality of the two artificial control drinks differs from that of the fully natural orange juice OJ, which may constitute a study limitation.

INTRODUCTION

Recent epidemiological studies have associated the frequent consumption of sugar-sweetened beverages with some adverse health effects, such as early death, weight gain and cancer [1; 2] Findings of such studies are often extrapolated to fruit juice consumption, including 100% fruit juices. However, these observational, epidemiological results contrast with experimental studies which have demonstrated clear differences in the metabolic response of the human body to fruit juices as compared with that seen for sugar-sweetened beverages[3; 4].

One characteristic of citrus foods is that they are a rich and exclusive source of dietary flavanones, a category of (poly)phenol compounds, mainly present as hesperidin in orange. Flavanone intake has been repeatedly associated with a lower incidence of mortality from CVD [5; 6]. Results from preclinical studies using different models of atherosclerosis also provide evidence for a role of citrus flavanones in cardiovascular protection, with a slowdown in atherosclerosis development [7].These atheroprotective effects have been related to the capacity of flavanones to modulate the expression of genes involved in cellular processes responsible for vascular dysfunction[8]. Evidence of the vascular protective effects of citrus flavanones have been reported in few randomised controlled clinical trials [4; 9; 10; 11]. However, published trial with orange flavanones do not yet allow firm conclusions to be drawn about their efficacy to modulate vascular function, mainly due to the high degree of discrepancies between the study designs.

The ability of citrus flavanones to exert beneficial effects depends on their bioavailability. The gut microbiota plays a key role in flavanone absorption, because naturally-occuring flavanones are present as molecules with glycosyl moieties, e.g. a rutinosyl, i.e. a α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranosyl moiety in hesperidin. The sugar moiety of hesperidin must be hydrolysed by bacterial glycosidases to yield a format which the human body can absorb i.e. the aglycone, hesperetin. Upon release, hesperetin can be absorbed by intestinal cells or may be further catabolized into diverse phenolic compounds by microbial action in the colon. After absorption of hesperetin and its microbial catabolites, they enter the blood circulation and are subject to further human metabolism. For instance, hepatic metabolism includes several conjugation reactions, e.g., sulphatation or glucuronidation[12; 13]. Hence, the extent of hesperetin released in the colon, the formation of colonic catabolites and their subsequent absorption could largely depend on the gut microbial composition, which has been shown to vary between subjects [14]. In agreement with this hypothesis, previous studies have reported a large interindividual variability in the urinary

excretion level of flavanones [15; 16] that could reflect differences in gut microbiota composition, but this remains to be demonstrated. A recent clinical study found that regular consumption of OJ can modulate the gut microbiota profile and these changes were associated with positive shifts in some metabolic outcomes[17].

Dietary (poly)phenols and their circulating metabolites encompass a huge diversity of compounds [18] whose health effects seem increasingly linked to their capacity to exert complex genomic modifications, such as changes to the expression of genes [19]. In preclinical studies, flavanones have been shown to induce changes in the expression of a number of genes in aorta and endothelial cells that relate to inflammation, and endothelial cell function [7; 20; 21; 22], revealing potential molecular mechanisms of action to explain their health properties. However, such mechanisms of action are still largely unexplored in humans [20]. Based on a huge body of experimental results demonstrating the anti-inflammatory effects of polyphenols and related mechanisms [23], these compounds are suggested as key players in the protective effects of their food sources for chronic inflammatory diseases. However, in humans, consumption of polyphenol-rich foods induces subtle changes in inflammatory and oxidative status that can only typically be measured using sensitive techniques, such as the lipidomic profiling of oxylipins [24]. Oxylipins are a superclass of lipid mediators comprising hundreds of metabolites which regulate a diversity of biological processes including inflammation, cell adhesion, migration and proliferation, blood clotting and vessel permeability [25].

Based on this state of the art, the present human randomised, controlled, double-blind, cross over intervention conducted on subjects predisposed to CVD aims to establish a cause-and-effect relationship between hesperidin intake and the vascular protective effects of drinking OJ naturally

rich in hesperidin. The study will also provide insights into the mechanisms responsible for the observed effects.

STUDY OBJECTIVES

Primary Objective

The primary objective is to assess the effect of a subchronic consumption (6 weeks) of a naturally flavanone-rich OJ or a control drink supplemented with orange flavanones on endothelial function, by assessing flow-mediated dilation (FMD) in subjects with a predisposition to CVD, based on age, waist circumference and body mass index.

Secondary Objectives

The secondary objectives are to:

- assess the effects of the intervention: (1) on other markers of vascular function using a range of well-established measurements in the macrocirculation (blood pressure, arterial stiffness) and in the microvasculature at skin level (microvascular reactivity by Flow Laser Doppler, FLD); (2) on the postprandial endothelial response in response to a challenge meal; (3) on biomarkers of cardiovascular risk, endothelial dysfunction and inflammation; (4) on anthropometric parameters and body composition; (5) on the vitamin C and carotenoids status; (6) on the gut microbiota composition (microbial communities profiling)
- measure the bioavailability of hesperidin and its metabolites in biofluids (plasma, urine)
- ascertain the underpinning molecular mechanisms of the vascular responsiveness (nutrigenomic analysis, oxylipin profiling).

METHOD AND ANALYSIS

Study Design

HESPER-HEALTH is a human dietary intervention study designed as a randomized, double-blind, controlled, cross-over trial with three arms that will be conducted at the Clinical Investigation Center, Inserm 1405 (PIC/CIC) of the University Hospital of Clermont-Ferrand, France. The sponsor is the University Hospital of Clermont-Ferrand. This trial will be carried out on subjects predisposed to CVD, based on age (40-65 years old) and on the overweight (waist circumference ≥ 80 cm for woman, ≥ 94 cm for man and with a BMI≤30 kg/m^2). Forty-two participants will be recruited and will receive the three treatments in a random order: (A) a commercially-available OJ naturally rich in hesperidin (ca. 600-650 mg/L), (B) a control beverage with a total sugar concentration identical to (A), and (C) a control beverage identical to (B) but supplemented with hesperidin at the level present in the natural OJ (A).

For each volunteer, the study is divided in three identical experimental periods of 45 days. These periods include a three day run in period during which time specific dietary recommendations will be followed, samplings and measurements will be performed at home. This run in period will be followed by a 6-weeks treatment period of consumption of one of the three beverages. A four to six week wash-out is planned between each of the three experimental periods. The protocol includes seven visits to PIC/CIC, including one visit (V1) at inclusion, and will last in total for 28 to 33 weeks (**Figure 1**). Over the last 24h prior to each visit, volunteers will be asked to collect stool samples and 24h urine samples to assess gut microbiota composition and flavanone bioavailability and metabolism, respectively.

At the beginning and end of each experimental period, overnight-fasted volunteers will be invited to attend the PIC/CIC (visits V2 to V7) for vascular function tests, blood sampling, anthropometric measurements and body composition analysis. Blood will be sampled for further assessment of plasma flavanones including metabolites, carotenoids and vitamin C, oxylipin profiling, systemic biomarkers of endothelial activation and inflammation, metabolic parameters, and for the analysis of the nutrigenomic response.

At each visit (V2 to V7), any adverse events (AE) will be followed up. In case of occurrence of a serious AE, the sponsor will be notified and in return he will notify the competent authoritees. All unexpected serious AE are reported in an annual safety report. After measurements and blood collection in the fasting state, a challenge meal together with the respective study drink will be administered to all subjects before further exploration. This will enable evaluatation of the acute postprandial effects (at T+3h, T+6h) of the study products on endothelial function (V2,V4,V6), to also be repeated after the 6 weeks intervention period (V3, V5, V7). The challenge meal will consist of fresh cream, sucrose and milk proteins, providing 900 kcal – a pro-oxidant and pro-inflammatory meal that is known to induce a transient endothelial dysfunction during the post prandial period [26].

During each experimental period, volunteers will be asked not to eat citrus foods and to consume no more than 250 mL/d of polyphenol-rich beverages (coffee, tea, fruit juices, wine, cocoa). To check that volunteers do not change their eating habits during the study, for each experimental period they will be asked to complete food records over three consecutive days defined by the investigator. The first food record will be completed prior to visit V2 to PIC/CIC and the two others

at mid-term of each intervention period. On the two days preceeding each visit at the PIC/CIC, volunteers will be asked not to consume polyphenol-rich foods and beverages. The day before each visit (V2-V7) at the PIC/CIC, they will have to consume exactly the same dinner without polyphenols at 8 pm. To ensure a good understanding of all these dietary instructions, participants meet a dietician during the inclusion visit (V1).

Inclusion and Exclusion criteria

These criteria are listed in the Table 1.

The reasons for a premature cessation of the study include withdrawal of consent, significant deviation from the protocol, incidental illness, occurrence of a serious adverse event, intolerance to the tested products, antibiotic treatment during the study, a non-observance of the nutritional protocol declared by the subject or highlighted by food records.

Study products

Three study beverages will be used in cross-over and double-blinding conditions. These include (A) an OJ from a commercially-available OJ concentrate (Sucocítrico Cutrale, Araraquara, São Paulo, Brazil) containing approx. 600-650 mg hesperidin per L, (B) a control drink with a total sugar concentration identical to that of (A), and (C) a control drink identical to (B) but supplemented with hesperidin at the level found in the OJ (Figure 2). The daily administered dose will be 330 mL to be distributed over two intakes (2 × 165 mL) - one in the morning during breakfast and the second during lunch. This will correspond to ca. 200-215 mg hesperidin per day (drinks A and C, resp.).

The control beverages (B) and (C) will be made isocaloric to (A), all containing an identical total sugar concentration (ca. 9.0 g/100 mL), comprising glucose, fructose, and sucrose. Thus, the amount of total sugar provided daily by each drink will amount to ca. 30 g. All drinks will contain citric acid at similar levels. To match the visual appearance of the OJ, food colorants as well as a clouding agent will be added to the control drinks. Flavour will be matched by adding a natural orange aroma to all beverages (A-C), including the OJ from concentrate to which orange aroma addition is required by European law. All the beverages will be pasteurized according to commercial practice and filled into 330 mL brown glass bottles with no visual distinction possible between bottles of different drinks. Despite careful colour and flavor matching, both the hesperidin-free (B, placebo) and the hesperidin-rich (C) control drink may be differentiated sensorially from the OJ by attentive subjects. In contrast, subjects and clinic staff will be unable to identify which of the artificial control drinks (B) and (C) is the one rich in or free from hesperidin.

Finally, bottles will be labelled and packaged for each cross-over period in a blinded fashion on the basis of the randomisation schedule. They will be stored at +4°C until distribution to the participants. Beverages will be distributed to volunteers at the first visit of each experimental period (V2, V4, V6) in bags containing 48 bottles per period. Volunteers will be asked to store the bottles in a dark place at room temperature or cooler. The produced beverages will be analytically characterized in detail, including, e.g., the levels of potassium [27], carotenoids[28], and soluble, insoluble [14] and total hesperidin [29]. Hesperidin levels in drinks (A) and (C) as well as levels of ascorbic and dehydroascorbic acid (vitamin C) in the OJ (A) will be monitored over the entire study period. Colony forming units of yeasts, total viable counts, lactic acid bacteria and moulds will also be assessed to ensure the microbiological safety of the products. To assess compliance, participants

will be asked to report daily on diaries their consumption of the study beverages and to return the dairies and the empty and non-consumed bottles at the end of each period (V3, V5, V7).

Assignment of intervention

Randomisation and Allocation : Subjects will be randomly assigned to treatment groups according to a pre-established list of randomisation designed by a clinical research supervisor, independently from the investigators and the sponsor. The treatment number will be allocated by order of entrance in inclusion. Subjects will be randomised to receive drink A, B, or C using a Latin-square random design. Subject allocation to treatments will take place after inclusion and will be based on a computer-generated randomisation list. Access to the randomisation list will be restricted to staff performing this task. The randomisation number will be allocated by order of entrance in inclusion. Under normal circumstances, the blinding should not be broken until all subjects have completed the trial and the database will be locked. However, the blinding can be broken should a specific emergency treatment at the site require knowledge of the treatment status of the subject. In such a case, the investigator can, using a sealed envelop and following an internal procedure, reveal the treatment assignment through the randomisation plan and then inform the sponsor.

Participant eligibility

Forty-two volunteers will be recruited from the existing PIC/CIC volunteer's database, by announcement in local press and media by paper and digital poster campaigns and by social networks. Each subject who meets the recruitment criteria (Table 1) will be pre-selected by the investigator and will be given a detailed explanation of the protocol. Participant who agree to participate will have a first visit (V1, **Figure 1**) at the PIC/CIC to provide their written consent to

participate and to have a medical examination, an interview with a dietitian and a blood checkup to ensure that all clinical criteria are met.

Sample size

Sample size calculation has been performed based on both our experience in FMD measurement and on the FMD response (main criteria) observed in previous dietary interventions[30,31]. The targeted statistical power was based on interindividual variability for FMD measurement of the operator (SD = 1.9%). Aiming for a statistical power greater than 80% and a two-tailed type I error at 0.017 (to take into account the three comparisons to be considered), the total number of subjects required to provide sufficient power to detect a minimal absolute difference of FMD equals 1.6% in a 3-sequences cross-over study (with an intra-class correlation coefficient at 0.5) is 36. Assuming unforeseen drop-outs and follow-up losses, 42 participants will be recruited.

STUDY ASSESSMENTS

The time table of the measurements that will be performed in the HESPER-HEALTH study is presented in **Figure 3**. The outcome assessors and data analysts will be blinded for treatments.

Primary outcome

Endothelial function is assessed using the non-invasive technique FMD which constitutes the gold standard to evaluate vascular endothelial function in humans. The FMD technique measures the diameter of the brachial artery by ultrasound before and after increasing shear stress by inducing a reactive hyperemia, with the degree of dilation reflecting mostly the arterial endothelial nitric oxide release. The procedure for the FMD measurement is full compliant with the reference method

described by Coretti et al. [32]. FMD is measured on the left brachial artery above the antecubital fossa using a high-resolution ultrasound system with a 7-12 MHz linear array 190 transducer (Vivid S5, GE Healthcare, Versailles, France). The use of a mechanical arm device (Vascular Imaging, Amsterdam, Netherlands) allows precise movements of the probe in the three dimensions. Images are analysed using automated edge detection software 197 (Hemodyn 3M apparatus, Dinap SRL, Buenos Aires, Argentina). FMD will be assessed at V2, V3, V4, V5, V6 and V7 under both fasting and postprandial conditions.

Secondary Outcomes

- ***Other vascular function measurements***

Endothelial function in the microcirculation will be assessed using Flowmetry by Laser Doppler (FLD), which is a non-invasive and validated technique for continuous measurement of the endothelial dependent microvascular reactivity. The laser-doppler system PeriFlux 5010 (Perimed) is used at the level of the hand to follow the response to a reactive hyperemia induced by a temporary occlusion of the brachial artery, using the same stimulus as for FMD measurement. FLD will be assessed during V2, V3, V4, V5, V6 and V7 in fasting and postprandial conditions.

Arterial stiffness will be evaluated by the carotid-femoral Pulse Wave Velocity (PWV) which is calculated from measurements of pulse transit time and the distance travelled between the carotid and femoral arteries using a validated non-invasive device (SphygmoCor; AtCor Medical Pty. Ltd.). PWV will be assessed after fasting at V2, V3, V4, V5, V6 and V7.

Blood pressure will be monitored by subjects at home using tensiometers (Mircrolife BP A200, Microlife) which will be loaned to them for the study duration. They will be asked to perform three repeated measurements in fasting condition during the three consecutive days preceding their visits

to the PIC/CPC (V2, V3, V4, V5, V6 and V7), and to record obtained values on a dedicated data sheet.

➢ ***Flavanone metabolism and bioavailability in biofluids***

Phase 2 metabolites of the *Citrus* flavanones and their microbial-derived catabolites of flavanones including conjugated forms like glucuronidated and sulfated ones will be analyzed in plasma and 24h urine samples according to the method based on those reported by Aschoff et al. [15] and Mullen et al. [33]. For plasma and urine analyses, acetonitrile and methanol will be used as extraction solvents as described in detail by Mullen et al. [33]. A subset of urine and plasma samples will be analyzed after enzymatic hydrolysis with glucuronidase and sulfatase as described by Aschoff et al. [15]. Identification and quantitation will be done by UHPLC-DAD-ESI-MS/MS for all samples collected (urine) or sampled (plasma) at V2, V3, V4, V5, V6 and V7.

➢ **S*ystemic biomarkers related to CVD risk, endothelial dysfunction and inflammation***

These include metabolic parameters (plasma glucose, insulin, Tri Acyl Glycerol (TAG), Total Cholesterol, High density lipoprotein cholesterol (HDL-chol), uric acid that will be measured by spectrometric and enzymatic methods; total fatty acids, by gas chromatography), soluble adhesion molecules and inflammatory markers (Intercellular Adhesion Molecule (ICAM), Vascular Cell Adhesion Molecule (VCAM) and e-selectin, Interleukin-6 (IL-6), Tumor Necrosis Factor Alpha (TNFalpha), high sensitivity- C Reactive Protein (hs-CRP), by using Elisa assays), plasma nitrites (by chemi-luminescence) and the release of endothelial extracellular vesicles (by flux cytometry). These analyzes will be performed in blood sampled at V2, V3, V4, V5, V6 and V7.

➢ ***Carotenoids and vitamin C status***

Carotenoids will be quantitated from human plasma after a protein-crash with ethanol and repeated extraction with hexane using the method described by Aschoff et al. [28]. Vitamin C status will be quantified in deproteinized plasma by HPLC-fluorescence detection as previously described [34]. These measures will be performed in blood sampled at V2, V3, V4, V5, V6 and V7.

➢ ***Anthropometry and body composition assessment***

Body Mass Index (BMI), waist to height ratio (waist circumference/height) and the percentages of fat mass, lean mass and water (obtained using a multi-frequency Bioelectrical Impedance Analyser) will be determined. These parameters will be recorded at V2, V3, V4, V5, V6 and V7.

➢ ***Gut microbiota composition assessment***

The composition of the gut microbiota will be determined by 16S metabarcoding. This method involves the amplification by PCR of variable regions of the 16S rDNA gene from fecal DNA followed by the preparation of DNA libraries and high throughput next-generation sequencing (Illumina technology). Analysis will be completed by an absolute quantification by qPCR of target groups of the human gut microbiota considered as beneficial to the host, or involved in polyphenol metabolism. These analyses will be performed in faeces samples collected during the 12h preceding V2, V3, V4, V5, V6 and V7.

Mechanistic outcomes

In the HESPER-HEALTH study, we aim to explore the molecular mechanisms of action underlying vascular effects of OJ and hesperidin consumption. To this end, we propose to perform global and integrated analyses of expression of genes (nutrigenomic analysis) in the blood and to analyse the

oxylipin profiling to identify changes in biological processes involved in inflammation and vascular dysfunction.

Nutrigenomic response assessment**:** RNA will be extracted from whole blood using PAXgene Blood RNA System. Global gene expression profile of both protein coding and protein non-coding RNAs (miRNAs, snoRNAs, lncRNAs) will be assessed using a microarray approach. For genes identified as differentially expressed, integrated multi-omic bioinformatic analyses will be performed to identify gene ontologies, gene network, cellular pathways and interactions between different types of RNAs. Potential transcription factors involved will be searched and capacity of flavanone metabolites to bind to transcription factors as well as cell signaling proteins regulating their activity will be predicted using 3D docking analysis. Analysis will be performed from blood sampled at V3, V5 and V7.

Oxylipin profiling: a comprehensive assessment of circulating total oxylipins (free and esterified forms) will be performed using a targeted and quantitative MS-based method (LC-MS/MS) as described previously [35]. Briefly, EDTA plasma will be mixed with 22 internal standards and an antioxidant solution preventing artificial oxylipin production during sample processing. Then total oxylipins will be extracted using solid phase extraction (SPE) following protein precipitation and alkaline hydrolysis. Extracted oxylipins will then be measured using electrospray ionization in negative ion mode and multiple reaction monitoring (MRM). Analysis will be performed in plasma sampled at V2, V3, V4, V5, V6 and V7.

Statistical analyses

All analyses will be performed with Stata software (version 15, StataCorp, College Station, USA) before the breaking of the randomiszation code, according to International Conference on Harmonization-Good Clinical Practice guideline. Continuous variables will be presented,

according to their statistical distribution, as mean and standard-deviation, or median and interquartile range. The Shapiro-Wilk test will be used to assess normality. Categorical data will be presented as exact number and percentage.

The primary outcome will be analysed using random-effects model (i.e. mixed linear model for continuous dependent variable) for a 3-treatment crossover study. The statistical model includes treatment group, sequence, period, carry-over on FMD baseline value as fixed effects. Subject nested in sequence will be included in the model as a random effect. A Sidak's type I error will be applied to take into account multiple comparisons. The *treatment group x period* interaction will be studied. If this interaction is not significant, the main effect of treatment will be assessed. If this interaction is significant, there will be a particular focus on the first period. The normality of residuals from random-effects model will be studied as aforementioned, with the Shapiro-Wilk statistic and visual inspection of residual plots. If appropriate, a transformation (for example logarithmic) of the primary outcome could be proposed to achieve its normality. The results will be expressed with effect-sizes and 95% confidence intervals.

The primary analysis will be completed by multivariable approach using the statistical model described above with, additionally, covariates determined according to univariate results and clinical relevance, including primarily waist circumference, BMI, weight at enrolment, age, gender and energy intake. The other continuous outcomes will be analyzed with the same statistical analysis plan. For categorical data, generalized mixed linear modelling will be carried out. Any change in the current statistical analysis plan during the study will be noted in the study records.

ETHICS AND DISSEMINATION

Approval

In accordance with the Declaration of Helsinki and French regulations on clinical trials, the study was evaluated by an independent ethics committee, chosen at random by the French ministry of research, namely the 'Comité de Protection des Personnes Sud-Est III, Bron, France' (registration number: 20.10.15.60521). The approval of the ethics committee was obtained on November 17, 2020. The first inclusion was on February 24, 2021. The study is declared to the French National Agency for the Safety of Medicines (ID-RCB : 2020-A01985-34). Any substantial change in the protocol or in the informed consent form will be presented to both authorities. The trial is also registered on ClinicalTrials.gov (NCT04731987).

Consent

Subjects will be informed by a fair and accessible form approved by the ethics committee. Subjects will be free to ask any question on all aspects of the study before giving the consent and informed that they are free to withdraw from the study at any time. The investigator will ensure that the written consent obtained from subjects prior to their participation in the study is free and informed (supplemental data 1).

Data quality and management

The data collected will be anonymized by a subject code assigned to each participant. All data from the interview, clinical examination and explorations will be reported in a paper Case Report Form (CRF) by experimented and trained study managers of the PIC/CIC and will constitute the data trial kept confidential. The database of the study will be designed and managed using the Research Electronic Data Capture (REDCap™, Vanderbilt University), a dedicated software allowing a secure and local storage of the data and also an audit trail by user authentication. Data entries will be made from the paper CRF with a double entry procedure. After reconciliation of the two entries

and elimination of transcription errors, data validation will be carried out to resolving discrepancies by the data manager. Then, database will be locked and securely sent to the biostatistician.

According to the "minimal risks and contraints" ranking of this study by the ethics commitee, the monocentric design and the low risk calculated by the sponsor, there is no data monitoring committee and no interim analyses planned for this study. Audits or inspections concerning the trial or investigation team activities could be carried out at anytime.

Data availability statement

data not available due to legal restriction.

Dissemination

The results will be communicated to participants, in peer-review journals, and presented at international conferences in the domain of Food, Nutrition & Health.

Patient and Public Involvement statement

patients and the public were not involved in the design or conduct of this protocol.

DISCUSSION

The controlled randomised HESPER-HEALTH study will use reliable and sensitive clinical biomarkers of human health to provide a comprehensive picture of the effects of a flavanone-rich OJ on different components of vascular function. It will also produce mechanistic insights to support the contribution of citrus flavanones in vascular health. Together, the results of the study will strengthen the level of evidence of the link between flavanone intake and the effects of a

moderate consumption of OJ on vascular endpoints in humans. The results may also improve understanding of the role of gut microbiota in the interindividual variability in the absorption and metabolism of citrus flavanones, and the putative prebiotic-like effect of flavanone-rich foods. The results from HESPER-HEALTH could help health professionals communicate science-driven dietary advice about fruit juice consumption to their patients. They should also be useful for the citrus sector to encourage the selection of orange varieties naturally rich in hesperidin, and to provide health-focused guidance on how to adapt processing methods to produce fruit juice richer in readily available flavanones.

Author contributions: MAV, DM, NM, RE, CG, CBS, PM, CG, LEM, RS, GP, CM contributed to the conceptualisation, design, and implementation of this research protocol; BP led to the development of the statistical analysis plan. All authors read and approved the final manuscript. All authors will have access to the final trial data set. Of note, the classical authorship rules will be applied in the future papers dealing with the results of the trial.

Acknowledgments : We thank the medical team of the PIC/CIC for carrying the clinical investigations and Lise Bernard at the Central Pharmacy of the University Hospital Clermont-Ferrand. We thank Dr. Volker Herdegen, Dietmar Gürster, Caroline Grimm, and Tobias Schardt from Eckes Granini (Niederolm, Germany) for providing a pilot production line and helping to produce and package the drinks to be tested. We thank Peter Bach, Tim Dreifke, Paul Luka Dreis, and Anna-Maria Schmelzer (all Geisenheim University) for their technical assistance during beverage development and production. We thank Sucocítrico Cutrale (Araraquara, São Paulo, Brazil) for donating the OJ concentrate. Döhler (Darmstadt, Germany) is gratefully acknowledged for donating the colorants, clouding agent, and flavouring used in control drink development and production. HealthTech Bio Actives (Beniel, Spain) is acknowledged for providing the hesperidin formulation.

Funding: This work was supported by a consortium of orange producers, juice manufacturers and packaging companies based in Europe and Brazil under the umbrella of the European Fruit Juice Association (AIJN). A representative from AIJN will give his opinion before any communication or publication.

Disclaimer: The views presented in this paper are those of the authors. Funding companies have played no role in the study beyond providing financial support.

Competing interest : None declared

508 **Table 1**: **Inclusion and exclusion criteria for participation in HesperHealth study**

Inclusion criteria	*Exclusion criteria*
Man or woman	Treated pre-diabetic or diabetic
40-65 years (inclusive)	Treated for hypertension
Post-menopausal woman	Use of statins or other medications for lowering cholesterol
Overweight (waist circumference ≥80 cm for women and ≥94 cm for men, with BMI ≤30)	Treated with antibiotics, antifungals, probiotics or prebiotics in the 3 months before the enrolment
	Menopausal hormone therapy
Ability to give informed consent to participate in research.	Diagnosed gastrointestinal illness
Willingness to accept randomization and undergo the testing and intervention procedures and deliver stool, blood and urine samples for testing	Any serious medical condition that precludes safe participation in the study
No aversion or intolerance to citrus foods	History of eating disorders
Accept to limit their total intake of flavonoid rich beverages (tea, coffee, cocoa, wine, fruit juice) to 250 ml/day	Digestive disorders with diarrhea during the 3 months preceding the beginning of the study
	Self-declared vegetarian, vegetalian, vegan
	History of substance or alcohol abuse
	Involvement in a weight loss program within the 3 past months or who had a bariatric surgery
	Current smokers (within the last 30 days)
	Use of dietary supplements currently or in the past one month
	Declarative strenuous exercise greater than 6 hours per week

509

FIGURE LEGENDS

Figure 1: General scheme of HESPER-HEALTH study progress.

V: visits in PIC/CIC; Drink A: OJ naturally rich in hesperidin; Drink B: control beverage with sugar concentrations identical to (A); Drink C: control beverage identical to (B) but supplemented with hesperidin at the level of (A); FR: 3 days food record.

Figure 2. HPLC-DAD chromatogram of an OJ from a commercially available OJ concentrate naturally rich in hesperidin (A), a control beverage with an identical total sugar concentration (B), and a control beverage additionally supplemented with hesperidin (C). Flavonoids were extracted according to IFU (2005) [29] and analysed by HPLC-DAD using a C18 column (250 × 4.6 mm, particle size 5.0 µm, Kinetex®, Phenomenex, Aschaffenburg, Germany) and an acetonitrile-based elution gradient.

Figure 3. Time table of the conduct and measurements of the HESPER-HEALTH study.

The crosses indicate the visits and types of samples taken (X) and the recommendations and measurements made for each of them (x).

ALAT, alanine amino transferase; ASAT, aspartate amino transferase; BP, blood pressure; DBP, diastolic blood pressure; FLD, flowmetry by laser doppler; FMD, flow mediated dilatation; Gamma GT, gamma glutamyl tranferase; HDL Chol, high density lipoprotein cholesterol; hsCRP, high sensitivity C reactive protein; ICAM, intercellular adhesion molecule; IL6, interleukin-6; PIC/CIC, plateforme d'investigation clinique/centre d'investigation clinique; PWV, pulse wave velocity; SBP, systolic blood pressure; TAG, tri acyl glycerol; TNFα, tumor necrosis factor alpha; TSH, thyroid stimulating hormone; VCAM, vascular cell adhesion molecule.

References

1. Chazelas E, Srour B, Desmetz E *et al.* (2019) Sugary drink consumption and risk of cancer: results from NutriNet-Sante prospective cohort. *BMJ* 366, l2408.
2. Collin LJ, Judd S, Safford M *et al.* (2019) Association of Sugary Beverage Consumption With Mortality Risk in US Adults: A Secondary Analysis of Data From the REGARDS Study. *JAMA network open* 2, e193121.
3. Busing F, Hagele FA, Nas A *et al.* (2019) High intake of orange juice and cola differently affects metabolic risk in healthy subjects. *Clin Nutr* 38, 812-819.
4. Morand C, Dubray C, Milenkovic D *et al.* (2011) Hesperidin contributes to the vascular protective effects of orange juice: a randomized crossover study in healthy volunteers. *The American journal of clinical nutrition* 93, 73-80.
5. Cassidy A, Bertoia M, Chiuve S *et al.* (2016) Habitual intake of anthocyanins and flavanones and risk of cardiovascular disease in men. *The American journal of clinical nutrition* 104, 587-594.
6. Mizrahi A, Knekt P, Montonen J *et al.* (2009) Plant foods and the risk of cerebrovascular diseases: a potential protection of fruit consumption. *The British journal of nutrition* 102, 1075-1083.
7. Chanet A, Milenkovic D, Claude S *et al.* (2013) Flavanone metabolites decrease monocyte adhesion to TNF-alpha-activated endothelial cells by modulating expression of atherosclerosis-related genes. *The British journal of nutrition* 110, 587-598.
8. Morand C, Barber-Chamoux N, Monfoulet L *et al.* (2019) Dietary (Poly)Phenols and Vascular Health. In *Recent Advances in Polyphenol Research*, vol. 6 [H Halbwirth, K Stich, V Cheynier and S Quideau, editors].
9. Habauzit V, Verny MA, Milenkovic D *et al.* (2015) Flavanones protect from arterial stiffness in postmenopausal women consuming grapefruit juice for 6 mo: a randomized, controlled, crossover trial. *The American journal of clinical nutrition* 102, 66-74.
10. Li L, Lyall GK, Martinez-Blazquez JA *et al.* (2020) Blood Orange Juice Consumption Increases Flow-Mediated Dilation in Adults with Overweight and Obesity: A Randomized Controlled Trial. *The Journal of nutrition* 00, 1–8.
11. Valls RM, Pedret A, Calderon-Perez L *et al.* (2020) Effects of hesperidin in orange juice on blood and pulse pressures in mildly hypertensive individuals: a randomized controlled trial (Citrus study). *European journal of nutrition*.
12. Cani PD, Everard A (2016) Talking microbes: When gut bacteria interact with diet and host organs. *Molecular nutrition & food research* 60, 58-66.
13. Tomas-Barberan FA, Selma MV, Espin JC (2016) Interactions of gut microbiota with dietary polyphenols and consequences to human health. *Current opinion in clinical nutrition and metabolic care* 19, 471-476.
14. Vallejo F, Larrosa M, Escudero E *et al.* (2010) Concentration and solubility of flavanones in orange beverages affect their bioavailability in humans. *Journal of agricultural and food chemistry* 58, 6516-6524.
15. Aschoff JK, Riedl KM, Cooperstone JL *et al.* (2016) Urinary excretion of Citrus flavanones and their major catabolites after consumption of fresh oranges and pasteurized orange juice: A randomized cross-over study. *Molecular nutrition & food research* 60, 2602-2610.
16. Tomas-Navarro M, Vallejo F, Sentandreu E *et al.* (2014) Volunteer stratification is more relevant than technological treatment in orange juice flavanone bioavailability. *Journal of agricultural and food chemistry* 62, 24-27.
17. Fidelix M, Milenkovic D, Sivieri K *et al.* (2020) Microbiota modulation and effects on metabolic biomarkers by orange juice: a controlled clinical trial. *Food & Function* 11, 1599-1610.
18. Kay CD, Clifford MN, Mena P *et al.* (2020) Recommendations for standardizing nomenclature for dietary (poly)phenol catabolites. *American Journal of Clinical Nutrition* 112, 1051-1068.

19. Krga I, Milenkovic D, Morand C *et al.* (2016) An update on the role of nutrigenomic modulations in mediating the cardiovascular protective effect of fruit polyphenols. *Food & Function* 7, 3656-3676.
20. Milenkovic D, Deval C, Dubray C *et al.* (2011) Hesperidin Displays Relevant Role in the Nutrigenomic Effect of Orange Juice on Blood Leukocytes in Human Volunteers: A Randomized Controlled Cross-Over Study. *Plos One* 6.
21. Milenkovic D, Deval C, Gouranton E *et al.* (2012) Modulation of miRNA Expression by Dietary Polyphenols in apoE Deficient Mice: A New Mechanism of the Action of Polyphenols. *Plos One* 7, 156-167.
22. Milenkovic D, Jude B, Morand C (2013) miRNA as molecular target of polyphenols underlying their biological effects. *Free Radical Biology and Medicine* 64, 40-51.
23. Yahfoufi N, Alsadi N, Jambi M *et al.* (2018) The Immunomodulatory and Anti-Inflammatory Role of Polyphenols. *Nutrients* 10.
24. Borkowski K, Yim SJ, Holt RR *et al.* (2019) Walnuts change lipoprotein composition suppressing TNF alpha-stimulated cytokine production by diabetic adipocyte. *Journal of Nutritional Biochemistry* 68, 51-58.
25. Gabbs M, Leng S, Devassy JG *et al.* (2015) Advances in Our Understanding of Oxylipins Derived from Dietary PUFAs. *Advances in Nutrition* 6, 513-540.
26. Burton-Freeman B (2010) Postprandial metabolic events and fruit-derived phenolics: a review of the science. *The British journal of nutrition* **104 Suppl 3**, S1-14.
27. IFU (2005) Determination of Sodium, Potassium, Calcium and Magnesium In *Methods of analysis*, International Federation of Fruit Juice Producers (Ed.) ed., vol. 58 [IFoFJP (Ed.), editor]. Zug, Switzerland.
28. Aschoff JK, Kaufmann S, Kalkan O *et al.* (2015) In vitro bioaccessibility of carotenoids, flavonoids, and vitamin C from differently processed oranges and orange juices [Citrus sinensis (L.) Osbeck]. *Journal of agricultural and food chemistry* 63, 578-587.
29. IFU (2005) Determination of Hesperidin and Naringin HPLC In *Methods of analysis*, International Federation of Fruit Juice Producers (Ed.) ed., vol. 58 [IFoFJP (Ed.), editor]. Zug, Switzerland.
30. Widlansky ME, Hamburg NM, Anter E *et al.*(2007) Acute EGCG supplementation reverses endothelial dysfunction in patients with coronary artery disease. Journal of American College of Nutrition 2, 95-102.
31. Heiss C, Kleinbongard P, Dejam A *et al.* (2005) Acute consumption of flavanol-rich cocoa and the reversal of endothelial dysfunction in smokers. *Journal of American College of Cardiology* 46, 1276-83.
32. Corretti MC, Anderson TJ, Benjamin EJ *et al.* (2002) Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: a report of the International Brachial Artery Reactivity Task Force. *Journal of the American College of Cardiology* **39**, 257-265.
34. Tessier F, BirlouezAragon I, Tjani C *et al.* (1996) Validation of a micromethod for determining oxidized and reduced vitamin C in plasma by HPLC-fluorescence. *International Journal for Vitamin and Nutrition Research* **66**, 166-170.
35. Mainka M, Dalle C, Petera M *et al.* (2020) Harmonized procedures lead to comparable quantification of total oxylipins across laboratories. *Journal of Lipid Research* **61**, 1424-1436.

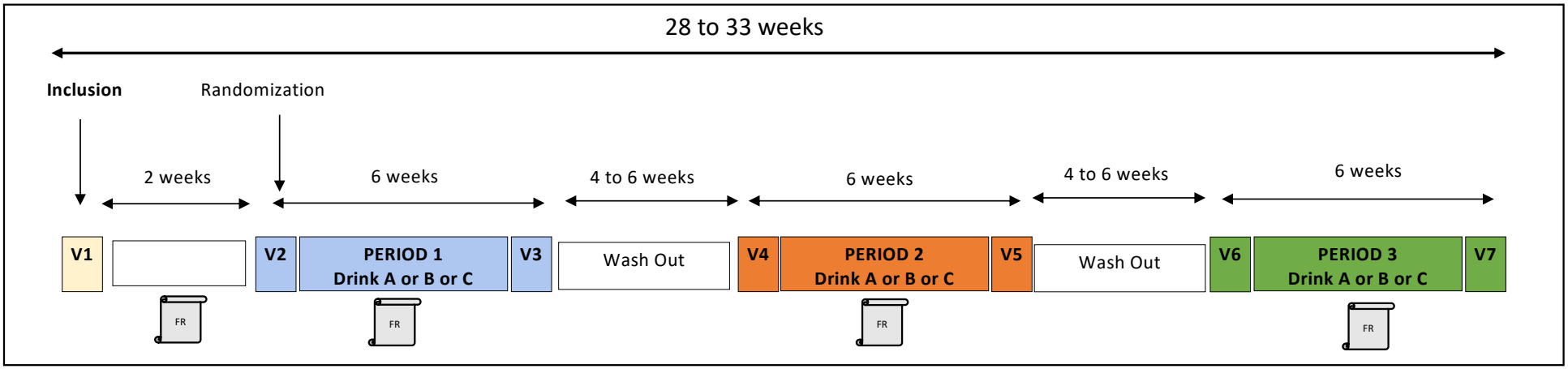
28 to 33 weeks
Inclusion
Randomization
2 weeks
6 weeks
4 to 6 weeks
6 weeks
4 to 6 weeks
6 weeks
V1
V2
PERIOD 1
Drink A or B or C
V3
Wash Out
V4
PERIOD 2
Drink A or B or C
V5
Wash Out
V6
PERIOD 3
Drink A or B or C
V7
FR
FR
FR
FR

Figure 1

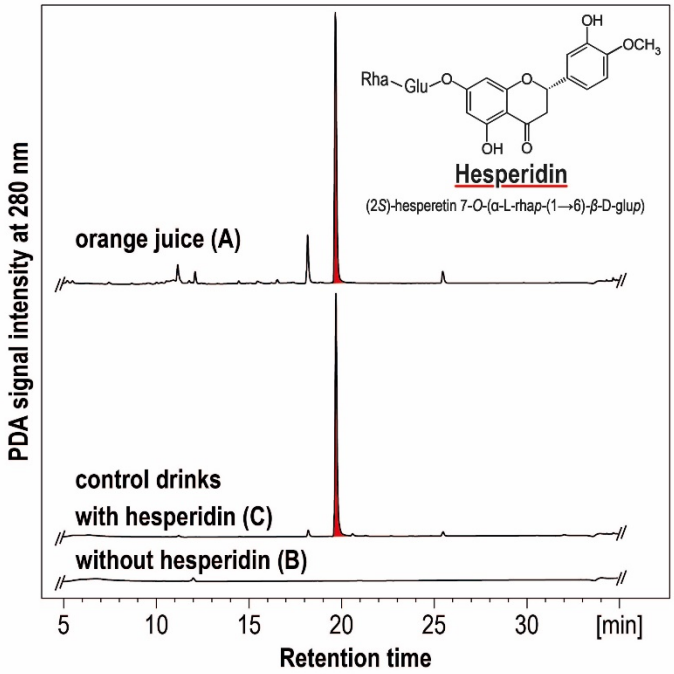

Figure 2.

		Eligibility assessment	*Pre-experimental period*					*Experimental period*					
		Visit 1	At home					**Visit 2, 4, 6**	At Home				**Visit 3, 5, 7**
Procedures		Inclusion	Day -5	Day -4	Day -3	Day -2	Day -1	Day 1	Day 2 to 38	Day 39	Day 40	Day 41	Day 42
PIC/CIC visit		**X**						**X**					**X**
Consent form signature, medical examination, dietician interwiew		x											
Anthropometry		x						x					x
BP		x											
Impedancemetry/Body composition								x					x
Food Record			x	x	x				x x x				
Diet low in polyphenols						x	x				x	x	
Standardized meal at diner							x					x	
24h urine collection	Flavanones urinary excretion						**X**					**X**	
Feces collection	Gut Microbiota analysis						**X**					**X**	
Randomisatiom								x (visit 2)					
Blood sampling		**X**						**X**					**X**
	Blood cell count, platelets	x											
	ASAT, ALAT, Gamma GT	x											
	Creatinin	x											
	TSH	x											
	Glucose, Insulin	x						x					x
	TAG, Total and HDL Chol	x						x					x
	Uric acid							x					x
	hsCRP	x						x					x
	I-CAM, V-CAM, e-selectin,IL6, TNFα							x					x
	Nitrites							x					x
	Vit C, Carotenoids							x					x
	Extracellular vesicles												x
	Flavanones							x					x
	Total FA							x					x
	Oxylipins profiling							x					x
	Nutrigenomic analysis												x
Vascular function assessment	Endothelial Function (FMD)							x					x
	Microvascular reactivity (FLD)							x					x
	Arterial Stiffness (PWV)							x					x
	BP self measurement (SBP+DBP)				x	x	x			x	x	x	

FIGURE 3 : Time table of the conduct and measurements of the HESPER-HEALTH study

CONSENT FORM TO PARTICIPATE IN RESEARCH INVOLVING HUMAN VOLUNTEERS

HESPER-HEALTH

Orange Juice and Hesperidin - Their Vascular Health Benefits: A Randomized Controlled Crossover Human Study

Principal investigator

Professor Gisèle Pickering Plateforme d'investigation Clinique / Centre d'Investigation Clinique Inserm 1405, Centre Hospitalier Universitaire de Clermont-Ferrand, 58 Rue Montalembert, 63000 Clermont-Ferrand

I undersigned

Mrs, Miss, Sir (cross out unnecessary terms) (name, first name) ……

Born __ __ / __ __ / __ __ __ __

Address ……

Declare that the Doctor (name, first name, telephone) …… .. …… ……
offered to participate in the aforementioned study; he explained me the protocol and detailed in particular:

- the objective, the method, and the duration of the study

- the constraints and potential risks incurred

- my right to refuse to participate and to withdraw my consent at any time without having to justify myself

- my obligation to register to French social security

- that, if I wish I would be informed by the investigating doctor at the end of the protocol of the overall results

- that an exclusion period of 7 days is defined in this protocol

- that the South-East III Committee for the Protection of Persons (CPP) issued a favorable opinion on February 2, 2021.

- that the promotor, the Clermont-Ferrand University Hospital, took out insurance covering this research.

- that I am not placed under judicial protection,

- that I must have sufficient time before signing this consent,

- that it was clear to me that I could oppose the conservation of my samples and their re-use for medical or scientific research programs aimed to improve scientific knowledge:

☐ I agree with the fact that the biological samples taken from me being stored and used for research purposes.

☐ I am opposed with the fact that the biological samples taken from me being kept and used for research purposes.

Study information collected by the investigator is treated confidentially. I accept that this data may be subject to anonymous computer processing. I have noted that the right of access provided for by the law of August 6, 2004 relating to data processing, files and freedoms is exercised at any time with the doctor who follows me in the context of research and who know my identity. I can exercise my right of rectification and opposition with this same doctor, who will contact the research sponsor. After having freely discussed and obtained answers to all my questions, I freely and voluntarily agree to participate in this research involving the human person under the conditions specified in the information and consent form.

Name and first name of the subject: …… …….

Date: _ _ / _ _ / _ _ _ _

Signature preceded by the words "Read and understood":

Name of the investigator: …….

Date: _ _ / _ _ / _ _ _ _

Signature:

This document must be produced in 2 copies, the original of which must be kept by the investigator, the first copy must be given to the person giving his consent.

SPIRIT 2013 CHECKLIST: HESPER-HEALTH STUDY

Administrative information			Line
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	L 1-2
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	L 426-429
	2b	All items from the World Health Organization Trial Registration Data Set	L 70-71; L 426-431
Protocol version	3	Date and version identifier	
Funding	4	Sources and types of financial, material, and other support	L 489-490; L492-496; L498-500
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	L 4-14; L480-482
	5b	Name and contact information for the trial sponsor	L 173
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	L170-173; L500-501
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	L 441-449
Introduction			
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	L 90-145
	6b	Explanation for choice of comparators	L 228-248
Objectives	7	Specific objectives or hypotheses	L 147-165

Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	L 169-179
Methods: Participants, interventions, and outcomes			
Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	L 170-173
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	Table 1
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	L181-218; Figure 1
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	L 222-225
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	L 259-261
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	L 300-394

Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	Table 2; L 181-196
Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	L 287-294
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	L 278-280
Methods: Assignment of interventions (for controlled trials)			
Allocation:			L 264-275
Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	L 264-272
Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	L268-275
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	L264-270
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	L247-248; L250-251; L298
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	L272-275
Methods: Data collection, management, and analysis			

Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	L300-394
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	L259-261
Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	L441-453
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	L397-418
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	L414-418
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	Not Applicable
Methods: Monitoring			
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	L 450-453

	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	L 452
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	L198-200
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	L452-453
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	L424-431
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	L429-430
Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	L434-438
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	Not applicable
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	L441-444
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	L506
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	L483
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	Not applicable

Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	L459-460
	31b	Authorship eligibility guidelines and any intended use of professional writers	L483-484
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	L456; L463
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	supplemental data 1
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	Not applicable