PEER REVIEW HISTORY

ARTICLE DETAILS

TITLE (PROVISIONAL)	Gut microbiota composition and arterial stiffness measured by pulse wave velocity. Case-control study protocol. (MIVAS study)
AUTHORS	salvado, rita; santos-minguez, sandra; Agudo-Conde, Cristina; Lugones-Sanchez, Cristina; Cabo-Laso, Angela; Mª Hernandez-Sanchez, Jesus; Benito, Rocio; Rodriguez-Sanchez, Emiliano; Gomez-Marcos, Manuel; Hernandez-Rivas, Jesus; Guimarães Cunha, Pedro; Garcia-Ortiz, Luis; Investigators, MIVAS

VERSION 1 – REVIEW

REVIEWER	JOSE GERALDO MILL Universidade Federal do Espírito Santo, Brazil
REVIEW RETURNED	22-Jul-2020

GENERAL COMMENTS	Congratulations for this research proposal. The only suggestion is related to the cut off of 10 m/s for pulse wave velocity (PWV). If this fixed cut off is used then the groups will show very different ages as PWV steadily increases at a rate of 1/m/s per decade in healthy subjects. I agree that the ESC established this cut off a decade ago. However, if 10 m/s indicates a higher value in a normotensive subject of 35 y, this is a 'normal' value for other normotensive subject with 70 y.Also, women show a 1m/s less PWV for the same age of men. I suggest to separate groups according to reference values adjusted for sex and age (Int J Cardiol 2018, Baldo MP et al). The fixed value to dicotomize the sample will be an important limitation of the study.All analysis should be adjusted to age and sex.

REVIEWER	Philip Wenzel, MD Department of Cardiology University Medical Center Mainz, Germany
REVIEW RETURNED	14-Oct-2020

GENERAL COMMENTS	In the manuscript "Gut microbiota composition and arterial stiffness measured by pulse wave velocity. Case-control study protocol. (MIVAS study)" Salvado and coworkers propose a mulicenter observational study to investigate the relationship between composition of gut microbiota and vascular stiffness. The topic is timely and important, since the role of novel cardiovascular risk factors is more and more appreciated. - Please reassess inclusion criteria. Hypertension and BMI are among the strongest confounders of vascular stiffness. The authors need to better control for these confounders by patient selection. otherwise, the planned number of 324 enrolled subjects appears far too low to allow any reliable conclusions on the

	relationship between gut microbiota composition and vascular stiffness. - please employ more parameters than just pulsewave velocity to assess vascular stiffness. Minor comments: - I am not sure whether the statement "At least 5 publications in first quartile scientific journals are planned." on pg 5 line 11 is necessary - The introduction is too long. Breaking down the introduction into several subunits by introducing sub-headings is very uncommon. Please refine and shorten signifcantly; you may want to move aspects of background information etc into the discussion if needed

VERSION 1 – AUTHOR RESPONSE

Reviewer: 1
Reviewer Name: JOSE GERALDO MILL
Institution and Country: Universidade Federal do Espírito Santo, Brazil
Please state any competing interests or state ‘None declared’: No competing interest to declare
Comments to the Author
Congratulations for this research proposal.

1.- The only suggestion is related to the cut off of 10 m/s for pulse wave velocity (PWV). If this fixed cut off is used then the groups will show very different ages as PWV steadily increases at a rate of 1/m/s per decade in healthy subjects. I agree that the ESC established this cut off a decade ago. However, if 10 m/s indicates a higher value in a normotensive subject of 35 y, this is a 'normal' value for other normotensive subject with 70 y. Also, women show a 1m/s less PWV for the same age of men. I suggest to separate groups according to reference values adjusted for sex and age (Int J Cardiol 2018, Baldo MP et al).

RESPONSE TO THE REVIEWER
We agree with the reviewer that PWV cut-off point of 10m/s, established by the ESC, has some limitations, even more so now that we have population reference values.
In addition to the document provided by the reviewer [1], we have also available cf-PWV values for the European reference population [2] and for the Spanish population, specifically from Salamanca [3], one of the towns where the study will be carried out. For this reason, we will analyse cf-PWV considering a cut-off point correspondent to the 90th percentile, for age and sex.
We have modified the text, taking into consideration the recommendations of both reviewers, resulting as follows in the Abstract; Strengths and limitations; and Design section (page 6):
Cases will be defined by the presence of at least one of the following: cf-PWV, CAVI or ba-PWV above the 90th percentile, for age and sex, of the reference population.

We have also adapted figure 1 to incorporate these changes.

2.- The fixed value to dicotomize the sample will be an important limitation of the study. All analysis should be adjusted to age and sex.

RESPONSE TO THE REVIEWER
The option of dichotomizing the value of cf-PWV was made to set a criteria in the definition of the case. However, we agree with the reviewer that the analysis with continuous variables offers greater power, therefore, in the statistical analysis section, the multiple regression analysis is described as a tool to analyse the associations between independent and dependent variables, quantitatively. All analyses will be adjusted for age, sex, and other confounding variables, such as hypertension, BMI and drugs that can influence the relationship between the microbiota and arterial stiffness.

In the statistical analysis we have qualified these aspects, as detailed below: (page 15, line 14-15)
Logistic regression will be performed to evaluate the association between the study factor (gut microbial diversity) and the dependent variable (arterial stiffness), adjusted for possible confounding variables (sex, age, BMI, hypertension). A multiple linear regression will also be performed to analyse the relationship of the study factor (gut microbiota) with the variables that analyse vascular structure and function quantitatively. This regression, and all the others multivariate analyses performed, will be adjusted for the same confounding variables as the logistic regression.

Reviewer: 2
Reviewer Name: Philip Wenzel, MD
Institution and Country: Department of Cardiology, University Medical Center Mainz, Germany
Please state any competing interests or state ‘None declared’: None declared
Comments to the Author
In the manuscript "Gut microbiota composition and arterial stiffness measured by pulse wave velocity. Case-control study protocol. (MIVAS study)" Salvado and coworkers propose
a mulicenter observational study to investigate the relationship between composition of gut microbiota and vascular stiffness. The topic is timely and important, since the role of novel cardiovascular risk factors is more and more appreciated.
1.-Please reassess inclusion criteria. Hypertension and BMI are among the strongest confounders of vascular stiffness. The authors need to better control for these confounders by patient selection.
RESPONSE TO THE REVIEWER
As the reviewer indicates, there are a number of factors that can influence both arterial stiffness and microbiota composition. All of them will be taken into account in the statistical analysis. We have already excluded patients with diabetes, we have been considering the exclusion of patients with hypertension and obesity, but the prevalence of these in the population is too high, and the prevalence of arterial stiffness would not permit the achievement of a sufficient number of subjects considered as cases.
However, all confounding variables will be controlled in the analysis and we consider that the use of percentiles for age and sex can partially minimize the possible bias of hypertension and obesity.
We have modified the statistical analysis section to incorporate these recommendations, resulting as follows:(page 16)
Logistic regression will be performed to evaluate the association between the study factor (gut microbial diversity) and the dependent variable (arterial stiffness), adjusted for possible confounding variables (sex, age, BMI, hypertension). A multiple linear regression will also be performed to analyse the relationship of the study factor (gut microbiota) with the variables that analyse vascular structure and function quantitatively. This regression, and all the others multivariate analyses performed, will be adjusted for the same confounding variables as the logistic regression
2.-Otherwise, the planned number of 324 enrolled subjects appears far too low to allow any reliable conclusions on the relationship between gut microbiota composition and vascular stiffness.
RESPONSE TO THE REVIEWER
The size of the sample, as the reviewer comments, is important to obtain a sufficient statistical power of the study and to be able to test the hypotheses raised. Though, the economic limitations are important, as this is an expensive project, mainly due to the costs of the microbiota analysis.

However, things have changed since March, when the protocol was written and at this moment, we have the potential to expand the sample size to eventually include 500 subjects. Therefore, we made the appropriate changes:

2.1.Abstract, methods section:

The study will be developed in Primary Health Care Centers. We will select 500 subjects (250 cases and 250 controls), between 45 and 74 years of age.

2.2.Strengths and limitations, point 2:

- 250 cases defined by the presence of at least one of the following: cf-PWV, CAVI or ba-PWV above the 90th percentile, for age and sex, of the reference population. Cases will be matched with 250 controls using propensity score.

2.3.The sample size paragraph would read as follows (page 7):

The sample size was estimated to detect a minimum odds ratio of 1.75 in the study factor (microbiota dysbiosis), considering vascular stiffness as a dependent variable and accepting an α risk of 0.05 and a β risk of 0.20, in a two-sided test, assuming a rate of losses due to technical difficulties or refusal to participate of 5%, and a rate of exposure of 0.3%, in the control group. Therefore, it will be necessary to include 500 subjects: 250 patients with arterial stiffness and 250 controls.

3.-Please employ more parameters than just pulse wave velocity to assess vascular stiffness.

RESPONSE TO THE REVIEWER

In this Project, in addition to the cf-PWV, other parameters are determined, such as the Central and peripheral Ax, using the Sphygmocor system. CAVI and ba-PWV will be determined using the Vasera device. Central and peripheral augmentation index will be measured with the wrist-worn device (Microsoft®). Therefore, we have considered using other additional parameters, as recommended by the reviewer, and we have modified the case definition to consider subjects that have at least one of the following: cf-PWV, CAVI or ba-PWV above the 90th percentile of the reference population for age and sex.

We have made the following modifications:

3.1. We have added the following paragraph to the introduction (page 4, at the end of the second paragraph):

There are several methods to measure arterial stiffness. Carotid to femoral pulse wave velocity (cf-PWV) is the gold standard,[1] others widely accepted are: CAVI, which measures the stiffness of the aorta, femoral artery and tibial artery; [2] ba-PWV, which uses brachial and tibial arterial waves. [3]The evaluation of carotid Intima–media thickness (IMT) can identify the presence of atherosclerotic plaques which traduces structural damage on the artery.

3.2. We have modified the last sentence of the section Carotid-femoral pulse wave velocity (cf-PWV) and Central Augmentation Index (CAIx) (page 13) that reads as follows:

Subclinical organ damage will be defined as cf-PWV, above the 90th percentile, for age and sex, of the reference population. [4]

3.3.We have also modified the last paragraph of the section Cardio-ankle Vascular Index (CAVI), brachial ankle PWV (ba-PWV) and Ankle Brachial Index (ABI) (page 13), as follows:

CAVI will be classified as: normal (CAVI <8), borderline (8≤CAVI <9) and abnormal (CAVI≥9) [5]. Subclinical organ damage will be defined as, CAVI or ba-PWV above the 90th percentile for age and sex of the reference population [4]. ABI≤0.9 will be considered abnormal. [6]

3.4.We have modified the text, considering the recommendations of both reviewers, in the Abstract, Strengths and limitations and Design section (page 6), that reads as follows:

Cases will be defined by the presence of at least one of the following: cf-PWV, CAVI or ba-PWV above the 90th percentile, for age and sex, of the reference population.

3.5. We have also modified figure 1 to incorporate these changes.

4.- Minor comments:
- I am not sure whether the statement "At least 5 publications in first quartile scientific journals are planned." on pg 5 line 11 is necessary

RESPONSE TO THE REVIEWER

We have deleted the phrase that the reviewer refers to, as we agree that it is not necessary.

5.- The introduction is too long. Breaking down the introduction into several subunits by introducing sub-headings is very uncommon. Please refine and shorten significantly; you may want to move aspects of background information etc into the discussion if needed

RESPONSE TO THE REVIEWER

We have shortened the introduction substantially, and removed the subtitles, leaving it as follows: (pages 4-6)
Cardiovascular diseases (CVD) are the leading cause of morbidity and mortality globally.[7] In the latest years, a big effort has been made to improve the identification of individuals at high risk of suffering a cardiovascular event, looking beyond classical risk factors, using biomarkers that reflect early functional or morphological changes, before overt disease manifests, allowing timely treatment of subclinical disease.[6]

Arterial stiffness has been proven to have a good predictive value for CVD,[8] in various populations, with different levels of risk: general population, elderly, patients with type 2 diabetes, hypertension or end stage renal disease.[9] Arterial stiffness reflects the aortic wall damage caused by several cardiovascular risk factors, over a long period of time, signaling the patients in which arterial risk factors were translated to real risk.[10] There are several methods to measure arterial stiffness. Carotid to femoral pulse wave velocity (cf-PWV) is the gold standard,[1] others widely accepted are: CAVI, which measures the stiffness of the aorta, femoral artery and tibial artery; [2] ba-PWV, which uses brachial and tibial arterial waves. [3] The evaluation of carotid Intima–media thickness (IMT) permit us to identify the presence of atherosclerotic plaques which traduces structural damage on the artery.
The major determinants of arterial stiffness are age and hypertension but gender and classical cardiovascular risk factors also play an important role.[10] Other factors, as genetic burden, systemic inflammatory diseases and gut microbiota,[11] have also been linked to pulse wave velocity. Gut microbiota composition has also been implicated on the genesis of hypertension,[12] obesity,[13] insulin resistance, metabolic syndrome[13] and type 2 diabetes.[14]
Gut microbiota is a new player in the pathophysiology of cardiovascular disease. There are 100 trillion bacteria in the human gut, with 3,3 million non-redundant genes, a hundred times the human genome, which gives human microbiome a huge metabolic potential. In adult's gut microbiota, the majority of the microbial populations belong to the bacteria domain, with approximately 90% of Bacteroidetes and Firmicutes phyla. Commensal gut microbiota has two main functions: intervenes in human immunologic response and contributes to energy harvest from no digestible starches.
The shift from a healthy microbiota toward dysbiosis mean that there is an increase in pathobionts and is likely to be triggered by environmental factors.[13] Although age and gut's genetically defined architecture are the most relevant factors influencing gut's microbiome composition[15], diet and lifestyle are likely the major causes of inter-individual variation in the composition of human gut's microbiome. There is evidence that the consumption of artificial sweeteners,[16] dietary emulsifiers,[17] a high-salt diet[18] and obesity[19] alter the gut microbiota, reduce microbial diversity and induce inflammation, whereas a diet rich in vegetables has been linked with a healthy microbial diversity[20 21].

Dysbiosis is characterized by a greater amount of pro-inflammatory species, that favor metabolic diseases development, caused by both diet-dependent and independent mechanisms. [13 22]Diet independent mechanisms are mediated by two major receptor families that detect microbes: Toll-like Receptors (TLRs) and Nod-like Receptors (NLRs), which sense the presence of intracellular microbes.[23] The activation of these receptors trigger inflammatory reactions, in the liver, white adipose tissue, brain, and other organs, and trigger metabolic diseases, such as insulin resistance.[24]

Diet dependent mechanisms result from microbial enzymatic activities. Some are beneficial, like microbial fermentation of polysaccharides, producing SCFA, and bile-acid. Others are detrimental, such as phosphatidylcholine metabolization by intestinal microorganisms, that results in the production of trimethylamine N-oxide (TMAO), which is associated with cardiovascular disease development and progression.[25-27]
Our hypothesis is that patients with arterial stiffness, have a different intestinal flora, when compared with healthy controls, ie, subject free of cardiovascular disease. We also hypothesize that gut microbiota will be different in subjects with different lifestyles, body composition, as well as with target organ damage and neurocognition.
The main objective of this study will be to analyse the relationship between intestinal microbiota composition and arterial stiffness, in a population without cardiovascular disease.
As secondary objectives we will consider the relationship of gut microbiota with other measures of vascular structure and function, end organ disease, cognition, cardiovascular risk factors, body composition and lifestyles. We will also analyse gender differences in intestinal microbiota composition and its relationship with vascular structure and function.

VERSION 2 – REVIEW

REVIEWER	JOSE GERALDO MILL Federal University of Espírito Santo, Brazil
REVIEW RETURNED	29-Dec-2020

GENERAL COMMENTS	I recommend acceptance of this article in the present form.

REVIEWER	Philip Wenzel, MD University Medical Center Mainz, Germany
REVIEW RETURNED	08-Dec-2020

GENERAL COMMENTS	The response to my comments is fine for me. The fact, that you can increase the number of participants to 500 is encouraging, since that will surely increase the power of your statistics. However, it still raises the question, as to how far a statistical power calculation was properly made; you will indeed need a lot of participants to be able to control for important and frequent confounders such as arterial hypertension. Since i am not a statistical expert, you may want to seek advice from a statistician and adopt the study aims and/or re-define the goals of the study accordingly