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# BMJ Open

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

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3 1 **Study protocol for a randomized controlled trial evaluating the role of Orange juice,**  
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5 2 **HESPERidin in vascular HEALTH benefits: The HESPER-HEALTH Study**  
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25 40

26  
27 41 **Key words :**

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29 42 Randomized controlled trial ; vascular function ; orange juice ; hesperidin ; flavanones

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31 43 bioavailability and metabolism

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## 48 **ABSTRACT**

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

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

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

## 72 **ARTICLE SUMMARY**

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### 74 **Strengths and Limitations of this study**

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

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90

## 91 **INTRODUCTION**

92

93 Recent epidemiological studies have associated the frequent consumption of sugar-sweetened  
94 beverages with some adverse health effects, such as early death, weight gain and cancer <sup>(1; 2)</sup>  
95 Findings of such studies are often extrapolated to fruit juice consumption, including 100% fruit

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3 96 juices. However, these observational, epidemiological results contrast with experimental studies  
4  
5 97 which have demonstrated clear differences in the metabolic response of the human body to fruit  
6  
7 98 juices as compared with that seen for sugar-sweetened beverages<sup>(3; 4)</sup>.

9  
10 99 One characteristic of citrus foods is that they are a rich and exclusive source of dietary  
11  
12 100 flavanones, a category of (poly)phenol compounds, mainly present as hesperidin in orange.  
13  
14 101 Flavanone intake has been repeatedly associated with a lower incidence of mortality from CVD <sup>(5;</sup>  
15  
16 102 <sup>6)</sup>. Results from preclinical studies using different models of atherosclerosis also provide evidence  
17  
18 103 for a role of citrus flavanones in cardiovascular protection, with a slowdown in atherosclerosis  
19  
20 104 development <sup>(7)</sup>. These atheroprotective effects have been related to the capacity of flavanones to  
21  
22 105 modulate the expression of genes involved in cellular processes responsible for vascular  
23  
24 106 dysfunction<sup>(8)</sup>. Evidence of the vascular protective effects of citrus flavanones have been reported  
25  
26 107 in few randomised controlled clinical trials <sup>(4; 9; 10; 11)</sup>. However, published trial with orange  
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28 108 flavanones do not yet allow firm conclusions to be drawn about their efficacy to modulate vascular  
29  
30 109 function, mainly due to the high degree of discrepancies between the study designs.

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33 110 The ability of citrus flavanones to exert beneficial effects depends on their bioavailability. The  
34  
35 111 gut microbiota plays a key role in flavanone absorption, because naturally-occurring flavanones are  
36  
37 112 present as molecules with glycosyl moieties, e.g. a rutinosyl, i.e. a  $\alpha$ -L-rhamnopyranosyl-(1→6)-  
38  
39 113  $\beta$ -D-glucopyranoosyl moiety in hesperidin. The sugar moiety of hesperidin must be hydrolysed by  
40  
41 114 bacterial glycosidases to yield a format which the human body can absorb i.e. the aglycone,  
42  
43 115 hesperetin. Upon release, hesperetin can be absorbed by intestinal cells or may be further  
44  
45 116 catabolized into diverse phenolic compounds by microbial action in the colon. After absorption of  
46  
47 117 hesperetin and its microbial catabolites, they enter the blood circulation and are subject to further  
48  
49 118 human metabolism. For instance, hepatic metabolism includes several conjugation reactions, e.g.,  
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51 119 sulphatation or glucuronidation<sup>(12; 13)</sup>. Hence, the extent of hesperetin released in the colon, the  
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3 120 formation of colonic catabolites and their subsequent absorption could largely depend on the gut  
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5 121 microbial composition, which has been shown to vary between subjects <sup>(14)</sup>. In agreement with this  
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7 122 hypothesis, previous studies have reported a large interindividual variability in the urinary  
8  
9 123 excretion level of flavanones <sup>(15; 16)</sup> that could reflect differences in gut microbiota composition,  
10  
11 124 but this remains to be demonstrated. A recent clinical study found that regular consumption of OJ  
12  
13 125 can modulate the gut microbiota profile and these changes were associated with positive shifts in  
14  
15 126 some metabolic outcomes<sup>(17)</sup>.  
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21 128 Dietary (poly)phenols and their circulating metabolites encompass a huge diversity of  
22  
23 129 compounds <sup>(18)</sup> whose health effects seem increasingly linked to their capacity to exert complex  
24  
25 130 genomic modifications, such as changes to the expression of genes <sup>(19)</sup>. In preclinical studies,  
26  
27 131 flavanones have been shown to induce changes in the expression of a number of genes in aorta and  
28  
29 132 endothelial cells that relate to inflammation, and endothelial cell function <sup>(7; 20; 21; 22)</sup>, revealing  
30  
31 133 potential molecular mechanisms of action to explain their health properties. However, such  
32  
33 134 mechanisms of action are still largely unexplored in humans <sup>(20)</sup>. Based on a huge body of  
34  
35 135 experimental results demonstrating the anti-inflammatory effects of polyphenols and related  
36  
37 136 mechanisms <sup>(23)</sup>, these compounds are suggested as key players in the protective effects of their  
38  
39 137 food sources for chronic inflammatory diseases. However, in humans, consumption of polyphenol-  
40  
41 138 rich foods induces subtle changes in inflammatory and oxidative status that can only typically be  
42  
43 139 measured using sensitive techniques, such as the lipidomic profiling of oxylipins <sup>(24)</sup>. Oxylipins are  
44  
45 140 a superclass of lipid mediators comprising hundreds of metabolites which regulate a diversity of  
46  
47 141 biological processes including inflammation, cell adhesion, migration and proliferation, blood  
48  
49 142 clotting and vessel permeability <sup>(25)</sup>.  
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3 144 Based on this state of the art, the present human randomised, controlled, double-blind, cross  
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5 145 over intervention conducted on subjects predisposed to CVD aims to establish a cause-and-effect  
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7 146 relationship between hesperidin intake and the vascular protective effects of drinking OJ naturally  
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10 147 rich in hesperidin. The study will also provide insights into the mechanisms responsible for the  
11  
12 148 observed effects.  
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## 16 17 150 **STUDY OBJECTIVES**

### 18 19 151 **Primary Objective**

20  
21 152 The primary objective of this trial is to assess the effect of a subchronic consumption (6 weeks) of  
22  
23 153 a naturally flavanone-rich OJ or a control drinks supplemented with orange flavanones on  
24  
25 154 endothelial function, by assessing flow-mediated dilation (FMD) in subjects with a predisposition  
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27 155 to CVD, based on age, body mass index and waist circumference.  
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### 32 33 157 **Secondary Objectives**

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35 158 The secondary objectives of the trial are to:

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37 159 ➤ assess the effects of the intervention: (1) on other markers of vascular function using a range  
38  
39 160 of well-established measurements in the macrocirculation (blood pressure, arterial  
40  
41 161 stiffness) and in the microvasculature at skin level (microvascular reactivity by Flow Laser  
42  
43 162 Doppler, FLD); (2) on the postprandial endothelial response in response to a challenge  
44  
45 163 meal; (3) on biomarkers of cardiovascular risk, endothelial dysfunction and inflammation;  
46  
47 164 (4) on anthropometric parameters and body composition; (5) on the vitamin C and  
48  
49 165 carotenoids status; (6) on the gut microbiota composition (microbial communities profiling)  
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51 166 ➤ measure the bioavailability of hesperidin and its metabolites in biofluids (plasma, urine)  
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3 167 ➤ ascertain the underpinning molecular mechanisms of the vascular responsiveness  
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5 168 (nutrigenomic analysis, oxylipins profiling).  
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## 12 13 171 **METHOD AND ANALYSIS**

### 14 15 16 172 **Study Design**

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19 173 HESPER-HEALTH is a human dietary intervention study designed as a randomized, double-blind,  
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21 174 controlled, cross-over trial with three arms that will be conducted at the Clinical Investigation  
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23 175 Center, Inserm 1405 (PIC/CIC) of the University Hospital of Clermont-Ferrand, France. This trial  
24  
25 176 will be carried out on subjects predisposed to CVD, based on age (40-65 years old) and overweight  
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27 177 (waist circumference  $\geq 80$  cm for woman,  $\geq 94$  cm for man). Forty-two participants will be  
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29 178 recruited and will receive the three treatments in a random order: (A) a commercially-available OJ  
30  
31 179 naturally rich in hesperidin (ca. 600-650 mg/L), (B) a control beverage with a total sugar  
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33 180 concentration identical to (A), and (C) a control beverage identical to (B) but supplemented with  
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35 181 hesperidin at the level present in the natural OJ (A).  
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42 183 For each volunteer, the study is divided in three identical experimental periods of 45 days. These  
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44 184 periods include a three day run in period during which time specific dietary recommendations will  
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46 185 be followed, samplings and measurements will be performed at home. This run in period will be  
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48 186 followed by a 6-weeks treatment period of consumption of one of the three beverages. A four to  
49  
50 187 six week wash-out is planned between each of the three experimental periods. The protocol  
51  
52 188 includes seven visits to PIC/CIC, including one visit (V1) at inclusion, and will last in total for 28  
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54 189 to 33 weeks (**Figure 1**). Over the last 24h prior to each visit, volunteers will be asked to collect  
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3 190 stool samples and 24h urine samples to assess gut microbiota composition and flavanone  
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5 191 bioavailability and metabolism, respectively.  
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10 193 At the beginning and end of each experimental period, overnight-fasted volunteers will be invited  
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12 194 to attend the PIC/CIC (visits V2 to V7) for vascular function tests, blood sampling, anthropometric  
13  
14 195 measurements and body composition analysis. Blood will be sampled for further assessment of  
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16 196 plasma flavanones including metabolites, carotenoids and vitamin C, oxylipin profiling, systemic  
17  
18 197 biomarkers of endothelial activation and inflammation, metabolic parameters, and for the analysis  
19  
20 198 of the nutrigenomic response.  
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26 200 At each visit (V2 to V7), after measurements and blood collection in the fasting state, a challenge  
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28 201 meal together with the respective study drink will be administered to all subjects before further  
29  
30 202 exploration. This will enable evaluation of the acute postprandial effects (at T+3h, T+6h) of the  
31  
32 203 study products on endothelial function (V2,V4,V6), to also be repeated after the 6 weeks  
33  
34 204 intervention period (V3, V5, V7). The challenge meal will consist of fresh cream, sucrose and milk  
35  
36 205 proteins, providing 900 kcal – a pro-oxidant and pro-inflammatory meal that is known to induce a  
37  
38 206 transient endothelial dysfunction during the post prandial period <sup>(26)</sup>.  
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44 208 During each experimental period, volunteers will be asked not to eat citrus foods and to consume  
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46 209 no more than 250 mL/d of polyphenol-rich beverages (coffee, tea, fruit juices, wine, cocoa). To  
47  
48 210 check that volunteers do not change their eating habits during the study, for each experimental  
49  
50 211 period they will be asked to complete food questionnaires over three defined days. The first  
51  
52 212 questionnaire will be completed prior to visit V2 to PIC/CIC and two others at mid-term of each  
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54 213 intervention period. On the two days preceding each visit at the PIC/CIC, volunteers will be asked  
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214 not to consume polyphenol-rich foods and beverages and they will have to consume exactly the  
215 same dinner without polyphenols at 8 pm the day before each visit at the PIC/CIC.

216

### 217 **Inclusion and Exclusion criteria**

218 These criteria are listed in the Table 1.

219

### 220 **Study products**

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

229

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

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3 238 between bottles of different drinks. Despite careful colour and flavor matching, both the hesperidin-  
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5 239 free (B, placebo) and the hesperidin-rich (C) control drink may be differentiated sensorially from  
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7 240 the OJ by attentive subjects. In contrast, subjects and clinic staff will be unable to identify which  
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9 241 of the artificial control drinks (B) and (C) is the one rich in or free from hesperidin.  
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12 242  
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14 243 Finally, bottles will be labelled and packaged for each cross-over period in a blinded fashion on  
15  
16 244 the basis of the randomisation schedule. They will be stored at +4°C until distribution to the  
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18 245 participants, who will be asked to store the beverages in a dark place at room temperature or cooler.  
19  
20 246 Hesperidin levels in drinks (A) and (C) as well as levels of ascorbic and dehydroascorbic acid  
21  
22 247 (vitamin C) in the OJ (A) will be monitored over the entire study period. Colony forming units of  
23  
24 248 yeasts, total viable counts, lactic acid bacteria and moulds will also be assessed to ensure the  
25  
26 249 microbiological safety of the products.  
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### 33 251 **Assignment of intervention**

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35 252 **Randomisation and Allocation** : Subjects will be randomly assigned to treatment groups  
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37 253 according to a pre-established list of randomisation designed by a PIC/CIC clinical research  
38  
39 254 supervisor, independently from the investigators and the sponsor. The treatment number will be  
40  
41 255 allocated by order of entrance in inclusion. Subjects will be randomised to receive drink A, B, or  
42  
43 256 C using a Latin-square random design. Subject allocation to treatments will take place after  
44  
45 257 inclusion and will be based on a computer-generated randomisation list. Access to the  
46  
47 258 randomisation list will be restricted to staff performing this task. The randomisation number will  
48  
49 259 be allocated by order of entrance in inclusion. Under normal circumstances, the blinding should  
50  
51 260 not be broken until all subjects have completed the trial and the database will be locked. However,  
52  
53 261 the blinding can be broken should a specific emergency treatment at the site require knowledge of  
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3 262 the treatment status of the subject. In such a case, the investigator can reveal the treatment  
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5 263 assignment through the randomisation plan and then inform the sponsor as soon as possible  
6  
7  
8 264 (Procedure ORAGA MO-028).  
9

10 265  
11  
12 266 **Participant eligibility**  
13  
14 267 Forty-two volunteers will be recruited from the existing PIC/CIC volunteer's database  
15  
16 268 supplemented by announcement in local press. Each subject who meets the recruitment criteria  
17  
18  
19 269 (**Table 1**) will be pre-selected by the investigator and will be given a detailed explanation of the  
20  
21 270 protocol. If the subject accepts, he or she will be asked to come to PIC/CIC for an initial study visit  
22  
23  
24 271 (V1, **Figure 1**) to give written consent to participate, and to have a medical examination, an  
25  
26 272 interview with a dietitian and a blood checkup to ensure that all clinical criteria are met.  
27

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30 274 **Sample size**  
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33 275 Sample size calculation has been performed based on the FMD response (main criteria) observed  
34  
35 276 in previous dietary interventions. The targeted statistical power was based on interindividual  
36  
37 277 variability for FMD measurement of the operator (SD = 1.9%). Aiming for a statistical power  
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39  
40 278 greater than 80% and a two-tailed type I error at 0.017 (to take into account the three comparisons  
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42 279 to be considered), the total number of subjects required to provide sufficient power to detect a  
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44  
45 280 minimal absolute difference of FMD equals 1.6% in a 3-sequences cross-over study (with an intra-  
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47 281 class correlation coefficient at 0.5) is 36. Assuming unforeseen drop-outs and follow-up losses, 42  
48  
49 282 participants will be recruited.  
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## 284 **OUTCOME MEASURES**

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

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### 287 **Primary outcome**

288 The primary outcome is endothelial function assessed at the level of of the brachial artery using

289 the non-invasive technique FMD which constitutes the gold standard to evaluate vascular

290 endothelial function in humans. The FMD technique measures the diameter of the brachial artery

291 by non-invasive ultrasound before and after increasing shear stress by inducing a reactive

292 hyperemia, with the degree of dilation reflecting mostly the arterial endothelial nitric oxide release.

293 The procedure for the FMD measurement is full compliant with the reference method described by

294 Coretti et al. <sup>(27)</sup>. FMD will be assessed at V2, V3, V4, V5, V6 and V7 under both fasting and

295 postprandial conditions.

296

### 297 **Secondary Outcomes**

#### 298 **➤ *Other vascular function measurements***

299 Endothelial function in the microcirculation will be assessed using Flowmetry by Laser Doppler

300 (FLD), which is a non-invasive and validated technique for continuous measurement of the

301 endothelial dependent microvascular reactivity. FLD will be assessed during V2, V3, V4, V5, V6

302 and V7 in fasting and postprandial conditions.

303 Arterial stiffness will be evaluated by the carotid-femoral Pulse Wave Velocity (PWV) which is

304 calculated from measurements of pulse transit time and the distance travelled between the carotid

305 and femoral arteries using a validated non-invasive device. PWV will be assessed after fasting at

306 V2, V3, V4, V5, V6 and V7.



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3 307 Blood pressure will be monitored by subjects at home using tensiometers which will be loaned to  
4  
5 308 them for the study duration. They will be asked to perform three repeated measurements in fasting  
6  
7 309 condition during the three consecutive days preceding their visits to the PIC/CPC (V2, V3, V4, V5,  
8  
9 310 V6 and V7), and to record obtained values on a dedicated data sheet.  
11

12 311  
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14 312 ➤ ***Flavanone metabolism and bioavailability in biofluids***  
15  
16 313 Phase 2 metabolites and microbial-derived catabolites of flavanones will be analyzed by LC-MS  
17  
18 314 in 24h urine and in plasma samples according to the method reported by Aschoff et al. <sup>(15)</sup>. Prior to  
19  
20 315 analysis, the above-mentioned analytes will be extracted from plasma samples using solid phase  
21  
22 316 extraction cartridges. These analyses will be done for the biological fluids collected (urine) or  
23  
24 317 sampled (plasma) at V2, V3, V4, V5, V6 and V7.  
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30 319 ➤ ***Systemic biomarkers related to CVD risk, endothelial dysfunction and inflammation***  
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33 320 These include metabolic parameters (plasma glucose, insulin, TAG, Total Cholesterol, HDL-chol,  
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35 321 uric acid that will be measured by spectrometric and enzymatic methods; total fatty acids, by gas  
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37 322 chromatography), soluble adhesion molecules and inflammatory markers (ICAM, VCAM and e-  
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39 323 selectin, IL-6, TNFalpha, hs-CRP, by using Elisa assays), plasma nitrites (by chemi-luminescence)  
40  
41 324 and the release of endothelial extracellular vesicles (by flux cytometry). These analyses will be  
42  
43 325 performed in blood sampled at V2, V3, V4, V5, V6 and V7.  
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49 327 ➤ ***Carotenoids and vitamin C status***  
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51 328 Carotenoids will be quantitated from human plasma after a protein-crash with ethanol and repeated  
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53 329 extraction with hexane using the method described by Aschoff et al. <sup>(28)</sup>. Vitamin C status will be  
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3 330 quantified in deproteinized plasma by HPLC-fluorescence detection as previously described <sup>(29)</sup>.  
4

5 331 These measures will be performed in blood sampled at V2, V3, V4, V5, V6 and V7.  
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8 332  
9 333 **➤ *Anthropometry and body composition assessment***

10  
11 334 Body Mass Index (BMI), waist to height ratio (waist circumference/height) and the percentages  
12  
13 335 of fat mass, lean mass and water (obtained using a multi-frequency Bioelectrical Impedance  
14  
15 336 Analyser) will be determined. These parameters will be recorded at V2, V3, V4, V5, V6 and V7.  
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19 337  
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21 338 **➤ *Gut microbiota composition assessment***

22  
23 339 The composition of the gut microbiota will be determined by 16S metabarcoding. This method  
24  
25 340 involves the amplification by PCR of variable regions of the 16S rDNA gene from fecal DNA  
26  
27 341 followed by the preparation of DNA libraries and high throughput next-generation sequencing  
28  
29 342 (Illumina technology). Analysis will be completed by an absolute quantification by qPCR of target  
30  
31 343 groups of the human gut microbiota considered as beneficial to the host, or involved in polyphenol  
32  
33 344 metabolism. These analyses will be performed in faeces samples collected during the 12h preceding  
34  
35 345 V2, V3, V4, V5, V6 and V7.  
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41 347 **Mechanistic outcomes**

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43 348 In the HESPER-HEALTH study, we aim to explore the molecular mechanisms of action underlying  
44  
45 349 vascular effects of OJ and hesperidin consumption. To this end, we propose to perform global and  
46  
47 350 integrated analyses of expression of genes (nutrigenomic analysis) in the blood and to analyse the  
48  
49 351 oxylipin profiling to identify changes in biological processes involved in inflammation and  
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51 352 vascular dysfunction.  
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3 353 **Nutrigenomic response assessment:** RNA will be extracted from whole blood using  
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5 354 PAXgene Blood RNA System. Global gene expression profile of both protein coding and protein  
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7 355 non-coding RNAs (miRNAs, snoRNAs, lncRNAs) will be assessed using a microarray approach.  
8  
9  
10 356 For genes identified as differentially expressed, integrated multi-omic bioinformatic analyses will  
11  
12 357 be performed to identify gene ontologies, gene network, cellular pathways and interactions between  
13  
14 358 different types of RNAs. Potential transcription factors involved will be searched and capacity of  
15  
16 359 flavanone metabolites to bind to transcription factors as well as cell signaling proteins regulating  
17  
18 360 their activity will be predicted using 3D docking analysis. Analysis will be performed from blood  
19  
20 361 sampled at V3, V5 and V7.  
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23  
24 362 **Oxylipin profiling:** a comprehensive assessment of circulating oxylipins will be performed  
25  
26 363 using a targeted MS-based method (LC-MS/MS) using 22 internal standards and providing  
27  
28 364 both qualitative (i.e. oxylipin signature) and quantitative information <sup>(30)</sup>. Analysis will be  
29  
30 365 performed in plasma sampled at V2, V3, V4, V5, V6 and V7.  
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### 35 367 **Statistical analyses**

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37 368 All analyses will be performed with Stata software (version 15, StataCorp, College Station, USA)  
38  
39 369 before the breaking of the randomization code, according to International Conference on  
40  
41 370 Harmonization-Good Clinical Practice guideline. Continuous variables will be presented,  
42  
43 371 according to their statistical distribution, as mean and standard-deviation, or median and  
44  
45 372 interquartile range. The Shapiro-Wilk test will be used to assess normality. Categorical data will  
46  
47 373 be presented as exact number and percentage.  
48  
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50 374 The primary outcome will be analysed using random-effects model (i.e. mixed linear model for  
51  
52 375 continuous dependent variable) for a 3-treatment crossover study. The full statistical model  
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54 376 includes treatment group, sequence, period, carry-over on FMD baseline value as fixed effects.  
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3 377 Subject nested in sequence will be included in the model as a random effect. A Sidak's type I error  
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5 378 will be applied to take into account multiple comparisons. The *treatment group x period* interaction  
6  
7 379 will be studied. If this interaction is not significant, the main effect of treatment will be assessed.  
8  
9  
10 380 If this interaction is significant, there will be a particular focus on the first period. The normality  
11  
12 381 of residuals from random-effects model will be studied as aforementioned, with the Shapiro-Wilk  
13  
14 382 statistic and visual inspection of residual plots. If appropriate, a transformation (for example  
15  
16 383 logarithmic) of the primary outcome could be proposed to achieve its normality. The results will  
17  
18 384 be expressed with effect-sizes and 95% confidence intervals.  
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21 385 The primary analysis will be completed by multivariable approach using the statistical model  
22  
23 386 described above with, additionally, covariates determined according to univariate results and  
24  
25 387 clinical relevance: BMI, gender, weight at enrolment and age. The other continuous outcomes will  
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27 388 be analyzed with the same statistical analysis plan. For categorical data, generalized mixed linear  
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29 389 modelling will be carried out.  
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## 35 392 **ETHICS AND DISSEMINATION**

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42 394 **Consent:** Subject will be informed in a complete and fair manner, in accessible terms, of the  
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44 395 objectives and constraints of the study, the possible risks incurred, the necessary surveillance and  
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46 396 security measures, their rights to refuse to participate in the study or the possibility to withdraw at  
47  
48 397 any time. The patient's free, informed and written consent will be collected by the investigator.  
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50 398 Document templates have been approved by the ethics committee and are to be used for the test  
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52 399 concerned, to the exclusion of any other documentation. One original copy co-signed by the  
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54 400 investigating doctor and the subject will be given to the volunteer.  
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5 402 **Data management:** Experienced and trained study coordinators will be dedicated to data  
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7 403 acquisition, coding, security and storage under the responsibility of the investigator. The study data  
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9 404 will be computerised in a coded manner, and in accordance with the information technology law  
10  
11 405 and freedom, by the Clermont-Ferrand CIC. The data will be entered into the computer files using  
12  
13 406 a double entry procedure meeting the standards set by good clinical practice. After comparing the  
14  
15 407 double entry, the computer data files and any corrections made to them will be stored and  
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17 408 retrievable on request.  
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24 410 **Data availability statement:** data not available due to legal restriction.  
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28 412 **Dissemination:** The results will be communicated in peer-review journals and presented at  
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30 413 international conferences in the domain of Food, Nutrition & Health.  
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35 415 **Patient and Public Involvement statement:** patients and the public were not involved in the  
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37 416 design or conduct of this protocol  
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## 42 419 **DISCUSSION**

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46 420 The controlled randomised HESPER-HEALTH study will use reliable and sensitive clinical  
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48 421 biomarkers of human health to provide a comprehensive picture of the effects of a flavanone-rich  
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50 422 OJ on different components of vascular function. It will also produce mechanistic insights of  
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52 423 relevance to support the contribution of citrus flavanones in vascular protective effects. Together,  
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54 424 the generated knowledge should strengthen the level of evidence of the link between flavanone  
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3 425 intake and the effects of a moderate consumption of OJ on vascular endpoints in humans. The  
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5 426 results will also improve understanding of the role of gut microbiota in the interindividual  
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7 427 variability in the absorption and metabolism of citrus flavanones, and the putative prebiotic-like  
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9 428 effect of flavanone-rich foods. Ultimately, results from HESPER-HEALTH could help health  
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11 429 professionals communicate science-driven dietary advice about fruit juice consumption to their  
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13 430 patients. They should also be useful for the citrus sector to encourage the selection of orange  
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15 431 varieties naturally rich in hesperidin, and to provide health-focused guidance on how to adapt  
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17 432 processing methods to produce fruit juice richer in flavanones.  
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3 433 **Author contributions:** MAV, DM, NM, RE, CG, CBS, PM, CG, LEM, RS, GP, CM contributed  
4  
5 434 to the conceptualisation, design, and implementation of this research protocol; BP led to the  
6  
7 435 development of the statistical analysis plan.

8  
9  
10 436 All authors read and approved the final manuscript.

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12 437  
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19  
20 441 from Eckes Granini (Niederolm, Germany) for providing a pilot production line and helping to  
21  
22 442 produce and package the drinks to be tested. We thank Peter Bach, Tim Dreifke, Paul Luka Dreis,  
23  
24 443 and Anna-Maria Schmelzer (all Geisenheim University) for their technical assistance during  
25  
26 444 beverage development and production. We thank Sucocítrico Cutrale (Araraquara, São Paulo,  
27  
28 445 Brazil) for donating the OJ concentrate. Döhler (Darmstadt, Germany) is gratefully acknowledged  
29  
30 446 for donating the colorants, clouding agent, and flavouring used in control drink development and  
31  
32 447 production. HealthTech Bio Actives (Beniel, Spain) is acknowledged for providing the hesperidin  
33  
34 448 formulation.

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37  
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39  
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41  
42 452 umbrella of the European Fruit Juice Association (AIJN).

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45  
46 454 **Disclaimer:** The views presented in this paper are those of the authors. Funding companies have  
47  
48 455 played no role in the study beyond providing financial support.

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3 457 **Competing interest** : None declared  
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For peer review only



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 $\leq$ 30; waist circumference $\geq$ 80 cm for women and $\geq$ 94 cm for men)	Treated with antibiotics, antifungals, probiotics or prebiotics in the 3 months before the enrolment
Ability to give informed consent to participate in research.	Menopausal hormone therapy
Willingness to accept randomization and undergo the testing and intervention procedures and deliver stool, blood and urine samples for testing	Diagnosed gastrointestinal illness
No aversion or intolerance to citrus foods	Any serious medical condition that precludes safe participation in the study
Accept to limit their total intake of flavonoid rich beverages (tea, coffee, cocoa, wine, fruit juice) to 250 ml/day	History of eating disorders
	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

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461 **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 P P s					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							x					x	
	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	

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3 466 **FIGURE LEGENDS**  
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7 468 **Figure 1: General scheme of HESPER-HEALTH study progress.**  
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9 469 V: visits in PIC/CIC; Drink A: OJ naturally rich in hesperidin; Drink B: control beverage with  
10 470 sugar concentrations identical to (A); Drink C: control beverage identical to (B) but supplemented  
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12 471 with hesperidin at the level of (A); FQ: 3 days food questionnaire.  
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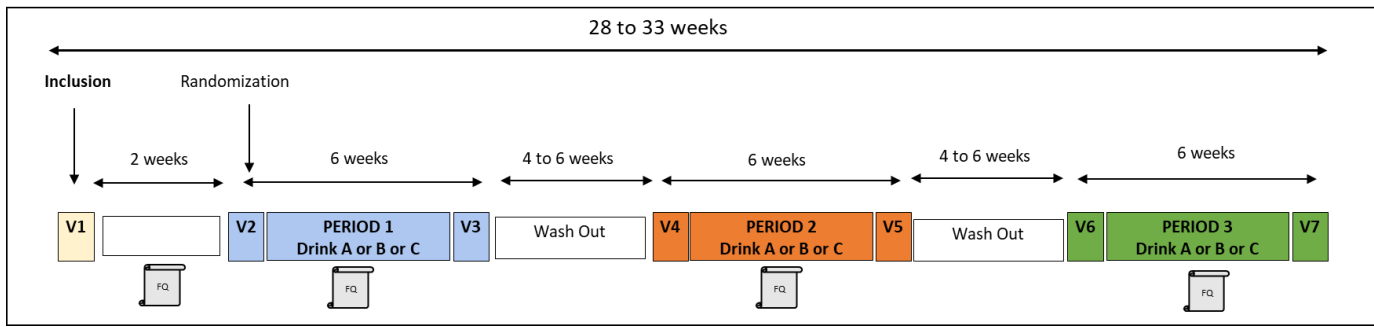
16 472  
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18 473 **Figure 2. HPLC-DAD chromatogram of an OJ from a commercially available OJ concentrate**  
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20 474 **naturally rich in hesperidin (A), a control beverage with an identical total sugar**  
21  
22 475 **concentration (B), and a control beverage additionally supplemented with hesperidin (C).**  
23  
24 476 Flavonoids were extracted according to IFU (2005)<sup>(31)</sup> and analysed by HPLC-DAD using a C18  
25  
26 477 column (250 × 4.6 mm, particle size 5.0 µm, Kinetex®, Phenomenex, Aschaffenburg, Germany)  
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28 478 and an acetonitrile-based elution gradient.  
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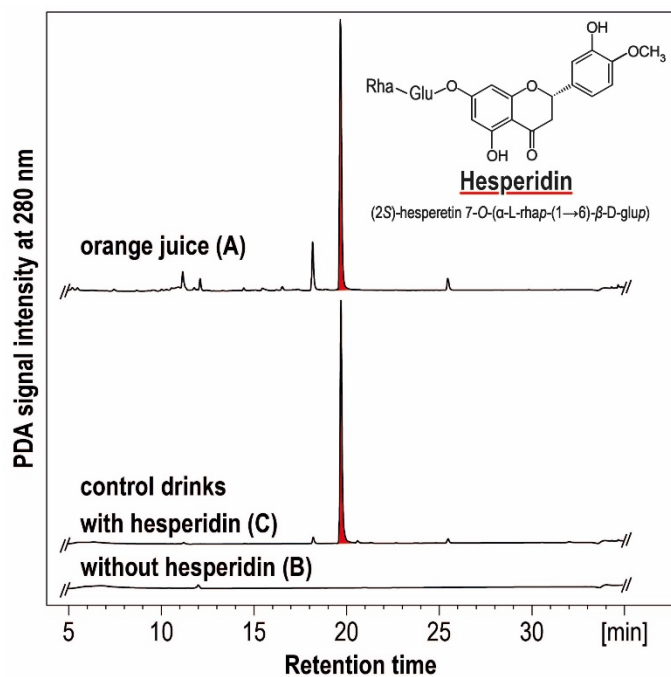
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**Figure 1**

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**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)<sup>(31)</sup> 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.

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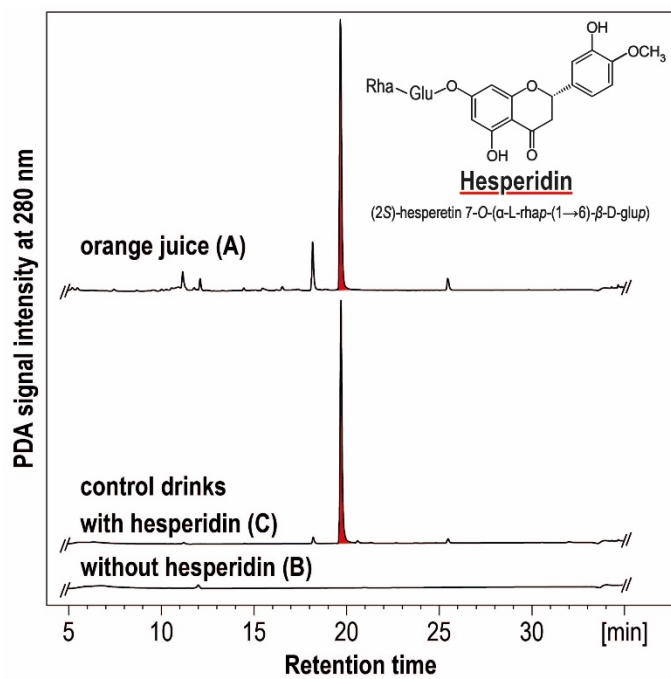


Figure 2.

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# BMJ Open

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

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<b>Primary Subject Heading</b>:	Nutrition and metabolism
Secondary Subject Heading:	Cardiovascular medicine
Keywords:	MOLECULAR BIOLOGY, NUTRITION & DIETETICS, VASCULAR MEDICINE

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3 1 **Study protocol for a randomized controlled trial evaluating the role of Orange juice,**  
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5 2 **HESPERidin in vascular HEALTH benefits: The HESPER-HEALTH Study**  
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27 41 **Key words :**

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29 42 Randomized controlled trial ; vascular function ; orange juice ; hesperidin ; flavanones

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31 43 bioavailability and metabolism

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## 48 ABSTRACT

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

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

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

## 72 **ARTICLE SUMMARY**

- 73
- 74 **Strengths and Limitations of this study**
- 75 ➤ This randomised controlled dietary intervention is carried out to assess the effects of OJ
  - 76 consumption on vascular function and to determine the contribution of hesperidin to these
  - 77 effects.
  - 78 ➤ This trial includes an analysis of hesperidin bioavailability and metabolism in biofluids to
  - 79 enable further correlation with the vascular response of individuals.
  - 80 ➤ The use of innovative approaches (i.e., oxylipin profiling and nutrigenomics) will provide
  - 81 insights on the molecular mechanisms underlying the vascular effects.
  - 82 ➤ By including an analysis of the gut microbiota, this study will clarify interactions between
  - 83 OJ, hesperidin and the microbiome.
  - 84 ➤ The sensoriality of the two artificial control drinks differs from that of the fully natural
  - 85 orange juice OJ, which may constitute a study limitation.

## 88 **INTRODUCTION**

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90 Recent epidemiological studies have associated the frequent consumption of sugar-sweetened

91 beverages with some adverse health effects, such as early death, weight gain and cancer <sup>(1; 2)</sup>

92 Findings of such studies are often extrapolated to fruit juice consumption, including 100% fruit

93 juices. However, these observational, epidemiological results contrast with experimental studies

94 which have demonstrated clear differences in the metabolic response of the human body to fruit

95 juices as compared with that seen for sugar-sweetened beverages<sup>(3; 4)</sup>.



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3 96 One characteristic of citrus foods is that they are a rich and exclusive source of dietary  
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5 97 flavanones, a category of (poly)phenol compounds, mainly present as hesperidin in orange.  
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7 98 Flavanone intake has been repeatedly associated with a lower incidence of mortality from CVD (5;  
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10 99 <sup>6</sup>). Results from preclinical studies using different models of atherosclerosis also provide evidence  
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12 100 for a role of citrus flavanones in cardiovascular protection, with a slowdown in atherosclerosis  
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14 101 development (7). These atheroprotective effects have been related to the capacity of flavanones to  
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16 102 modulate the expression of genes involved in cellular processes responsible for vascular  
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18 103 dysfunction (8). Evidence of the vascular protective effects of citrus flavanones have been reported  
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20 104 in few randomised controlled clinical trials (4; 9; 10; 11). However, published trial with orange  
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22 105 flavanones do not yet allow firm conclusions to be drawn about their efficacy to modulate vascular  
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24 106 function, mainly due to the high degree of discrepancies between the study designs.  
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28 107 The ability of citrus flavanones to exert beneficial effects depends on their bioavailability. The  
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30 108 gut microbiota plays a key role in flavanone absorption, because naturally-occurring flavanones are  
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32 109 present as molecules with glycosyl moieties, e.g. a rutosyl, i.e. a  $\alpha$ -L-rhamnopyranosyl-(1→6)-  
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34 110  $\beta$ -D-glucopyranosyl moiety in hesperidin. The sugar moiety of hesperidin must be hydrolysed by  
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36 111 bacterial glycosidases to yield a format which the human body can absorb i.e. the aglycone,  
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38 112 hesperetin. Upon release, hesperetin can be absorbed by intestinal cells or may be further  
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40 113 catabolized into diverse phenolic compounds by microbial action in the colon. After absorption of  
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42 114 hesperetin and its microbial catabolites, they enter the blood circulation and are subject to further  
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44 115 human metabolism. For instance, hepatic metabolism includes several conjugation reactions, e.g.,  
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46 116 sulphatation or glucuronidation (12; 13). Hence, the extent of hesperetin released in the colon, the  
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48 117 formation of colonic catabolites and their subsequent absorption could largely depend on the gut  
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50 118 microbial composition, which has been shown to vary between subjects (14). In agreement with this  
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52 119 hypothesis, previous studies have reported a large interindividual variability in the urinary  
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3 120 excretion level of flavanones <sup>(15; 16)</sup> that could reflect differences in gut microbiota composition,  
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5 121 but this remains to be demonstrated. A recent clinical study found that regular consumption of OJ  
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7 122 can modulate the gut microbiota profile and these changes were associated with positive shifts in  
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9 123 some metabolic outcomes<sup>(17)</sup>.

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14 125 Dietary (poly)phenols and their circulating metabolites encompass a huge diversity of  
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16 126 compounds <sup>(18)</sup> whose health effects seem increasingly linked to their capacity to exert complex  
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18 127 genomic modifications, such as changes to the expression of genes <sup>(19)</sup>. In preclinical studies,  
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20 128 flavanones have been shown to induce changes in the expression of a number of genes in aorta and  
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22 129 endothelial cells that relate to inflammation, and endothelial cell function <sup>(7; 20; 21; 22)</sup>, revealing  
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24 130 potential molecular mechanisms of action to explain their health properties. However, such  
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26 131 mechanisms of action are still largely unexplored in humans <sup>(20)</sup>. Based on a huge body of  
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28 132 experimental results demonstrating the anti-inflammatory effects of polyphenols and related  
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30 133 mechanisms <sup>(23)</sup>, these compounds are suggested as key players in the protective effects of their  
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32 134 food sources for chronic inflammatory diseases. However, in humans, consumption of polyphenol-  
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34 135 rich foods induces subtle changes in inflammatory and oxidative status that can only typically be  
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36 136 measured using sensitive techniques, such as the lipidomic profiling of oxylipins <sup>(24)</sup>. Oxylipins are  
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38 137 a superclass of lipid mediators comprising hundreds of metabolites which regulate a diversity of  
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40 138 biological processes including inflammation, cell adhesion, migration and proliferation, blood  
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42 139 clotting and vessel permeability <sup>(25)</sup>.

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47 141 Based on this state of the art, the present human randomised, controlled, double-blind, cross  
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49 142 over intervention conducted on subjects predisposed to CVD aims to establish a cause-and-effect  
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51 143 relationship between hesperidin intake and the vascular protective effects of drinking OJ naturally

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3 144 rich in hesperidin. The study will also provide insights into the mechanisms responsible for the  
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5 145 observed effects.  
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## 10 147 **STUDY OBJECTIVES**

### 12 148 **Primary Objective**

14 149 The primary objective is to assess the effect of a subchronic consumption (6 weeks) of a naturally  
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17 150 flavanone-rich OJ or a control drink supplemented with orange flavanones on endothelial function,  
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19 151 by assessing flow-mediated dilation (FMD) in subjects with a predisposition to CVD, based on  
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21 152 age, waist circumference and body mass index.  
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### 26 154 **Secondary Objectives**

28 155 The secondary objectives are to:

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31 156 ➤ assess the effects of the intervention: (1) on other markers of vascular function using a range  
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33 157 of well-established measurements in the macrocirculation (blood pressure, arterial  
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35 158 stiffness) and in the microvasculature at skin level (microvascular reactivity by Flow Laser  
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37 159 Doppler, FLD); (2) on the postprandial endothelial response in response to a challenge  
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39 160 meal; (3) on biomarkers of cardiovascular risk, endothelial dysfunction and inflammation;  
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41 161 (4) on anthropometric parameters and body composition; (5) on the vitamin C and  
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43 162 carotenoids status; (6) on the gut microbiota composition (microbial communities profiling)  
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46 163 ➤ measure the bioavailability of hesperidin and its metabolites in biofluids (plasma, urine)  
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49 164 ➤ ascertain the underpinning molecular mechanisms of the vascular responsiveness  
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51 165 (nutrigenomic analysis, oxylipin profiling).  
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5 168 **METHOD AND ANALYSIS**6  
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8 169 **Study Design**

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11 170 HESPER-HEALTH is a human dietary intervention study designed as a randomized, double-blind,  
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13 171 controlled, cross-over trial with three arms that will be conducted at the Clinical Investigation  
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15 172 Center, Inserm 1405 (PIC/CIC) of the University Hospital of Clermont-Ferrand, France. The  
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17 173 sponsor is the University Hospital of Clermont-Ferrand. This trial will be carried out on subjects  
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19 174 predisposed to CVD, based on age (40-65 years old) and on the overweight (waist circumference  
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21 175  $\geq 80$  cm for woman,  $\geq 94$  cm for man and with a  $BMI \leq 30$  kg/m<sup>2</sup>). Forty-two participants will be  
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23 176 recruited and will receive the three treatments in a random order: (A) a commercially-available OJ  
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25 177 naturally rich in hesperidin (ca. 600-650 mg/L), (B) a control beverage with a total sugar  
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27 178 concentration identical to (A), and (C) a control beverage identical to (B) but supplemented with  
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29 179 hesperidin at the level present in the natural OJ (A).  
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36 181 For each volunteer, the study is divided in three identical experimental periods of 45 days. These  
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38 182 periods include a three day run in period during which time specific dietary recommendations will  
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40 183 be followed, samplings and measurements will be performed at home. This run in period will be  
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42 184 followed by a 6-weeks treatment period of consumption of one of the three beverages. A four to  
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44 185 six week wash-out is planned between each of the three experimental periods. The protocol  
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46 186 includes seven visits to PIC/CIC, including one visit (V1) at inclusion, and will last in total for 28  
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48 187 to 33 weeks (**Figure 1**). Over the last 24h prior to each visit, volunteers will be asked to collect  
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50 188 stool samples and 24h urine samples to assess gut microbiota composition and flavanone  
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52 189 bioavailability and metabolism, respectively.  
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5 191 At the beginning and end of each experimental period, overnight-fasted volunteers will be invited  
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7 192 to attend the PIC/CIC (visits V2 to V7) for vascular function tests, blood sampling, anthropometric  
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9 193 measurements and body composition analysis. Blood will be sampled for further assessment of  
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11 194 plasma flavanones including metabolites, carotenoids and vitamin C, oxylipin profiling, systemic  
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13 195 biomarkers of endothelial activation and inflammation, metabolic parameters, and for the analysis  
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15 196 of the nutrigenomic response.  
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21 198 At each visit (V2 to V7), any adverse events (AE) will be followed up. In case of occurrence of a  
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23 199 serious AE, the sponsor will be notified and in return he will notify the competent authorities. All  
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25 200 unexpected serious AE are reported in an annual safety report. After measurements and blood  
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27 201 collection in the fasting state, a challenge meal together with the respective study drink will be  
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29 202 administered to all subjects before further exploration. This will enable evaluation of the acute  
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31 203 postprandial effects (at T+3h, T+6h) of the study products on endothelial function (V2,V4,V6), to  
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33 204 also be repeated after the 6 weeks intervention period (V3, V5, V7). The challenge meal will consist  
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35 205 of fresh cream, sucrose and milk proteins, providing 900 kcal – a pro-oxidant and pro-inflammatory  
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37 206 meal that is known to induce a transient endothelial dysfunction during the post prandial period  
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42 207 (26).  
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47 209 During each experimental period, volunteers will be asked not to eat citrus foods and to consume  
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49 210 no more than 250 mL/d of polyphenol-rich beverages (coffee, tea, fruit juices, wine, cocoa). To  
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51 211 check that volunteers do not change their eating habits during the study, for each experimental  
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53 212 period they will be asked to complete food records over three consecutive days defined by the  
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55 213 investigator. The first food record will be completed prior to visit V2 to PIC/CIC and the two others

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3 214 at mid-term of each intervention period. On the two days preceding each visit at the PIC/CIC,  
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5 215 volunteers will be asked not to consume polyphenol-rich foods and beverages. The day before each  
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7 216 visit (V2-V7) at the PIC/CIC, they will have to consume exactly the same dinner without  
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10 217 polyphenols at 8 pm. To ensure a good understanding of all these dietary instructions, participants  
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12 218 meet a dietician during the inclusion visit (V1).  
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### 17 220 **Inclusion and Exclusion criteria**

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19 221 These criteria are listed in the Table 1.  
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21 222 The reasons for a premature cessation of the study include withdrawal of consent, significant  
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23 223 deviation from the protocol, incidental illness, occurrence of a serious adverse event, intolerance  
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26 224 to the tested products, antibiotic treatment during the study, a non-observance of the nutritional  
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28 225 protocol declared by the subject or highlighted by food records.  
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### 33 227 **Study products**

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35 228 Three study beverages will be used in cross-over and double-blinding conditions. These include  
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37 229 (A) an OJ from a commercially-available OJ concentrate (Sucocítrico Cutrale, Araraquara, São  
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39 230 Paulo, Brazil) containing approx. 600-650 mg hesperidin per L, (B) a control drink with a total  
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42 231 sugar concentration identical to that of (A), and (C) a control drink identical to (B) but  
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44 232 supplemented with hesperidin at the level found in the OJ (Figure 2). The daily administered dose  
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46 233 will be 330 mL to be distributed over two intakes ( $2 \times 165$  mL) - one in the morning during  
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48 234 breakfast and the second during lunch. This will correspond to ca. 200-215 mg hesperidin per day  
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51 235 (drinks A and C, resp.).  
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3 237 The control beverages (B) and (C) will be made isocaloric to (A), all containing an identical total  
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5 238 sugar concentration (ca. 9.0 g/100 mL), comprising glucose, fructose, and sucrose. Thus, the  
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7 239 amount of total sugar provided daily by each drink will amount to ca. 30 g. All drinks will contain  
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10 240 citric acid at similar levels. To match the visual appearance of the OJ, food colorants as well as a  
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12 241 clouding agent will be added to the control drinks. Flavour will be matched by adding a natural  
13  
14 242 orange aroma to all beverages (A-C), including the OJ from concentrate to which orange aroma  
15  
16 243 addition is required by European law. All the beverages will be pasteurized according to  
17  
18 244 commercial practice and filled into 330 mL brown glass bottles with no visual distinction possible  
19  
20 245 between bottles of different drinks. Despite careful colour and flavor matching, both the hesperidin-  
21  
22 246 free (B, placebo) and the hesperidin-rich (C) control drink may be differentiated sensorially from  
23  
24 247 the OJ by attentive subjects. In contrast, subjects and clinic staff will be unable to identify which  
25  
26 248 of the artificial control drinks (B) and (C) is the one rich in or free from hesperidin.  
27  
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32  
33 250 Finally, bottles will be labelled and packaged for each cross-over period in a blinded fashion on  
34  
35 251 the basis of the randomisation schedule. They will be stored at +4°C until distribution to the  
36  
37 252 participants. Beverages will be distributed to volunteers at the first visit of each experimental period  
38  
39 253 (V2, V4, V6) in bags containing 48 bottles per period. Volunteers will be asked to store the bottles  
40  
41 254 in a dark place at room temperature or cooler. The produced beverages will be analytically  
42  
43 255 characterized in detail, including, e.g., the levels of potassium <sup>(27)</sup>, carotenoids<sup>(28)</sup>, and soluble,  
44  
45 256 insoluble <sup>(14)</sup> and total hesperidin <sup>(29)</sup>. Hesperidin levels in drinks (A) and (C) as well as levels of  
46  
47 257 ascorbic and dehydroascorbic acid (vitamin C) in the OJ (A) will be monitored over the entire study  
48  
49 258 period. Colony forming units of yeasts, total viable counts, lactic acid bacteria and moulds will also  
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51 259 be assessed to ensure the microbiological safety of the products. To assess compliance, participants  
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3 260 will be asked to report daily on diaries their consumption of the study beverages and to return the  
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5 261 dairies and the empty and non-consumed bottles at the end of each period (V3, V5, V7).  
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### 9 10 263 **Assignment of intervention**

11  
12 264 **Randomisation and Allocation** : Subjects will be randomly assigned to treatment groups according  
13  
14 265 to a pre-established list of randomisation designed by a clinical research supervisor, independently  
15  
16 266 from the investigators and the sponsor. The treatment number will be allocated by order of entrance  
17  
18 267 in inclusion. Subjects will be randomised to receive drink A, B, or C using a Latin-square random  
19  
20 268 design. Subject allocation to treatments will take place after inclusion and will be based on a  
21  
22 269 computer-generated randomisation list. Access to the randomisation list will be restricted to staff  
23  
24 270 performing this task. The randomisation number will be allocated by order of entrance in inclusion.  
25  
26 271 Under normal circumstances, the blinding should not be broken until all subjects have completed  
27  
28 272 the trial and the database will be locked. However, the blinding can be broken should a specific  
29  
30 273 emergency treatment at the site require knowledge of the treatment status of the subject. In such a  
31  
32 274 case, the investigator can, using a sealed envelop and following an internal procedure, reveal the  
33  
34 275 treatment assignment through the randomisation plan and then inform the sponsor.  
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### 40 276 41 42 277 **Participant eligibility**

43  
44 278 Forty-two volunteers will be recruited from the existing PIC/CIC volunteer's database, by  
45  
46 279 announcement in local press and media by paper and digital poster campaigns and by social  
47  
48 280 networks. Each subject who meets the recruitment criteria (Table 1) will be pre-selected by the  
49  
50 281 investigator and will be given a detailed explanation of the protocol. Participant who agree to  
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52 282 participate will have a first visit (V1, **Figure 1**) at the PIC/CIC to provide their written consent to  
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3 283 participate and to have a medical examination, an interview with a dietitian and a blood checkup  
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5 284 to ensure that all clinical criteria are met.  
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10 286 **Sample size**  
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12 287 Sample size calculation has been performed based on both our experience in FMD measurement  
13 and on the FMD response (main criteria) observed in previous dietary interventions<sup>(30,31)</sup>. The  
14 288 targeted statistical power was based on interindividual variability for FMD measurement of the  
15 289 operator (SD = 1.9%). Aiming for a statistical power greater than 80% and a two-tailed type I error  
16 290 at 0.017 (to take into account the three comparisons to be considered), the total number of subjects  
17 291 required to provide sufficient power to detect a minimal absolute difference of FMD equals 1.6%  
18 292 in a 3-sequences cross-over study (with an intra-class correlation coefficient at 0.5) is 36. Assuming  
19 293 unforeseen drop-outs and follow-up losses, 42 participants will be recruited.  
20 294  
21 295

## 22 296 **STUDY ASSESSMENTS**

23 297 The time table of the measurements that will be performed in the HESPER-HEALTH study is  
24 298 presented in **Figure 3**. The outcome assessors and data analysts will be blinded for treatments.  
25 299

### 26 300 **Primary outcome**

27 301 Endothelial function is assessed using the non-invasive technique FMD which constitutes the gold  
28 302 standard to evaluate vascular endothelial function in humans. The FMD technique measures the  
29 303 diameter of the brachial artery by ultrasound before and after increasing shear stress by inducing a  
30 304 reactive hyperemia, with the degree of dilation reflecting mostly the arterial endothelial nitric oxide  
31 305 release. The procedure for the FMD measurement is full compliant with the reference method  
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3 306 described by Coretti et al. (32). FMD is measured on the left brachial artery above the antecubital  
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5 307 fossa using a high-resolution ultrasound system with a 7-12 MHz linear array 190 transducer (Vivid  
6  
7 308 S5, GE Healthcare, Versailles, France). The use of a mechanical arm device (Vascular Imaging,  
9  
10 309 Amsterdam, Netherlands) allows precise movements of the probe in the three dimensions. Images  
11  
12 310 are analysed using automated edge detection software 197 (Hemodyn 3M apparatus, Dinap SRL,  
13  
14 311 Buenos Aires, Argentina). FMD will be assessed at V2, V3, V4, V5, V6 and V7 under both fasting  
16  
17 312 and postprandial conditions.  
18  
19 313

## 21 314 **Secondary Outcomes**

### 24 315 **➤ Other vascular function measurements**

26 316 Endothelial function in the microcirculation will be assessed using Flowmetry by Laser Doppler  
27  
28 317 (FLD), which is a non-invasive and validated technique for continuous measurement of the  
29  
30 318 endothelial dependent microvascular reactivity. The laser-doppler system PeriFlux 5010 (Perimed)  
31  
32 319 is used at the level of the hand to follow the response to a reactive hyperemia induced by a  
33  
34 320 temporary occlusion of the brachial artery, using the same stimulus as for FMD measurement. FLD  
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36 321 will be assessed during V2, V3, V4, V5, V6 and V7 in fasting and postprandial conditions.  
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40 322 Arterial stiffness will be evaluated by the carotid-femoral Pulse Wave Velocity (PWV) which is  
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42 323 calculated from measurements of pulse transit time and the distance travelled between the carotid  
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44 324 and femoral arteries using a validated non-invasive device (SphygmoCor; AtCor Medical Pty.  
45  
46 325 Ltd.). PWV will be assessed after fasting at V2, V3, V4, V5, V6 and V7.  
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48 326

49 326 Blood pressure will be monitored by subjects at home using tensiometers (Microlife BP A200,  
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51 327 Microlife) which will be loaned to them for the study duration. They will be asked to perform three  
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53 328 repeated measurements in fasting condition during the three consecutive days preceding their visits  
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3 329 to the PIC/CPC (V2, V3, V4, V5, V6 and V7), and to record obtained values on a dedicated data  
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5 330 sheet.

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10 332 ➤ ***Flavanone metabolism and bioavailability in biofluids***  
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12 333 Phase 2 metabolites of the *Citrus* flavanones and their microbial-derived catabolites of flavanones  
13  
14 334 including conjugated forms like glucuronidated and sulfated ones will be analyzed in plasma and  
15  
16 335 24h urine samples according to the method based on those reported by Aschoff et al. <sup>(15)</sup> and Mullen  
17  
18 336 et al. <sup>(33)</sup>. For plasma and urine analyses, acetonitrile and methanol will be used as extraction  
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20 337 solvents as described in detail by Mullen et al. <sup>(33)</sup>. A subset of urine and plasma samples will be  
21  
22 338 analyzed after enzymatic hydrolysis with glucuronidase and sulfatase as described by Aschoff et  
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24 339 al. <sup>(15)</sup>. Identification and quantitation will be done by UHPLC-DAD-ESI-MS/MS for all samples  
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26 340 collected (urine) or sampled (plasma) at V2, V3, V4, V5, V6 and V7.  
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33 342 ➤ ***Systemic biomarkers related to CVD risk, endothelial dysfunction and inflammation***  
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36 343 These include metabolic parameters (plasma glucose, insulin, Tri Acyl Glycerol (TAG), Total  
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38 344 Cholesterol, High density lipoprotein cholesterol (HDL-cho), uric acid that will be measured by  
39  
40 345 spectrometric and enzymatic methods; total fatty acids, by gas chromatography), soluble adhesion  
41  
42 346 molecules and inflammatory markers (Intercellular Adhesion Molecule (ICAM), Vascular Cell  
43  
44 347 Adhesion Molecule (VCAM) and e-selectin, Interleukin-6 (IL-6), Tumor Necrosis Factor Alpha  
45  
46 348 (TNFalpha), high sensitivity- C Reactive Protein (hs-CRP), by using Elisa assays), plasma nitrites  
47  
48 349 (by chemi-luminescence) and the release of endothelial extracellular vesicles (by flux cytometry).  
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50 350 These analyzes will be performed in blood sampled at V2, V3, V4, V5, V6 and V7.  
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3 352 ➤ *Carotenoids and vitamin C status*  
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5 353 Carotenoids will be quantitated from human plasma after a protein-crash with ethanol and repeated  
6  
7 354 extraction with hexane using the method described by Aschoff et al. <sup>(28)</sup>. Vitamin C status will be  
8  
9 355 quantified in deproteinized plasma by HPLC-fluorescence detection as previously described <sup>(34)</sup>.  
10  
11  
12 356 These measures will be performed in blood sampled at V2, V3, V4, V5, V6 and V7.  
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14 357  
15 358 ➤ *Anthropometry and body composition assessment*  
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18 359 Body Mass Index (BMI), waist to height ratio (waist circumference/height) and the percentages  
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20 360 of fat mass, lean mass and water (obtained using a multi-frequency Bioelectrical Impedance  
21  
22 361 Analyser) will be determined. These parameters will be recorded at V2, V3, V4, V5, V6 and V7.  
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25 362  
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27 363 ➤ *Gut microbiota composition assessment*  
28

29 364 The composition of the gut microbiota will be determined by 16S metabarcoding. This method  
30  
31 365 involves the amplification by PCR of variable regions of the 16S rDNA gene from fecal DNA  
32  
33 366 followed by the preparation of DNA libraries and high throughput next-generation sequencing  
34  
35 367 (Illumina technology). Analysis will be completed by an absolute quantification by qPCR of target  
36  
37 368 groups of the human gut microbiota considered as beneficial to the host, or involved in polyphenol  
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39 369 metabolism. These analyses will be performed in faeces samples collected during the 12h preceding  
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41  
42 370 V2, V3, V4, V5, V6 and V7.  
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47 372 **Mechanistic outcomes**  
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49 373 In the HESPER-HEALTH study, we aim to explore the molecular mechanisms of action underlying  
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51 374 vascular effects of OJ and hesperidin consumption. To this end, we propose to perform global and  
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53 375 integrated analyses of expression of genes (nutrigenomic analysis) in the blood and to analyse the  
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3 376 oxylipin profiling to identify changes in biological processes involved in inflammation and  
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5 377 vascular dysfunction.

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7 378 **Nutrigenomic response assessment:** RNA will be extracted from whole blood using PAXgene  
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9 379 Blood RNA System. Global gene expression profile of both protein coding and protein non-coding  
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11 380 RNAs (miRNAs, snoRNAs, lncRNAs) will be assessed using a microarray approach. For genes  
12  
13 381 identified as differentially expressed, integrated multi-omic bioinformatic analyses will be  
14  
15 382 performed to identify gene ontologies, gene network, cellular pathways and interactions between  
16  
17 383 different types of RNAs. Potential transcription factors involved will be searched and capacity of  
18  
19 384 flavanone metabolites to bind to transcription factors as well as cell signaling proteins regulating  
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21 385 their activity will be predicted using 3D docking analysis. Analysis will be performed from blood  
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23 386 sampled at V3, V5 and V7.

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26 387 **Oxylipin profiling:** a comprehensive assessment of circulating total oxylipins (free and esterified  
27  
28 388 forms) will be performed using a targeted and quantitative MS-based method (LC-MS/MS) as  
29  
30 389 described previously <sup>(35)</sup>. Briefly, EDTA plasma will be mixed with 22 internal standards and an  
31  
32 390 antioxidant solution preventing artificial oxylipin production during sample processing. Then total  
33  
34 391 oxylipins will be extracted using solid phase extraction (SPE) following protein precipitation and  
35  
36 392 alkaline hydrolysis. Extracted oxylipins will then be measured using electrospray ionization in  
37  
38 393 negative ion mode and multiple reaction monitoring (MRM). Analysis will be performed in plasma  
39  
40 394 sampled at V2, V3, V4, V5, V6 and V7.

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45 396 **Statistical analyses**  
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47 397 All analyses will be performed with Stata software (version 15, StataCorp, College Station, USA)  
48  
49 398 before the breaking of the randomization code, according to International Conference on  
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51 399 Harmonization-Good Clinical Practice guideline. Continuous variables will be presented,  
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3 400 according to their statistical distribution, as mean and standard-deviation, or median and  
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5 401 interquartile range. The Shapiro-Wilk test will be used to assess normality. Categorical data will  
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7 402 be presented as exact number and percentage.  
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10 403 The primary outcome will be analysed using random-effects model (i.e. mixed linear model for  
11  
12 404 continuous dependent variable) for a 3-treatment crossover study. The statistical model includes  
13  
14 405 treatment group, sequence, period, carry-over on FMD baseline value as fixed effects. Subject  
15  
16 406 nested in sequence will be included in the model as a random effect. A Sidak's type I error will be  
17  
18 407 applied to take into account multiple comparisons. The *treatment group x period* interaction will  
19  
20 408 be studied. If this interaction is not significant, the main effect of treatment will be assessed. If this  
21  
22 409 interaction is significant, there will be a particular focus on the first period. The normality of  
23  
24 410 residuals from random-effects model will be studied as aforementioned, with the Shapiro-Wilk  
25  
26 411 statistic and visual inspection of residual plots. If appropriate, a transformation (for example  
27  
28 412 logarithmic) of the primary outcome could be proposed to achieve its normality. The results will  
29  
30 413 be expressed with effect-sizes and 95% confidence intervals.  
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35 414 The primary analysis will be completed by multivariable approach using the statistical model  
36  
37 415 described above with, additionally, covariates determined according to univariate results and  
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39 416 clinical relevance, including primarily waist circumference, BMI, weight at enrolment, age, gender  
40  
41 417 and energy intake. The other continuous outcomes will be analyzed with the same statistical  
42  
43 418 analysis plan. For categorical data, generalized mixed linear modelling will be carried out. Any  
44  
45 419 change in the current statistical analysis plan during the study will be noted in the study records.  
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## 52 53 422 **ETHICS AND DISSEMINATION**

### 54 55 56 423 **Approval**

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3 424 In accordance with the Declaration of Helsinki and French regulations on clinical trials, the study  
4  
5 425 was evaluated by an independent ethics committee, chosen at random by the French ministry of  
6  
7 426 research, namely the ‘Comité de Protection des Personnes Sud-Est III, Bron, France’ (registration  
8  
9 427 number: 20.10.15.60521). The approval of the ethics committee was obtained on November 17,  
10  
11 428 2020. The first inclusion was on February 24, 2021. The study is declared to the French National  
12  
13 429 Agency for the Safety of Medicines (ID-RCB : 2020-A01985-34). Any substantial change in the  
14  
15 430 protocol or in the informed consent form will be presented to both authorities. The trial is also  
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17 431 registered on ClinicalTrials.gov (NCT04731987).  
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### 433 **Consent**

434 Subjects will be informed by a fair and accessible form approved by the ethics committee. Subjects  
435 will be free to ask any question on all aspects of the study before giving the consent and informed  
436 that they are free to withdraw from the study at any time. The investigator will ensure that the  
437 written consent obtained from subjects prior to their participation in the study is free and informed  
438 (supplemental data 1).  
439

440

### 441 **Data quality and management**

442 The data collected will be anonymized by a subject code assigned to each participant. All data from  
443 the interview, clinical examination and explorations will be reported in a paper Case Report Form  
444 (CRF) by experimented and trained study managers of the PIC/CIC and will constitute the data  
445 trial kept confidential. The database of the study will be designed and managed using the Research  
446 Electronic Data Capture (REDCap™, Vanderbilt University), a dedicated software allowing a  
447 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

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2  
3 448 and elimination of transcription errors, data validation will be carried out to resolving discrepancies  
4  
5 449 by the data manager. Then, database will be locked and securely sent to the biostatistician.  
6  
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8 450 According to the "minimal risks and constraints" ranking of this study by the ethics committee, the  
9  
10 451 monocentric design and the low risk calculated by the sponsor, there is no data monitoring  
11  
12 452 committee and no interim analyses planned for this study. Audits or inspections concerning the  
13  
14 453 trial or investigation team activities could be carried out at anytime.  
15  
16

#### 17 454 18 19 455 **Data availability statement**

20  
21 456 data not available due to legal restriction.  
22  
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24 457

#### 25 26 458 **Dissemination**

27  
28 459 The results will be communicated to participants, in peer-review journals, and presented at  
29  
30 460 international conferences in the domain of Food, Nutrition & Health.  
31  
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33 461

#### 34 35 462 **Patient and Public Involvement statement**

36  
37 463 patients and the public were not involved in the design or conduct of this protocol.  
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## 43 44 466 **DISCUSSION**

45  
46 467 The controlled randomised HESPER-HEALTH study will use reliable and sensitive clinical  
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48 468 biomarkers of human health to provide a comprehensive picture of the effects of a flavanone-rich  
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50 469 OJ on different components of vascular function. It will also produce mechanistic insights to  
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52 470 support the contribution of citrus flavanones in vascular health. Together, the results of the study  
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54 471 will strengthen the level of evidence of the link between flavanone intake and the effects of a  
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3 472 moderate consumption of OJ on vascular endpoints in humans. The results may also improve  
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5 473 understanding of the role of gut microbiota in the interindividual variability in the absorption and  
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7 474 metabolism of citrus flavanones, and the putative prebiotic-like effect of flavanone-rich foods. The  
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10 475 results from HESPER-HEALTH could help health professionals communicate science-driven  
11  
12 476 dietary advice about fruit juice consumption to their patients. They should also be useful for the  
13  
14 477 citrus sector to encourage the selection of orange varieties naturally rich in hesperidin, and to  
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17 478 provide health-focused guidance on how to adapt processing methods to produce fruit juice richer  
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19 479 in readily available flavanones.  
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3 480 **Author contributions:** MAV, DM, NM, RE, CG, CBS, PM, CG, LEM, RS, GP, CM contributed  
4  
5 481 to the conceptualisation, design, and implementation of this research protocol; BP led to the  
6  
7 482 development of the statistical analysis plan. All authors read and approved the final manuscript.  
8  
9  
10 483 All authors will have access to the final trial data set. Of note, the classical authorship rules will be  
11  
12 484 applied in the future papers dealing with the results of the trial.  
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16  
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18  
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20  
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22  
23 489 from Eckes Granini (Niederolm, Germany) for providing a pilot production line and helping to  
24  
25  
26 490 produce and package the drinks to be tested. We thank Peter Bach, Tim Dreifke, Paul Luka Dreis,  
27  
28 491 and Anna-Maria Schmelzer (all Geisenheim University) for their technical assistance during  
29  
30 492 beverage development and production. We thank Sucocítrico Cutrale (Araraquara, São Paulo,  
31  
32 493 Brazil) for donating the OJ concentrate. Döhler (Darmstadt, Germany) is gratefully acknowledged  
33  
34 494 for donating the colorants, clouding agent, and flavouring used in control drink development and  
35  
36  
37 495 production. HealthTech Bio Actives (Beniel, Spain) is acknowledged for providing the hesperidin  
38  
39 496 formulation.  
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43  
44 498 **Funding:** This work was supported by a consortium of orange producers, juice manufacturers and  
45  
46 499 packaging companies based in Europe and Brazil under the umbrella of the European Fruit Juice  
47  
48 500 Association (AIJN). A representative from AIJN will give his opinion before any communication  
49  
50 501 or publication.  
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3 503 **Disclaimer:** The views presented in this paper are those of the authors. Funding companies have  
4  
5 504 played no role in the study beyond providing financial support.  
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10 506 **Competing interest :** None declared

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For peer review only

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

<i>Inclusion criteria</i>	<i>Exclusion criteria</i>
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 $\geq 80$ cm for women and $\geq 94$ cm for men, with BMI $\leq 30$ )	Treated with antibiotics, antifungals, probiotics or prebiotics in the 3 months before the enrolment
Ability to give informed consent to participate in research.	Menopausal hormone therapy
Willingness to accept randomization and undergo the testing and intervention procedures and deliver stool, blood and urine samples for testing	Diagnosed gastrointestinal illness
No aversion or intolerance to citrus foods	Any serious medical condition that precludes safe participation in the study
Accept to limit their total intake of flavonoid rich beverages (tea, coffee, cocoa, wine, fruit juice) to 250 ml/day	History of eating disorders
	Digestive disorders with diarrhea during the 3 months preceding the beginning of the study
	Self-declared vegetarian, vegetarian, 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

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3 510 **FIGURE LEGENDS**  
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7 512 **Figure 1: General scheme of HESPER-HEALTH study progress.**  
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9 513 V: visits in PIC/CIC; Drink A: OJ naturally rich in hesperidin; Drink B: control beverage with  
10 514 sugar concentrations identical to (A); Drink C: control beverage identical to (B) but supplemented  
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12 515 with hesperidin at the level of (A); FR: 3 days food record.  
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18 517 **Figure 2. HPLC-DAD chromatogram of an OJ from a commercially available OJ concentrate**  
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20 518 **naturally rich in hesperidin (A), a control beverage with an identical total sugar**  
21  
22 519 **concentration (B), and a control beverage additionally supplemented with hesperidin (C).**  
23  
24

25 520 Flavonoids were extracted according to IFU (2005) <sup>(29)</sup> and analysed by HPLC-DAD using a C18  
26  
27 521 column (250 × 4.6 mm, particle size 5.0 µm, Kinetex<sup>®</sup>, Phenomenex, Aschaffenburg, Germany)  
28  
29 522 and an acetonitrile-based elution gradient.  
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34 524 **Figure 3. Time table of the conduct and measurements of the HESPER-HEALTH study.**  
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36 525 The crosses indicate the visits and types of samples taken (X) and the recommendations and  
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38 526 measurements made for each of them (x).  
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41 527 ALAT, alanine amino transferase; ASAT, aspartate amino transferase; BP, blood pressure; DBP, diastolic  
42  
43 528 blood pressure; FLD, flowmetry by laser doppler; FMD, flow mediated dilatation; Gamma GT, gamma  
44  
45 529 glutamyl tranferase; HDL Chol, high density lipoprotein cholesterol; hsCRP, high sensitivity C reactive  
46  
47 530 protein; ICAM, intercellular adhesion molecule; IL6, interleukin-6; PIC/CIC, plateforme d'investigation  
48  
49 531 clinique/centre d'investigation clinique; PWV, pulse wave velocity; SBP, systolic blood pressure; TAG, tri  
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51 532 acyl glycerol; TNF $\alpha$ , tumor necrosis factor alpha; TSH, thyroid stimulating hormone; VCAM, vascular cell  
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53 533 adhesion molecule.  
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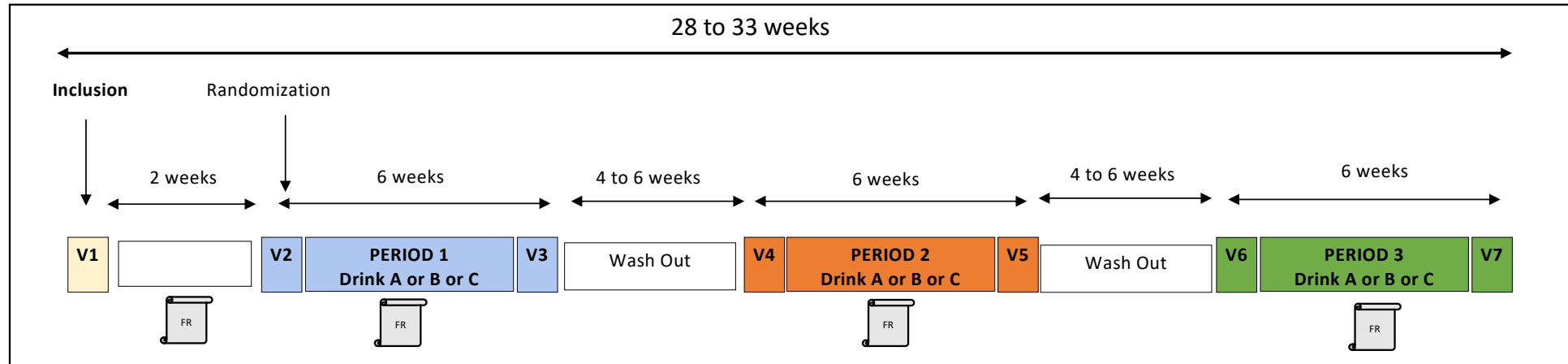


Figure 1



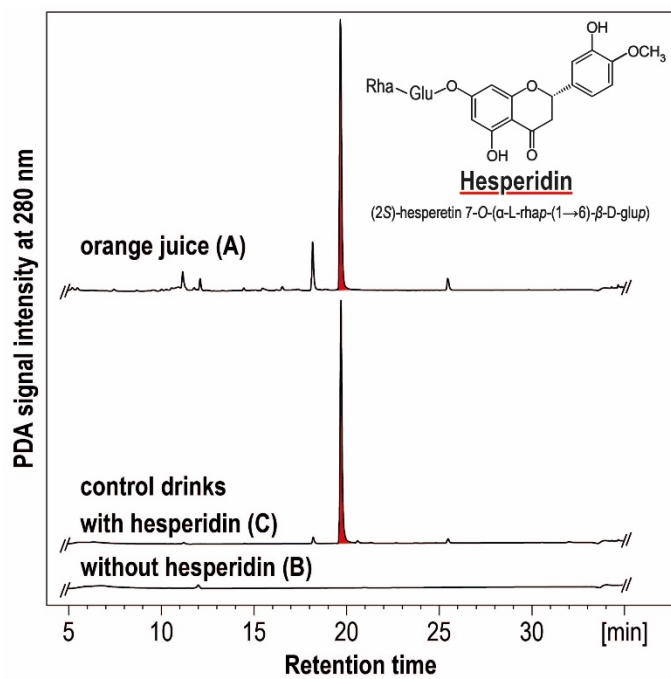


Figure 2.

		Eligibility assessment	Pre-experimental period					Experimental period					
		Visit 1	At home					Visit 2, 4, 6			At Home		Visit 3, 5, 7
<b>Procedures</b>		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
<b>PIC/CIC visit</b>		X						X					X
Consent form signature, medical examination, dietician interview		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	
<b>24h urine collection</b>								X					X
<b>Feces collection</b>								X					X
<b>Randomisation</b>								x (visit 2)					
<b>Blood sampling</b>		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					x
hsCRP		x						x					x
I-CAM, V-CAM, e-selectin, IL6, TNF $\alpha$								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
<b>Vascular function assessment</b>								x					x
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

1  
2  
3 **CONSENT FORM TO PARTICIPATE IN RESEARCH INVOLVING HUMAN VOLUNTEERS**  
4  
5  
6

7 **HESPER-HEALTH**

8  
9 **Orange Juice and Hesperidin - Their Vascular Health Benefits: A Randomized Controlled Crossover**  
10 **Human Study**  
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12

13  
14 **Principal investigator**

15 **Professor Gisèle Pickering** Plateforme d'investigation Clinique / Centre d'Investigation Clinique Inserm  
16 1405, Centre Hospitalier Universitaire de Clermont-Ferrand, 58 Rue Montalembert, 63000 Clermont-  
17 Ferrand  
18

19 I undersigned

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

21 Born \_\_\_ / \_\_\_ / \_\_\_\_\_

22 Address .....

23 Declare that the Doctor (name, first name, telephone) ..... ..

24  
25 offered to participate in the aforementioned study; he explained me the protocol and detailed in  
26 particular:

- 27  
28  
29  
30  
31  
32  
33 - the objective, the method, and the duration of the study  
34  
35 - the constraints and potential risks incurred  
36  
37 - my right to refuse to participate and to withdraw my consent at any time without having to  
38 justify myself  
39  
40 - my obligation to register to French social security  
41  
42 - that, if I wish I would be informed by the investigating doctor at the end of the protocol of  
43 the overall results  
44  
45 - that an exclusion period of 7 days is defined in this protocol  
46  
47 - that the South-East III Committee for the Protection of Persons (CPP) issued a favorable  
48 opinion on February 2, 2021.  
49  
50 - that the promotor, the Clermont-Ferrand University Hospital, took out insurance covering  
51 this research.  
52  
53 - that I am not placed under judicial protection,  
54  
55 - that I must have sufficient time before signing this consent,  
56  
57 - that it was clear to me that I could oppose the conservation of my samples and their re-use  
58 for medical or scientific research programs aimed to improve scientific knowledge:  
59  
60

- 1  
2  
3  I agree with the fact that the biological samples taken from me being stored and used  
4 for research purposes.  
5  I am opposed with the fact that the biological samples taken from me being kept and  
6 used for research purposes.  
7  
8  
9

10 Study information collected by the investigator is treated confidentially. I accept that this data  
11 may be subject to anonymous computer processing. I have noted that the right of access provided for  
12 by the law of August 6, 2004 relating to data processing, files and freedoms is exercised at any time  
13 with the doctor who follows me in the context of research and who know my identity. I can exercise  
14 my right of rectification and opposition with this same doctor, who will contact the research sponsor.  
15 After having freely discussed and obtained answers to all my questions, I freely and voluntarily agree  
16 to participate in this research involving the human person under the conditions specified in the  
17 information and consent form.  
18  
19  
20  
21  
22  
23  
24

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

26 Date: \_\_/\_\_/\_\_\_\_

27  
28 Signature preceded by the words "Read and understood":  
29  
30  
31  
32  
33  
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35  
36  
37  
38  
39

40 Name of the investigator: .....

41 Date: \_\_/\_\_/\_\_\_\_

42  
43 Signature:  
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58 This document must be produced in 2 copies, the original of which must be kept by the  
59 investigator, the first copy must be given to the person giving his consent.  
60

## 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
<b>Introduction</b>			
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
<b>Methods: Participants, interventions, and outcomes</b>			
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
<b>Methods: Assignment of interventions (for controlled trials)</b>			
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
<b>Methods: Data collection, management, and analysis</b>			

1 2 3 4 5 6 7 8 9 10 11 12 13 14	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
15 16 17 18 19 20		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
21 22 23 24 25 26 27 28 29	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
30 31 32 33 34 35	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
36 37 38		20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	L414-418
39 40 41 42 43 44		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
45	<b>Methods: Monitoring</b>			
46 47 48 49 50 51 52 53 54 55 56 57 58 59 60	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
<b>Ethics and dissemination</b>			
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
<b>Appendices</b>			
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