PEER REVIEW HISTORY

ARTICLE DETAILS

TITLE (PROVISIONAL)	Study protocol for a randomized controlled trial evaluating the role of Orange juice, HESPERidin in vascular HEALTH benefits: The HESPER-HEALTH Study
AUTHORS	Verny, Marie-Anne; Milenkovic, Dragan; MACIAN, Nicolas; Pereira, Bruno; Evrard, Rémy; Gilcher, Caroline; Steingass, Christof B.; Mosoni, Pascale; Gladine, Cécile; Monfoulet, Laurent-Emmanuel; Schweiggert, Ralf; PICKERING, Gisèle; MORAND, Christine

VERSION 1 – REVIEW

REVIEWER	Tomas-Barberan, Francisco CEBAS CSIC
REVIEW RETURNED	04-Jul-2021

GENERAL COMMENTS	This is an interesting study that needs to be done. This was one of the coclusions of the COST action POSITIVein which part of the authors participated. The study is in general very well planed with enough description to be repeated, except for some relevant aspects regarding the juices and beverages used for the intervention. It would be desirable to have a clare description of the orange flavanones in the juice in both phases; soluble phase and unsoluble phase, as this can be a key factor for the interaction with intestinal microbiota and, finally, with their bioavailability and metabolism and the effects on cardiometabolic health biomarkers. This has been highlighted in refeernce 14 of this manuscript. This analysis of flavanones in supernatant and pellet should be done in the commercial juice and in the drink C in which hesperidin is added to the placebo drink in order to know the degree of solubility of hesperdin in both beverages. Another point is regardig the analysis of flavanones in plasma and urine. These analysed will be done after enzymatic hydrolysis with glucuronidase and sulphatase. It is a pity that the original conjugates are not analysed as these can also differ between individuals This ia probably a limitation of the study that should be recognized. Minor corrections. line 84 correct interractions line 113 correct glucopyranoosyl line 153 'or a control drinks' chenge to drink line 178 please provide more details about the way hesperidin is present in the juice (soluble/insoluble) and more details about the processing ofthe juice in the industry (pasteurization a which temperature and time?; or any other treatments).

	Line 181 hesperidin in the soluble and pellet phase should be measured in beverage C the same way as in the juice A Line 313 pleae provide some more detailsabot the LC-MS methods used. Even if they are mentioned in a previous publication with a reference, they should be briefly presented in the present paper. The same for the oxylipin analysis LC-MS-MS. line 423, not only richer. Orange juices can be processed for more soluble and therefore bioavailable flavanones (see ref. 14). Quantity is not the only factor affecting flavanone bioavailability.

REVIEWER	Radavelli-Bagatini, Simone Edith Cowan University, Medical and Health Science
REVIEW RETURNED	09-Aug-2021

GENERAL COMMENTS	Dear authors, It was a pleasure to review your manuscript. Your study protocol is interesting and relevant. However, I would like to suggest rewording some paragraphs to improve flow. I also think the methodology and design of the study require clarification. I have attached a copy of the manuscript with some comments and suggestions enclosed, as well as a list for the suggested changes. I hope this helps improve your manuscript. Thank you for the opportunity to review this work. Kind regards, Simone BMJ Open bmjopen-2021-053321 - Study protocol for a randomized controlled trial evaluating the role of Orange juice, HESPERidin in vascular HEALTH benefits: The HESPER-HEALTH Study Dear editor, Thank you for the opportunity to review this manuscript. The manuscript by Marie-Anne Verny and colleagues titled “Study protocol for a randomized controlled trial evaluating the role of Orange juice, HESPERidin in vascular HEALTH benefits: The HESPER-HEALTH Study” has been designed to investigate the vasculo-protective effects of orange juice (OJ). Potential mechanisms could be linked to hesperidin, flavanones with antioxidant and anti-inflammatory properties, found in oranges. This is a well written study protocol, and their research question is relevant and of interest. I have added some main queries and a few other minor comments below. Major 1. In the Methods, I missed seeing a section on self-reported information, such as the use of food questionnaire to assess food intake, or which instruments will be used to assess physical activity, if any. Still in relation to food questionnaire, which one is being used and has it been validated? 2. Is there a process evaluation plan for this study? Please see below some minor comments to be considered. Minor Is this an ongoing study? I have not found any dates reporting the plan of the study (i.e., recruitment start date) The use of 2 columns for page line numbers is a bit confusing Line 61: Further down the paper you have also included "BMI" (Line 155)

	Line 68: A sentence about the statistics would be helpful Line 139: “usingsensitive” need a space Line 145: You have used past tense in this sentence. Has data collection for this RCT finished? Line 155: In the abstract (line 61) you say "based on age and waist circumference" only – no mentioned about BMI Lines 176-177: Overweight based on BMI? Or do you mean with increased WC? Line 211: Are these validated questionnaires? Are they FFQs? Could you please include a reference or, if not validated, a brief explanation? Line 224: I suggest presenting a complete comparative nutritional content - how many calories do the drinks add to the diet, and the amount of other constituents? Lines 231-232: Presenting the full nutritional content of all beverages would be very helpful Line 244: How will the bottles be distributed (15L per intervention)? How many bottles will participants take home and in which visits? A brief explanation would improve clarity. Lines 270-272: Suggest rewording the whole sentence Line 276: Please add reference Line 286: You probably need a subheading for Study assessments here Line 289: Please provide make and other details of equipment used Line 300: Please provide make and other details of equipment used Line 303: Please provide make and other details of equipment used Line 307: Please provide make and other details of equipment used Line 319: Please spell out acronyms at first mention (i.e., TAG, ICAM, VCAM) Line 387: Have you considered also adjusting for energy intake and physical activity? Line 394: Please specify if this is verbally or detailed in a written participant information form Lines 394-400: Suggest rewording this paragraph Lines 402-408: Suggest rewording this paragraph Line 416: Full stop Lines 428: Suggest rewording Line 436: No need for a new paragraph. Please combine with paragraph above Line 450: Funding – “financial contribution from a” could be removed Table 1: In table 1, having a line with headings for "inclusion" and "exclusion" criteria would improve clarity Table 1 (line 9): I believe this is to avoid the impact of estrogen on FMD? However, women recruited will likely be older than men. Could you have assessed endothelial function avoiding the ovulation period? Table 1 (line 39): How will exercise be estimated? How will compliance be ensured? I don't think this has been included in the methods. Table 2: Table should have a label with all abbreviations, so reader don't need to go back to the text for acronyms Table 2: The headings for "visits" is confusing (first line). It is not clear which days belong to each visits Table 2 (line 12): Is this measured at visit 1? Table 2 (line 13): No food questionnaire in any of the last 3 visits?

	Table 2 (line 14): Sorry, can you please clarify what this is? Table 2 (line 18): Is this correct that some blood samples have repeated measures, and others don't? Table 2 (lines 30-31): I believe you need a "x" for these at baseline Figure legend (Figure 1): “3 Day food questionnaire” - Is this a food "frequency" questionnaire? Figure 1 (page 29): Figure 1 is very clear. I would suggest listing all the assessments (in addition to the FQ) to the figure, as it is a great way to visualise the whole study (including all study assessments)

VERSION 1 – AUTHOR RESPONSE

Reviewer: 1

Francisco Tomas-Barberan, CEBAS CSIC

Comments to the Author:
This is an interesting study that needs to be done. This was one of the coclusions of the COST action POSITIVein which part of the authors participated.
The study is in general very well planed with enough description to be repeated, except for some relevant aspects regarding the juices and beverages used for the intervention. It would be desirable to have a clare description of the orange flavanones in the juice in both phases; soluble phase and unsoluble phase, as this can be a key factor for the interaction with intestinal microbiota and, finally, with their bioavailability and metabolism and the effects on cardiometabolic health biomarkers. This has been highlighted in refeernce 14 of this manuscript. This analysis of flavanones in supernatant and pellet should be done in the commercial juice and in the drink C in which hesperidin is added to the placebo drink in order to know the degree of solubility of hesperdin in both beverages.
We fully agree with the reviewer. Please note that we had not yet included all analytical details regarding the characterization of the test beverages due to word count limitations. A thorough characterization of the beverages has definitely been planned, including measurements of potassium, dietary fiber, carotenoids as well as the mentioned soluble and insoluble fractions of total hesperidin
To follow the reviewer’s suggestion and to clarify that the beverages will be characterized in detail, we have added the following information to the manuscript in Lines 254-256: *“… at room temperature or cooler. The produced beverages will be analytically characterized in detail, including, e.g., the levels of potassium [(27))], carotenoids (Aschoff et al. [(28)]), and soluble, insoluble (Vallejo et al. [(14)]) and total hesperidin ([(29)]. …”*

Another point is regardig the analysis of flavanones in plasma and urine. These analysed will be done after enzymatic hydrolysis with glucuronidase and sulphatase. It is a pity that the original conjugates are not analysed as these can also differ between individuals This ia probably a limitation of the study that should be recognized.
We fully agree that analyzing the original conjugates is important and we had actually planned to do so. We thank the reviewer for pointing out that the current reference, which we had cited for the method, did only include analyses of the deconjugated forms, while the text has been missing the reference for the conjugated forms (Mullen et al. [(33)]).
Therefore, we have now changed the manuscript in Lines 334-340, as outlined in detail in our response to the comment referring to "Line 313" below.

Minor corrections. line 82 correct
interractions line 110 correct
glucopyranosyl
line 153 'or a control drinks' change to drink
These typographic errors have been corrected in the manuscript

line 178 please provide more details about the way hesperidin is present in the juice (soluble/insoluble) and more details about the processing of the juice in the industry (pasteurization a which temperature and time?; or any other treatments).
Line 181 hesperidin in the soluble and pellet phase should be measured in beverage C the same way as in the juice A
As mentioned above, we fully agree and will measure soluble, insoluble and total hesperidin according to Vallejo et al. [14] and IFU, 2005 [29], respectively. The results will be presented alongside the results of the study. Please note that our juice processing will follow commercial practice as mentioned already in Lines 229, 244. More processing details will be reported when the results of the trial will be published.

Line 313 please provide some more details about the LC-MS methods used. Even if they are mentioned in a previous publication with a reference, they should be briefly presented in the present paper.
The same for the oxylipin analysis LC-MS-MS.
In order to meet the reviewer request and not to exceed too much the number of words recommended by the journal for a protocol paper, some highlights are provided about these methods in the revised manuscript. However, all the methodologies will be described in details in future papers which will present the results of the study.
Regarding flavanone analyses by LC-MS, we have now provided a more detailed description in the revised manuscript in Lines 336-340.
Regarding oxylipin profiling, the paragraph has been completed with more details on methods (Lines 387-394).

line 423, not only richer. Orange juices can be processed for more soluble and therefore bioavailable flavanones (see ref. 14). Quantity is not the only factor affecting flavanone bioavailability.
We are aware of this fact and will measure soluble and insoluble hesperidin as mentioned above. However, we would like to refrain from too much discussion about the difference between soluble and insoluble hesperidin in this paper on the study protocol, as it might mislead the readers, because we do not vary the ratios of soluble to insoluble hesperidin in differently processed orange juices. We use a commercial batch of orange juice from concentrate. Nevertheless, in the criticized former line 423, we would like to make the following change to highlight that we are aware of the point raised by the reviewer: Line 479: *"…fruit juice richer in flavanones…" was changed to "…fruit juice richer in readily bioavailable flavanones…"*

REVIEWER 2

Dr. Simone Radavelli-Bagatini, Edith Cowan University, Medical Research Foundation (MRF)

Dear editor,
Thank you for the opportunity to review this manuscript.
The manuscript by Marie-Anne Verny and colleagues titled "Study protocol for a randomized controlled trial evaluating the role of Orange juice, HESPERidin in vascular HEALTH benefits: The HESPER-HEALTH Study" has been designed to investigate the vasculo-protective effects of orange juice (OJ). Potential mechanisms could be linked to hesperidin, flavanones with antioxidant and anti-inflammatory properties, found in oranges.
This is a well written study protocol, and their research question is relevant and of interest. I have added some main queries and a few other minor comments below.

Comments to the Author:
Dear authors,
It was a pleasure to review your manuscript. Your study protocol is interesting and relevant. However, I would like to suggest rewording some paragraphs to improve flow. I also think the methodology and design of the study require clarification. I have attached a copy of the manuscript with some

comments and suggestions enclosed, as well as a list for the suggested changes. I hope this helps improve your manuscript.
Thank you for the opportunity to review this work.
Kind regards,
Simone

Major

1. In the Methods, I missed seeing a section on self-reported information, such as the use of food questionnaire to assess food intake, or which instruments will be used to assess physical activity, if any. Still in relation to food questionnaire, which one is being used and has it been validated

At the inclusion visit (V1), each participant meets a dietician who provides them instructions to record their food intakes over three consecutive days and to follow the dietary recommendations (forbidden foods, limitation of polyphenol rich food intake) and the special dietary guidelines for the 48h period preceding each experimental visit (V2-V7)). At the end of the protocol, the food records will be analyzed by the dietician to evaluate nutrients and energy intakes. This information has been included in the revised manuscript (Lines 212-213). The level of physical activity is only assessed at inclusion by the medical investigator on the basis of the declarative (Table1). The objective is not to include subjects with an intense and regular physical activity (>6H/week). During the study, the physical activity is no longer followed.
Last, as indicated Lines 259-261, volunteers have also to make self-report to further assess compliance.

2. Is there a process evaluation plan for this study?

There is no process evaluation specific to this trial. However, the investigator team follows a set of quality indicators, common to all studies, in line with Good Clinical Practice.

Please see below some minor comments to be considered.
Minor
Is this an ongoing study? I have not found any dates reporting the plan of the study (i.e., recruitment start date)
The first inclusion was on February 24th,2021. This information has been added in the revised manuscript, in the approval subsection that has been more detailed (Lines 424-431).

The use of 2 columns for page line numbers is a bit confusing
We are sorry but this double numbering results from the conversion at the submission step of the word file of the paper by the journal website.

Line 61: Further down the paper you have also included "BMI" (Line 155)
This has been corrected

Line 68: A sentence about the statistics would be helpful
The statistics are described in a special section the manuscript and it does not seem us essential to talk about statistics in the abstract which already has 300 words.

Line 145: You have used past tense in this sentence. Has data collection for this RCT finished?
To avoid any confusion, the paragraph has been modified (Lines 141-145).
"Based on this state of the art, the present human randomized, controlled, double-blind, crossover intervention is conducted on subjects predisposed to CVD and it aims to establish a cause-and-effect relationship between hesperidin intake and the vascular protective effects of…".

Line 155: In the abstract (line 61) you say "based on age and waist circumference" only – no mentioned about BMI
Lines 176-177: Overweight based on BMI? Or do you mean with increased WC?
In this study, we focus on subjects with a predisposition to develop cardiovascular diseases (World Health Organization. 2000; Obesity: Preventing and Managing the Global Epidemic: Report on a WHO Consultation). For that we will work on a middle-aged population (40-65), overweight, i.e. presenting an

excess of abdominal fat (waist circumference ≥80 cm for women, and ≥94 cm for men, associated with an increased risk to develop cardiovascular diseases) and with a BMI≤30. This criteria has been clarified in the manuscript (Line 152, 175).

Line 211: Are these validated questionnaires? Are they FFQs? Could you please include a reference or, if not validated, a brief explanation?
We don't use FFQ but as explained above, we perform dietary records over three consecutive days. This has been clarified in the text (Lines 212-213).
These dietary records will allow the dietician to control the non-consumption of forbidden foods (citrus), the observance of the limit in polyphenols rich food intakes and will be used to estimate the nutrients (lipids, carbohydrates, proteins) and energy intakes.

Line 224: I suggest presenting a complete comparative nutritional content - how many calories do the drinks add to the diet, and the amount of other constituents?
Lines 231-232: Presenting the full nutritional content of all beverages would be very helpful Although we agree that a complete nutritional content will be helpful for interpreting the results of the study, we do not agree with the reviewer's suggestion to provide a full characterization table already at this stage, also because part of the analyses (e.g., dietary fiber, carotenoids etc.) have not yet been finished. However, we believe that the information provided should suffice for the readers at this stage to understand the concept of the study. Please also note that the used orange juice will be a commercial batch, i.e. also having a composition identical or highly similar to what is found in the markets. In lines 234 and 239, we also have provided quantities of hesperidin and sugar, which are the most relevant information about the study beverages and also allow any reader to estimate the caloric value of the juice and the other study beverages.

Line 244: How will the bottles be distributed (15L per intervention)? How many bottles will participants take home and in which visits? A brief explanation would improve clarity. Beverages will be distributed to volunteers at the first visit of each experimental period (V2, V4, V6) in bags containing 48 bottles (45 + 3 additional in case of need (breakage, loss etc.) per period. This information has been added in the manuscript (Lines 252-253).

Lines 270-272: Suggest rewording the whole sentence
The sentence has been reworded accordingly (Lines 281-284).

Line 276: Please add reference
The sentence has been completed and 2 references added in the revised manuscript (Lines 287-288).

Line 286: You probably need a subheading for Study assessments here
Line 289: Please provide make and other details of equipment used
Line 300: Please provide make and other details of equipment used
Line 303: Please provide make and other details of equipment used
Line 307: Please provide make and other details of equipment used
Line 319: Please spell out acronyms at first mention (i.e., TAG, ICAM, VCAM)
All these comments have been considered in the revised manuscript

Line 387: Have you considered also adjusting for energy intake and physical activity? The physical activity is not assessed during the protocol. Additional adjustments could be foreseen, energy intake could be one of them.

Line 394: Please specify if this is verbally or detailed in a written participant information form. Lines 394-400: Suggest rewording this paragraph
The consent paragraph (Lines 434-438) has been reworded as follows:
"Subjects will be informed by a fair and accessible form approved by the ethic committee. Subjects will be free to ask any question on all aspects of the study before giving the consent and informed that they are free to withdraw from the study at any time. The investigator will ensure that the written consent obtained from subjects prior to their participation in the study is free and informed."

Lines 402-408: Suggest rewording this paragraph
The section data management has been largely reworded and completed (Lines 441-449).

Line 416: Full stop
Lines 428: Suggest rewording
Line 436: No need for a new paragraph. Please combine with paragraph above Line 450:
Funding – “financial contribution from a” could be removed
All these remarks have been taken into account in the revised manuscript.

Table 1: In table 1, having a line with headings for "inclusion" and "exclusion" criteria would improve clarity
The table 1 has been modified accordingly.

Table 1 (line 9): I believe this is to avoid the impact of estrogen on FMD? However, women recruited will likely be older than men. Could you have assessed endothelial function avoiding the ovulation period?
Our objective was to recruit “healthy” men and women presenting the same level of risk of developing CVD. It is established that after menopausal, the risk for women to develop CVD met that of men, so we enroll menopausal women.

Table 1 (line 39): How will exercise be estimated?
exclusion criteria of declarative strenuous exercise : >6h/week.

How will compliance be ensured? I don't think this has been included in the methods.
A sentence has been added in the revised manuscript(Lines 259-261) :
“Participants will be asked to report daily on diaries their consumption of the study beverages and to return the dairies and the empty and non-consumed products at the end of each period (V3, V5, V7)”.

Table 2: Table should have a label with all abbreviations, so reader don't need to go back to the text for acronyms
The abbreviations have been detailed at the end of the table
Table 2: The headings for "visits" is confusing (first line). It is not clear which days belong to each visits
Table 2 (line 12): Is this measured at visit 1?
Table 2 (line 13): No food questionnaire in any of the last 3 visits?
Table 2 (line 14): Sorry, can you please clarify what this is?
Table 2 (line 18): Is this correct that some blood samples have repeated measures, and others don't?
The Table 2 has been revised to be more understandable

Table 2 (lines 30-31): I believe you need a "x" for these at baseline :
As this a randomized cross over study, this design will allow us to have a situation where each volunteer is his own control. As published previously by ours or by other groups (https://doi.org/10.1093/ajcn/86.5.1369), the analysis of the expression of genes in blood sampled at the end of each experimental period is appropriate to identify changes in gene expression between the dietary treatments.

Figure legend (Figure 1): “3 Day food questionnaire” - Is this a food "frequency" questionnaire?
As mentioned above, it is not a FFQ but a dietary record on 3 consecutive days. This has been modified in the figure 1 of the revised manuscript

Figure 1 (page 29): Figure 1 is very clear. I would suggest listing all the assessments (in addition to the FQ) to the figure, as it is a great way to visualise the whole study (including all study assessments)
All the study assessments are detailed in the text. A figure including all the measures performed will be presented in a next publication presenting the study results.

VERSION 2 – REVIEW

REVIEWER	Radavelli-Bagatini, Simone Edith Cowan University, Medical and Health Science
REVIEW RETURNED	22-Oct-2021

GENERAL COMMENTS	Dear authors, Thank you for the clarifications provided. This is a very interesting and relevant study protocol. All the best