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Gut microbiota composition and arterial stiffness measured by pulse wave velocity. Case-control study protocol. (MIVAS study)

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Title: Gut microbiota composition and arterial stiffness measured by pulse wave velocity. Case-control study protocol. (MIVAS study)

Short title: Gut microbiota and arterial stiffness

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Article Summary

Introduction

Intestinal microbiota is arising as a new element in the physiopathology of cardiovascular diseases. A healthy microbiota includes a balanced representation of bacteria with health promotion functions (symbiotes). The aim of the study is to analyze the relationship between intestinal microbiota composition and arterial stiffness, measured by carotid-femoral pulse wave velocity (cf-PWV).

Methods and analysis

An observational case-control study will be developed. Cases will be defined by a cf-PWV>10m/s. Controls will be selected from the same population as cases. The study will be developed in a Primary Health Care Center. We will select 324 subjects (162 cases and 162 controls), between 45 and 74 years of age. Cases will be selected from a database that combines data from EVA Study (Spain) and Guimarães/Vizela Study (Portugal). Measurements: PWV will be measure using the SphygmoCor® System and gut microbiome composition in faecal samples will be determined by 16S rRNA sequencing. Lifestyle will be assessed by food frequency questionnaire, adherence to the Mediterranean diet, and IPAQ questionnaire. Body composition will be evaluated by bioimpedance; cardio-ankle vascular index and ankle-brachial index will be determined using Vasera® device and carotid intimal median thickness by ultrasonography.

Strengths and limitations

- Multicentric and multicountry study
- Observational case-control study that evaluates 324 patients between 40 and 74 years.
- Analyses both gut microbiota and arterial stiffness in humans
- 162 cases defined by PWV>10m/s determined using the SphygmoCor System, matched with 162 controls using propensity score.
- Analyses the composition of the gut microbiome in faecal samples by 16S rRNA sequencing.

Keywords: gut microbiota. arterial stiffness, pulse wave velocity, case-control study, protocol

Ethics and dissemination

The study has been approved by Committee of ethics of research with medicines of the health area of Salamanca" in 14/12/2018 (cod. 2018-11-136) and the "Ethics committee for health

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of Guimaraes" (Portugal) in 15/10/2019 (ref: 67/2019). All study participants will sign an informed consent form agreeing to participate in the study, in compliance with the declaration of Helsinki and the WHO standards for observational studies.The results of this study will allow a better description of gut microbiota in patients with arterial stiffness. At least 5 publications in first quartile scientific journals are planned.

Trial Registration Number: NCT03900338

https://clinicaltrials.gov/ct2/show/NCT03900338

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INTRODUCTION

Cardiovascular diseases (CVD) are the leading cause of morbidity and mortality globally.[1] 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.[2]

Arterial stiffness has been proven to have a good predictive value for CVD,[3] in various populations, with different levels of risk: general population, elderly, patients with type 2 diabetes, hypertension or end stage renal disease.[4] 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.[5] Carotid to femoral pulse wave velocity (cf-PWV) is the gold standard to measure arterial stiffness.[6] The major determinants of arterial stiffness are age and hypertension but gender and classical cardiovascular risk factors also play an important role.[5] Other factors, as genetic burden, systemic inflammatory diseases and gut microbiota,[7] have also been linked to pulse wave velocity. Gut microbiota composition has also been implicated on the genesis of hypertension,[8] obesity,[9] insulin resistance, metabolic syndrome[9] and type 2 diabetes.[10]

Gut microbiota definition and function

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* and the remaining from *Actinobacteria* and *Proteobacteria phyla*. In addition, *Fusobacteria* and *Verrucomicrobia* can

also be detected.[11]

Commensal gut microbiota has two main functions: intervenes in human immunologic response and contributes to energy harvest from no digestible starches. Gut microbiota competes with pathogenic microorganisms for nutrients and attachment sites, promotes epithelial cell proliferation and differentiation, to maintain an intact mucosal surface, and promotes the development of gut lymphoid tissue.[12] Gut microbiota metabolizes cholesterol, several vitamins, such as choline, and complex carbohydrates and plant polysaccharides indigestible for human enzymes producing energy and end products such as short-chain fatty acids (SCFA).[13]

Dysbiosis and cardiovascular disease

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.[9] Although age and gut's genetically defined architecture are the most relevant factors influencing gut's microbiome composition[14], 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,[15] dietary emulsifiers,[16] a high-salt diet[17] and obesity[18] alter the gut microbiota, reduce microbial diversity and induce inflammation, whereas a diet rich in vegetables has been linked with a healthy microbial diversity[19 20]. Dysbiosis is characterized by a greater amount of pro-inflammatory species, that favor metabolic diseases development, caused by both diet-dependent and independent mechanisms.[9 13]

Diet independent mechanisms are mediated by two major receptor families that detect microbes: Toll-like Receptors (TLRs), which control the extracellular compartment, and Nod-like Receptors (NLRs), which sense the presence of intracellular microbes.[21] Lifestyle and dietetic factors, like alcohol and energy-dense Western diets, can increase gut permeability

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and subsequent translocation of molecules produced by gut microbiota that can activate peripheral TLRs and NLR resulting in inflammatory reactions, in the liver, white adipose tissue, brain, and other organs, and trigger metabolic diseases, such as insulin resistance.[22] This inflammation favors microbial penetration, which can contribute to the explanation of why obesity and other metabolic diseases are proinflammatory states.[9 13]

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, with conversion to trimethylamine (TMA), subsequently metabolized to trimethylamine N-oxide (TMAO), which is associated with cardiovascular disease development and progression.[23-25]

Our hypothesis is that patients with arterial stiffness, assessed by carotid femoral pulse wave velocity, 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 cardiovascular disease free population.

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.

METHODS AND ANALYSIS

Design and setting

This is an observational, multicentric, case-control study. Cases will be defined by a cf-PWV >10m/s. It will be an international study including patients from two neighbor countries: Portugal, through the Life and Health Sciences Research Institute, Minho University (Braga) and Spain, through the Primary Care Research Unit of Salamanca (APISAL) belonging to the Biomedical Research Institute of Salamanca (IBSAL) and Cancer Research Institute of Salamanca (CIC) that will also perform gut microbiota analysis.

STUDY POPULATION

We will select 324 subjects, aged 45 to 74 years, free of cardiovascular disease. We will recruit patients from a database that combines data from EVA study[26] (Spain) and Guimarães/Vizela Study[27] (Portugal), and if necessary, we will incorporate new patients who meet the inclusion criteria to complete the sample (Figure 1). Controls will be selected from the same population as cases. The methodology of these studies was published elsewhere.[26 27]

Selection criteria

Inclusion criteria: Patients between 45 and 74 years-old, who agree to participate in the study and do not meet any of the exclusion criteria.

Exclusion criteria: history of CVD (ischemic heart disease or stroke, peripheral arterial disease or heart failure), diabetes mellitus, renal failure in terminal stages (glomerular filtration rate below 30%), chronic inflammatory diseases, inflammatory bowel disease, body mass index >40kg/m^2, oncologic disease diagnosed in the last 5 years and/or under treatment, terminal conditions, antibiotic use within the last 15 days and those who refuse to sign the informed consent.

Sample size

The sample size was estimated to detect a minimum odds ratio of 2 in the study factor (microbiota dysbiosis), considering vascular stiffness (cf-PWV >10m/sec) 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 324 subjects, 162 with arterial stiffness and 162 controls.

VARIABLES AND MEASUREMENT INSTRUMENTS

General and potentially effect-modifying variables such as age, gender, occupation, family history of CVD, hypertension, dyslipidemia, hypothyroidism and drug use will be documented.

Anthropometric measurements

Body weight will be measured twice, using a homologated electronic scale (Seca® 770 medical scale and measurement systems, Birmingham, UK) after calibration (precision±0.1kg), with the patient wearing light clothing and barefoot. Body mass index (BMI) will be calculated as weight (kg) divided by height (m) squared. Waist circumference will be measured using a flexible graduated measuring tape, with the patient in the standing position, without clothing. Body composition will be determined by the Inbody 230 monitor (InBody® Co., Ltd. Seoul, Korea), which gives information of body composition analysis.

Office or Clinical Blood Pressure (BP) will involve three measurements of systolic BP (SBP) and diastolic BP (DBP), using the average of the last two, with a validated OMRON model by following the recommendations of the European Society of Hypertension (ESH).[28]

Habits and lifestyles

Diet: Adherence to the Mediterranean diet, will be measured using the validated 14-point Mediterranean Diet Adherence Screener (MEDAS),[29] developed by the PREDIMED study group.. Each question will be scored as 0 or 1. Adequate adherence to the Mediterranean diet will be assumed when the total score is ≥9 points.

With the APP developed in the EVIDENT study[30] (registry number 00/2014/2207), food consumption will be recorded during a usual week.

The dietary habits of participants will be assessed using a semi-quantitative 137-item food-frequency questionnaire (FFQ), previously validated in Spain[31] and Portugal[32]. The FFQ is based on typical portion sizes that will be multiplied by the consumption frequency for each food. This estimated frequency refers to the previous year, from the time of the interview, and is divided into 9 intake frequency categories ranging from never to more than 6 servings/day.

Physical activity: Two questionnaires will be used to assess physical activity:

International Physical Activity Questionnaire-Short Form (IPAQ-SF). The short form (9 items) categorizes physical activity for the last 7 days in three levels of intensity: (1) intense physical activity, (2) moderate-intensity activity, (3) light.[33]

Questionnaire hours seated (Marshall): Measures the amount of time spent sitting, at work, in the displacements and at home, during week days and weekend.[34]

Tobacco and alcohol consumption: Standardize questionnaire will be used to asses tobacco and alcohol use.

Laboratory measurements

Venous blood samples will be taken between 08:00 and 09:00 am, after participants have fasted and abstained from smoking, alcohol and caffeinated beverages consumption, for 12 hours. Fasting plasma glucose, creatinine, uric acid, liver function and lipids levels will be measured using standard enzymatic automated methods.. A blood sample of each participant will be frozen for posterior evaluation of total biliary acids, deoxycholic acid and SCFA concentration (mg/ml).

Gut microbiota measurements

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Stool samples will be collected by participants with the OMNIgene GUT (OMR -200) kit that permits transport and store with preservation of stabilized DNA, at room temperature, for 60 days. This kit allows obtaining high quality DNA suitable for 16S rRNA microbiome profiling and ensures microbiota profiles accurately represent the in vivo state. All specimens will be sent to the Cancer Research Center in Salamanca.

DNA will be extracted using the FastDNA Soil method, according to the manufacturer's instructions [FastDNA® SPIN Kit for Soil (MP Biomedicals, USA)]. DNA will be extracted from 100-150ul of different feces samples. Each sample will be added to a Lysing Matrix E tube (each tube contains 1.4mm ceramic spheres, 0.1mm silica spheres and one 4mm glass beads) and mixed with different buffers to solubilize membrane proteins, extra-cellular proteins and contaminants in the samples. Afterwards, the sample mix will be homogenize in the Cell disrupter Thermo Savant Fastprep FP120 at 6m/second for 40 seconds. This allows a mechanical disruption of cell walls of the present organisms. DNA will then be eluted with 50ul of DNase/Pyrogen-Free Water (DES), after successive washing steps through the silica Binding Matrix where the purified nucleic acids were retained. Finally, purified DNA will be measured in the Nanodrop, to check quality and quantity.

DNA yield (ng µl−1) will be quantified spectrophotometrically with a NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific, USA). It will also be quantified fluorometrically in a TapeStation 2200, using Genomic DNA ScreenTapes (Agilent, USA) and in a Qubit 4.0 fluorometer (Invitrogen, USA). DNA extraction efficiency will be calculated as the total amount of DNA extracted per biomass (µg g wet wt−1). NanoDrop will also be used to estimate the purity of the extracted DNA. Low absorption ratios at 260/280nm (<1.7) will be used as an indicator of protein impurities, and low absorption ratios at 260/230nm (<2) will be used as an indicator of contamination from polysaccharides. The TapeStation system

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performs electrophoresis in so-called ScreenTapes and outputs images of DNA integrity as well as a DNA Integrity Number (DIN) based on the sizes of the isolated DNA. The DIN ranges from 1 to 10, and a high DIN indicates large DNA fragments, whereas a low DIN indicates more fragmented DNA. DIN determines the fragmentation of a genomic DNA sample by assessing the distribution of signal across the size range using a proprietary algorithm.

Amplicon and Illumina sequencing of bacterial 16S rRNA genes

Amplicon sequences of bacterial 16S rRNA genes, which target v3-v4 regions, will be obtained using Illumina predesigned primer pair, as previously described[35]:

S-D-Bact-0341-b-S-17, 5'-CCTACGGGNGGCWGCAG-3' and *S-D-Bact-0785-a-A-21, 5'-GACTACHVGGGTATCTAATCC-3',* with a length of 465 bp.

Since v5-v6 regions have also been considered the most functional regions, together with v4[36], and two of the most relevant regions for phylogenetic classification, we will also analyze these regions. The v5-v6 primers were obtained from[37] being the sequences: V5F_Nextera 5'-RGGATTAGATACCC-3' and V6R_Nextera 5'- CGACRRCCATGCANCACCT-3', with a length of 281bp. Both primers pair, targeting the variable regions v3-v4 and v5-v6 are equipped with Illumina adapter overhang nucleotide sequences:

- *Forward overhang: 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-[locus-specific sequence]*
- *Reverse overhang: 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-[locus-specific sequence].*

The resulting amplicons will be purified using Agencourt AMPure XP (Beckman Coulter) as recommended by the manufacturer. They will then be amplified in a second PCR where the indexes will be added. The index is unique for each sample of each patient. Once the samples are indexed and identified, the last purification using the Agentcourt AMPure XP kit will be performed. At this point, amplicon libraries of each sample are generated. These libraries should be quantified in the Qubit, normalized and pooled in equimolar amounts. The pooled

samples will be sequenced, using an Illumina MiSeq with 2 × 300bp v3 chemistry, to obtain high quality scores.

Bioinformatics

Raw sequence data will be analyzed using an *in-house* pipeline. This pipeline includes base pair quality filtering, alignment and comparison to a reference database. Quality passing-filter readings will be clustered into operational taxonomic units (OTUs). Then, we will compare control *versus* cases samples. All the microbiome differences will be then interpreted and classified. Quality control will be carried out on a *per sample* basis, discarding paired-ends with an overlap of less than 200nt and removing chimeric sequences using de novo chimaera detection in USEARCH.[38]

Vascular structure and function

Carotid-femoral pulse wave velocity (cf-PWV) and Central Augmentation Index (CAIx): These parameters will be estimated using the SphygmoCor System (AtCor Medical Pty Ltd, Head Office, West Ryde, Australia). With the patient sitting and resting his/her arm on a rigid surface, pulse wave analysis will be performed with a sensor in the radial artery, using a mathematical transformation to estimate the aortic pulse wave. CAIx will be estimated from aortic wave morphology using the following formula: increase in central pressure×100/pulse pressure, and it will be adjusted for a heart rate of 75 bpm. Carotid and femoral artery pulse waves will be analyzed, with the patient in a supine position, using the SphygmoCor System, estimating the delay, as compared with the ECG wave, and calculating cf-PWV. Distance measurements will be taken with a measuring tape from the sternal notch to the carotid and femoral arteries at the sensor location and will be multiplied by 0.8. Subclinical organ damage will be defined as a cf-PWV>10m/s.[28]

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Cardio-ankle Vascular Index (CAVI), brachial ankle PWV (ba-PWV) and Ankle Brachial Index (ABI): These parameters will be estimated using the Vasera device VS-2000 (Fukuda Denshi Co., Ltd. Tokyo, Japan). CAVI values will be automatically calculated by substituting the stiffness parameters in the following equation to detect the vascular elasticity and the cardio ankle PWV: stiffness parameter $\beta=2\rho\times1/(Ps–Pd)\times\ln(Ps/Pd)\times PWV^2$, where ρ is the blood density, Ps and Pd are SBP and DBP in mmHg, and PWV is measured between the aortic valve and ankle. The average coefficient of the variation of CAVI is <5%, which is small enough for clinical use and confirms that CAVI has favorable reproducibility.[39] The ba-PWV will be estimated using the following equation: ba-PWV= ((0.5934×height(cm)+14.4724))/tba, where tba is the time the same waves were transmitted to the ankle.[40] For this study, the mean ABI, CAVI and ba-PWV obtained will be considered. CAVI will be classified as: normal (CAVI<8), borderline (8≤CAVI<9) and abnormal (CAVI≥9); ba-PWV≥17.5[41] and ABI≤0.9 will be considered abnormal.[42]

Central and peripheral augmentation index by the wrist-worn device: Participants will be examined in a seated position, after 10 minutes of rest with his/her arm supported on a firm surface, at heart height. The wrist-worn device has been developed by Microsoft Research (Redmond, Washington, United States) and was recently validated[43]. We will use this device to make a short recording of the radial pulse wave, from which PAIx and CAIx will be obtained. PAIx will be calculated as (second peak SBP (SBP2) - DBP)/(first peak SBP - DBP) x 100, to yield a percentage (%) value. From the estimated morphology of the aortic wave, by mathematical transformation specific to the wrist-worn device, CAIx will be calculated using the following formula: central augmentation pressure x 100/pulse pressure.

Assessment of vascular structure by Carotid IMT (C-IMT): Carotid ultrasound will be performed by investigators trained for this purpose before starting the study, to assess C-IMT.

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Measurements will be made of the common carotid after the examination of a 10 mm longitudinal section at a distance of 1cm from the bifurcation, performing measurements in the proximal and in the distal wall in the lateral, anterior and posterior projections, following an axis perpendicular to the artery to discriminate two lines, one for the intima blood interface and the other for the media-adventitious interface. The measurements will be obtained with the participant lying down, with the head extended and slightly turned opposite to the examined carotid artery. Pathologic intima media thickening: IMT > 0.9mm, or atheromatous plaque diameter greater than 1.5mm, or focal increase of 0.5mm or 50% of the adjacent GIM.[28]

Renal and Cardiac assessment

Kidney damage will be assessed by the estimated glomerular filtration rate using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI)[44] equation and albumin-creatinine ratio, following the criteria of the ESH[28]. Cardiac examination will be performed using a electrocardiogram device (ECG). ECG left ventricular hypertrophy will be defined as a Sokolow-Lyon index >3.5mV, or Cornell VDP>244mV×ms.[28]

Cognitive assessment

The **Montreal Cognitive Assessment (MoCA)**, a screening tool of dementia, validated in Spain[45] and Portugal[46] will be applied. The MoCA was designed as a rapid screening instrument for mild cognitive dysfunction. It assesses different cognitive domains: attention and concentration, executive functions, memory, language, visuo-constructional skills, conceptual thinking, calculations and orientation. Time estimated for MoCA administration is approximately 10 minutes. The total possible score is 30 points; a score of 26 or above is considered normal.

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Investigators applying the different tests will be blinded to participants clinical data. All assessments will be carried out within 10 days.

STATISTICAL ANALYSIS

Data input will be performed using the REDCap (System Electronic Data Capture),[47] with a questionnaire previously designed for the project. Normal distribution of variables will be verified using the Kolmogorov-Smirnov test. Quantitative variables will be displayed as mean±SD, if normally distributed, or as median (IQR), if asymmetrically distributed, and qualitative variables will be expressed as frequencies. Analysis of difference of means between variables of two categories will be carried out using a Student' t-test or a Mann-Whitney U test, as appropriate, while qualitative variables will be analyzed using a χ^2 test. To analyze the relationship between qualitative variables of more than two categories, and quantitative variables, an analysis of variance and the least significant difference test will be used in the post hoc tests; a Kruskal-Wallis test will be used in cases where the variables are not normally distributed. The relationship of quantitative variables to each other will be tested using Pearson or Spearman correlation, as appropriate. Analysis of covariance (ANCOVA) will be performed to adjust for the variables that can affect the results as confounders. Logistic regression will be performed to evaluate the association between the study factor (gut microbial diversity) and the dependent variable (arterial stiffness assed by PWV), adjusted for possible confounding variables (sex, age, BMI, MAP and family relatedness). A multiple linear regression will also be performed to analyze the relationship of the study factor (gut microbiota) with the variables that analyze vascular structure and function quantitatively. This regression will be adjusted for the same confounding variables as the logistic regression.

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Data will be analyzed using the SPSS V.23.0 statistical package (SPSS Inc, Chicago, Illinois, USA). A value of $p<0.05$ will be considered statistically significant. The statisticians/researchers who perform the different analyses will be blinded to participants clinical data.

QUALITY CONTROL

Different processes will be carried out to guarantee study data quality and thus maximize validity and reliability of measurements and results. For this purpose, field work operation manuals have been prepared. Educational leaflets will be developed to ensure correct stool collection. All of these actions will assure an adequate performance at each procedure. Monthly meetings will be held, with the investigators of both Centers, to analyze the entire process, and annual reports on study progress will be prepared.

PROJECT SCHEDULE

This project will be performed in 3 years. In the first 2 years we will perform sample selection and inclusion and data collection using the previously mentioned questionnaires. During the third year, analysis and dissemination of the results will be held.

ETHICS AND DISSEMINATION

Ethical considerations

The study was approved by the "Committee of ethics of research with medicines of the health area of Salamanca" in 14/12/2018 (cod. 2018-11-136) and the "Ethics committee for health of Guimaraes" (Portugal) in 15/10/2019 (ref: 67/2019). Participants must provide informed consent, in accordance with the Declaration of Helsinki. Confidentiality of participants data will always be guaranteed, in accordance with the Regulation (EU) 2016/679 of the European Parliament and of the Council of 27 April 2016, on the protection of natural persons with

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regard to the processing of personal data and on the free movement of such data, and repealing Directive 95/46/EC.

A SPIRIT checklist is available for this protocol. The clinical trial has been registered at ClinicalTrials.gov, with the identifier NCT03900338.

Dissemination plan

Data will be available to members of the research group and members of the Iberian Network on Arterial Structure, Central Hemodynamics and Neurocognition, following the criteria previously defined by the management team.

The research group plans to achieve rapid and widespread dissemination of results to ensure maximum visibility of this study. To this end, results of the study will be published in open-access scientific journals with peer review. At least one publication of the main results and others with the secondary results are planned. This will be complemented by the presentation of the results of the study at relevant scientific conferences and seminars, of national and international scope. Also, a doctoral thesis based on this project will be prepared. Appropriate dissemination will likewise be carried out through social networks and other media.

Patients or the public WERE NOT involved in the design, or conduct, or reporting, or dissemination plans of our research.

DISCUSSION

In recent years, there has been an increase in attention to gut microbiota richness and complexity. The detailed evaluation on the biochemical role of gut microbiome unveils its contribution to local and systemic inflammation and to the development of metabolic diseases, by both diet dependent and independent mechanisms.[21 23] A relation between microbiota and arterial stiffness, an early marker of vascular lesion, is expected.[48] A recent study from London found an inverse association between gut microbiome diversity and

arterial stiffness, in women.[49] Another study, from Moscow, reported a relation between metabolic dysfunctions, gut microbiota low diversity and increased representation of opportunistic pathogens.[50]

We propose to analyze microbiota in patients with documented arterial stiffness. We believe that results from this study will provide novel data that will contribute to the understanding of microbiota role in the development of cardiovascular diseases. That knowledge may help to develop non-pharmacological approaches and strategies to prevent CVD, through lifestyle modification.

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Figure legends

Figure 1: Study Flow chart

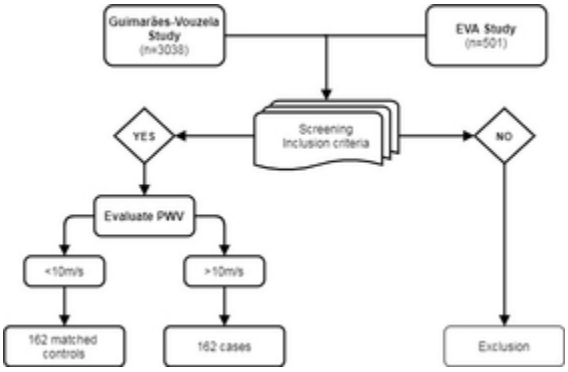

Figure 1: Study Flow chart

23x15mm (300 x 300 DPI)

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Gut microbiota composition and arterial stiffness measured by pulse wave velocity. Case-control study protocol. (MIVAS study)

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Title: Gut microbiota composition and arterial stiffness measured by pulse wave velocity. Case-control study protocol. (MIVAS study)

Short title: Gut microbiota and arterial stiffness

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Article Summary

Introduction

Intestinal microbiota is arising as a new element in the physiopathology of cardiovascular diseases. A healthy microbiota includes a balanced representation of bacteria with health promotion functions (symbiotes). The aim of the study is to analyze the relationship between intestinal microbiota composition and arterial stiffness.

Methods and analysis

An observational case-control study will be developed. Cases will be defined by the presence of at least one of the following: carotid-femoral pulse wave velocity (cf-PWV), cardio-ankle Vascular Index (CAVI), brachial ankle pulse wave velocity (ba or ba-PWV above the 90th percentile, for age and sex, of the reference population;. Controls will be selected from the same population as cases. 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. Cases will be selected from a database that combines data from EVA Study (Spain) and Guimarães/Vizela Study (Portugal). Measurements: cf-PWV will be measure using the SphygmoCor® System, CAVI, ba-PWV and ankle-brachial index will be determined using Vasera® device. Gut microbiome composition in faecal samples will be determined by 16S rRNA sequencing. Lifestyle will be assessed by food frequency questionnaire, adherence to the Mediterranean diet, and IPAQ questionnaire. Body composition will be evaluated by bioimpedance.

Ethics and dissemination

The study has been approved by Committee of ethics of research with medicines of the health area of Salamanca" in 14/12/2018 (cod. 2018-11-136) and the "Ethics committee for health of Guimaraes" (Portugal) in 15/10/2019 (ref: 67/2019). All study participants will sign an informed consent form agreeing to participate in the study, in compliance with the declaration of Helsinki and the WHO standards for observational studies. The results of this study will allow a better description of gut microbiota in patients with arterial stiffness.

Trial Registration Number: NCT03900338 (https://clinicaltrials.gov/ct2/show/NCT03900338)

Keywords: gut microbiota. arterial stiffness, pulse wave velocity, case-control study, protocol

Strengths and limitations

- Observational multicentric and multi-country case-control study evaluating the relationship of gut microbiota and arterial stiffness, in 500 subjects from 40 to 74 years of age.
- 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.
- Analyses the composition of the gut microbiome in faecal samples by 16S rRNA sequencing.
- It is not a random sample, and therefore it cannot be said that the representation is population-based.
- Some of the drugs taken by the included subjects could modify the microbiota.

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INTRODUCTION

Cardiovascular diseases (CVD) are the leading cause of morbidity and mortality globally.[1] 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.[2]

Arterial stiffness has been proven to have a good predictive value for CVD,[3] in various populations, with different levels of risk: general population, elderly, patients with type 2 diabetes, hypertension or end stage renal disease.[4] 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.[5] There are several methods to measure arterial stiffness. Carotid to femoral pulse wave velocity (cf-PWV) is the gold standard,[6] others widely accepted are: CAVI, which measures the stiffness of the aorta, femoral artery and tibial artery; [7] ba-PWV, which uses brachial and tibial arterial waves. [8]

The evaluation of carotid Intima–media thickness (IMT) can 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.[5] Other factors, as genetic burden, systemic inflammatory diseases and gut microbiota,[9] have also been linked to pulse wave velocity. Gut microbiota composition has also been implicated on the genesis of hypertension,[10] obesity,[11] insulin resistance, metabolic syndrome[11] and type 2 diabetes.[12]

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

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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.[11] Although age and gut's genetically defined architecture are the most relevant factors influencing gut's microbiome composition[13], 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,[14] dietary emulsifiers,[15] a high-salt diet[16] and obesity[17] alter the gut microbiota, reduce microbial diversity and induce inflammation, whereas a diet rich in vegetables has been linked with a healthy microbial diversity[18 19].

Dysbiosis is characterized by a greater amount of pro-inflammatory species, that favor metabolic diseases development, caused by both diet-dependent and independent mechanisms. [11 20]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.[21] 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.[22]

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.[23-25]

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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.

METHODS AND ANALYSIS

Design and setting

This is an observational, multicentric, case-control study. 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. It will be an international study including patients from two neighbor countries: Portugal, through the Life and Health Sciences Research Institute, Minho University (Braga) and Spain, through the Primary Care Research Unit of Salamanca (APISAL) belonging to the Biomedical Research Institute of Salamanca (IBSAL) and Cancer Research Institute of Salamanca (CIC) that will also perform gut microbiota analysis.

STUDY POPULATION

We will select 500 subjects, aged 45 to 74 years, free of cardiovascular disease. We will recruit patients from a database that combines data from EVA study[26] (Spain) and Guimarães/Vizela Study[27] (Portugal), and if necessary, we will incorporate new patients who meet the inclusion criteria to complete the sample (Figure 1). Controls will be selected from the same population as cases. The methodology of these studies was published elsewhere.[26 27]

Selection criteria

Inclusion criteria: Patients between 45 and 74 years-old, who agree to participate in the study and do not meet any of the exclusion criteria.

Exclusion criteria: history of CVD (ischemic heart disease or stroke, peripheral arterial disease or heart failure), diabetes mellitus, renal failure in terminal stages (glomerular filtration rate below 30%), chronic inflammatory diseases, inflammatory bowel disease, body mass index >40kg/m^2, oncologic disease diagnosed in the last 5 years and/or under treatment, terminal conditions, antibiotic use within the last 15 days and those who refuse to sign the informed consent.

Sample size

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 with arterial stiffness and 250 controls.

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Patient and Public Involvement

Patients will not participate in the study design, however they will actively participate in the study recruitment by disseminating the study objectives and inclusion criteria through their organizations. At the end of the study, in addition to sending a detailed report with the results of each patient, a dissemination session will be organized for all patients included in the study. Some of the participants will take part in this conference to share their experience and their personal evaluation of the study results.

VARIABLES AND MEASUREMENT INSTRUMENTS

General and potentially effect-modifying variables such as age, gender, occupation, family history of CVD, hypertension, dyslipidemia, hypothyroidism and drug use will be documented.

Anthropometric measurements

Body weight will be measured twice, using a homologated electronic scale (Seca® 770 medical scale and measurement systems, Birmingham, UK) after calibration (precision±0.1kg), with the patient wearing light clothing and barefoot. Body mass index (BMI) will be calculated as weight (kg) divided by height (m) squared. Waist circumference will be measured using a flexible graduated measuring tape, with the patient in the standing position, without clothing. Body composition will be determined by the Inbody 230 monitor (InBody® Co., Ltd. Seoul, Korea), which gives information of body composition analysis.

Office or Clinical Blood Pressure (BP) will involve three measurements of systolic BP (SBP) and diastolic BP (DBP), using the average of the last two, with a validated OMRON model by following the recommendations of the European Society of Hypertension (ESH).[28]

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Habits and lifestyles

Diet: Adherence to the Mediterranean diet, will be measured using the validated 14-point Mediterranean Diet Adherence Screener (MEDAS),[29] developed by the PREDIMED study group. Each question will be scored as 0 or 1. Adequate adherence to the Mediterranean diet will be assumed when the total score is ≥9 points.

With the APP developed in the EVIDENT study[30] (registry number 00/2014/2207), food consumption will be recorded during a usual week.

The dietary habits of participants will be assessed using a semi-quantitative 137-item food-frequency questionnaire (FFQ), previously validated in Spain[31] and Portugal[32]. The FFQ is based on typical portion sizes that will be multiplied by the consumption frequency for each food. This estimated frequency refers to the previous year, from the time of the interview, and is divided into 9 intake frequency categories ranging from never to more than 6 servings/day.

Physical activity: Two questionnaires will be used to assess physical activity:

International Physical Activity Questionnaire-Short Form (IPAQ-SF). The short form (9 items) categorizes physical activity for the last 7 days in three levels of intensity: (1) intense physical activity, (2) moderate-intensity activity, (3) light.[33]

Questionnaire hours seated (Marshall): Measures the amount of time spent sitting, at work, in the displacements and at home, during week days and weekend.[34]

Tobacco and alcohol consumption: Standardize questionnaire will be used to assess tobacco and alcohol use.

Laboratory measurements

Venous blood samples will be taken between 08:00 and 09:00 am, after participants have fasted and abstained from smoking, alcohol and caffeinated beverages consumption, for 12

hours. Fasting plasma glucose, creatinine, uric acid, liver function and lipids levels will be measured using standard enzymatic automated methods. A blood sample of each participant will be frozen for posterior evaluation of total biliary acids, deoxycholic acid and SCFA concentration (mg/ml).

Gut microbiota measurements

Stool samples will be collected by participants with the OMNIgene GUT (OMR -200) kit that permits transport and store with preservation of stabilized DNA, at room temperature, for 60 days. This kit allows obtaining high quality DNA suitable for 16S rRNA microbiome profiling and ensures microbiota profiles accurately represent the in vivo state. All specimens will be sent to the Cancer Research Center in Salamanca.

DNA will be extracted using the FastDNA Soil method, according to the manufacturer's instructions [FastDNA® SPIN Kit for Soil (MP Biomedicals, USA)]. DNA will be extracted from 100-150ul of different feces samples. Each sample will be added to a Lysing Matrix E tube (each tube contains 1.4mm ceramic spheres, 0.1mm silica spheres and one 4mm glass beads) and mixed with different buffers to solubilize membrane proteins, extra-cellular proteins and contaminants in the samples. Afterwards, the sample mix will be homogenized in the Cell disrupter Thermo Savant Fastprep FP120 at 6m/second for 40 seconds. This allows a mechanical disruption of cell walls of the present organisms. DNA will then be eluted with 50ul of DNase/Pyrogen-Free Water (DES), after successive washing steps through the silica Binding Matrix where the purified nucleic acids were retained. Finally, purified DNA will be measured in the Nanodrop, to check quality and quantity.

DNA yield (ng µl−1) will be quantified spectrophotometrically with a NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific, USA). It will also be quantified fluorometrically in a TapeStation 2200, using Genomic DNA ScreenTapes (Agilent, USA) and in a Qubit 4.0

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fluorometer (Invitrogen, USA). DNA extraction efficiency will be calculated as the total amount of DNA extracted per biomass (µg g wet wt−1). NanoDrop will also be used to estimate the purity of the extracted DNA. Low absorption ratios at 260/280nm (<1.7) will be used as an indicator of protein impurities, and low absorption ratios at 260/230nm (<2) will be used as an indicator of contamination from polysaccharides. The TapeStation system performs electrophoresis in so-called ScreenTapes and outputs images of DNA integrity as well as a DNA Integrity Number (DIN) based on the sizes of the isolated DNA. The DIN ranges from 1 to 10, and a high DIN indicates large DNA fragments, whereas a low DIN indicates more fragmented DNA. DIN determines the fragmentation of a genomic DNA sample by assessing the distribution of signal across the size range using a proprietary algorithm.

Amplicon and Illumina sequencing of bacterial 16S rRNA genes

Amplicon sequences of bacterial 16S rRNA genes, which target v3-v4 regions, will be obtained using Illumina predesigned primer pair, as previously described[35]:

S-D-Bact-0341-b-S-17, 5'-CCTACGGGNGGCWGCAG-3' and *S-D-Bact-0785-a-A-21, 5'-GACTACHVGGGTATCTAATCC-3',* with a length of 465 bp.

Since v5-v6 regions have also been considered the most functional regions, together with v4[36], and two of the most relevant regions for phylogenetic classification, we will also analyze these regions. The v5-v6 primers were obtained from[37] being the sequences: V5F_Nextera 5'-RGGATTAGATACCC-3' and V6R_Nextera 5'- CGACRRCCATGCANCACCT-3', with a length of 281bp. Both primers pair, targeting the variable regions v3-v4 and v5-v6 are equipped with Illumina adapter overhang nucleotide sequences:

- *Forward overhang: 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-[locus-specific sequence]*
- *Reverse overhang: 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-[locus-specific sequence].*

The resulting amplicons will be purified using Agencourt AMPure XP (Beckman Coulter) as recommended by the manufacturer. They will then be amplified in a second PCR where the indexes will be added. The index is unique for each sample of each patient. Once the samples are indexed and identified, the last purification using the Agentcourt AMPure XP kit will be performed. At this point, amplicon libraries of each sample are generated. These libraries should be quantified in the Qubit, normalized and pooled in equimolar amounts. The pooled samples will be sequenced, using an Illumina MiSeq with 2 × 300bp v3 chemistry, to obtain high quality scores.

Bioinformatics

Raw sequence data will be analyzed using an *in-house* pipeline. This pipeline includes base pair quality filtering, alignment and comparison to a reference database. Quality passing-filter readings will be clustered into operational taxonomic units (OTUs). Then, we will compare control *versus* cases samples. All the microbiome differences will be then interpreted and classified. Quality control will be carried out on a *per sample* basis, discarding paired-ends with an overlap of less than 200nt and removing chimeric sequences using de novo chimaera detection in USEARCH.[38]

Vascular structure and function

Carotid-femoral pulse wave velocity (cf-PWV) and Central Augmentation Index (CAIx): These parameters will be estimated using the SphygmoCor System (AtCor Medical Pty Ltd, Head Office, West Ryde, Australia). With the patient sitting and resting his/her arm on a rigid surface, pulse wave analysis will be performed with a sensor in the radial artery, using a mathematical transformation to estimate the aortic pulse wave. CAIx will be estimated from aortic wave morphology using the following formula: increase in central pressure×100/pulse pressure, and it will be adjusted for a heart rate of 75 bpm. Carotid and femoral artery pulse

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waves will be analyzed, with the patient in a supine position, using the SphygmoCor System, estimating the delay, as compared with the ECG wave, and calculating cf-PWV. Distance measurements will be taken with a measuring tape from the sternal notch to the carotid and femoral arteries at the sensor location and will be multiplied by 0.8. Subclinical organ damage will be defined as cf-PWV, above the 90th percentile, for age and sex, of the reference population.[39]

Cardio-ankle Vascular Index (CAVI), brachial ankle PWV (ba-PWV) and Ankle Brachial Index (ABI): These parameters will be estimated using the Vasera device VS-2000 (Fukuda Denshi Co., Ltd. Tokyo, Japan). CAVI values will be automatically calculated by substituting the stiffness parameters in the following equation to detect the vascular elasticity and the cardio ankle PWV: stiffness parameter $\beta=2\rho\times1/(Ps–Pd)\times\ln(Ps/Pd)\times PWV^2$, where ρ is the blood density, Ps and Pd are SBP and DBP in mmHg, and PWV is measured between the aortic valve and ankle. The average coefficient of the variation of CAVI is <5%, which is small enough for clinical use and confirms that CAVI has favorable reproducibility.[40] The ba-PWV will be estimated using the following equation: ba-PWV= ((0.5934×height(cm)+14.4724))/tba, where tba is the time the same waves were transmitted to the ankle.[41] For this study, the mean ABI, CAVI and ba-PWV obtained will be considered. CAVI will be classified as: normal (CAVI <8), borderline (8≤CAVI <9) and abnormal (CAVI≥9) [42]. Subclinical organ damage will be defined, CAVI or ba-PWV above the 90th percentile of the reference population [39].ABI≤0.9 will be considered abnormal[2].

Central and peripheral augmentation index by the wrist-worn device: Participants will be examined in a seated position, after 10 minutes of rest with his/her arm supported on a firm surface, at heart height. The wrist-worn device has been developed by Microsoft Research

(Redmond, Washington, United States) and was recently validated[43]. We will use this device to make a short recording of the radial pulse wave, from which PAIx and CAIx will be obtained. PAIx will be calculated as (second peak SBP (SBP2) - DBP)/(first peak SBP - DBP) x 100, to yield a percentage (%) value. From the estimated morphology of the aortic wave, by mathematical transformation specific to the wrist-worn device, CAIx will be calculated using the following formula: central augmentation pressure x 100/pulse pressure.

Assessment of vascular structure by Carotid IMT (C-IMT): Carotid ultrasound will be performed by investigators trained for this purpose before starting the study, to assess C-IMT. Measurements will be made of the common carotid after the examination of a 10 mm longitudinal section at a distance of 1cm from the bifurcation, performing measurements in the proximal and in the distal wall in the lateral, anterior and posterior projections, following an axis perpendicular to the artery to discriminate two lines, one for the intima blood interface and the other for the media-adventitious interface. The measurements will be obtained with the participant lying down, with the head extended and slightly turned opposite to the examined carotid artery. Pathologic intima media thickening: IMT > 0.9mm, or atheromatous plaque diameter greater than 1.5mm, or focal increase of 0.5mm or 50% of the adjacent GIM.[28]

Renal and Cardiac assessment

Kidney damage will be assessed by the estimated glomerular filtration rate using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI)[44] equation and albumin-creatinine ratio, following the criteria of the ESH[28]. Cardiac examination will be performed using a electrocardiogram device (ECG). ECG left ventricular hypertrophy will be defined as a Sokolow-Lyon index >3.5mV, or Cornell VDP>244mV×ms.[28]

Cognitive assessment

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The **Montreal Cognitive Assessment (MoCA)**, a screening tool of dementia, validated in Spain[45] and Portugal[46] will be applied. The MoCA was designed as a rapid screening instrument for mild cognitive dysfunction. It assesses different cognitive domains: attention and concentration, executive functions, memory, language, visuo-constructional skills, conceptual thinking, calculations and orientation. Time estimated for MoCA administration is approximately 10 minutes. The total possible score is 30 points; a score of 26 or above is considered normal.

Investigators applying the different tests will be blinded to participants clinical data. All assessments will be carried out within 10 days.

STATISTICAL ANALYSIS

Data input will be performed using the REDCap (System Electronic Data Capture),[47] with a questionnaire previously designed for the project. Normal distribution of variables will be verified using the Kolmogorov-Smirnov test. Quantitative variables will be displayed as mean±SD, if normally distributed, or as median (IQR), if asymmetrically distributed, and qualitative variables will be expressed as frequencies. Analysis of difference of means between variables of two categories will be carried out using a Student' t-test or a Mann-Whitney U test, as appropriate, while qualitative variables will be analyzed using a χ^2 test. To analyze the relationship between qualitative variables of more than two categories, and quantitative variables, an analysis of variance and the least significant difference test will be used in the post hoc tests; a Kruskal-Wallis test will be used in cases where the variables are not normally distributed. The relationship of quantitative variables to each other will be tested using Pearson or Spearman correlation, as appropriate. Analysis of covariance (ANCOVA) will be performed to adjust for the variables that can affect the results as confounders. 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 analyze the relationship of the study ctor (gut microbiota) with the variables that analyze 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.

Data will be analyzed using the SPSS V.23.0 statistical package (SPSS Inc, Chicago, Illinois, USA). A value of $p<0.05$ will be considered statistically significant. The statisticians/researchers who perform the different analyses will be blinded to participants clinical data.

QUALITY CONTROL

Different processes will be carried out to guarantee study data quality and thus maximize validity and reliability of measurements and results. For this purpose, field work operation manuals have been prepared. Educational leaflets will be developed to ensure correct stool collection. All of these actions will assure an adequate performance at each procedure. Monthly meetings will be held, with the investigators of both Centers, to analyze the entire process, and annual reports on study progress will be prepared.

PROJECT SCHEDULE

This project will be performed in 3 years. In the first 2 years we will perform sample selection and inclusion and data collection using the previously mentioned questionnaires. During the third year, analysis and dissemination of the results will be held.

ETHICS AND DISSEMINATION

Ethical considerations

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The study was approved by the "Committee of ethics of research with medicines of the health area of Salamanca" in 14/12/2018 (cod. 2018-11-136) and the "Ethics committee for health of Guimaraes" (Portugal) in 15/10/2019 (ref: 67/2019). Participants must provide informed consent, in accordance with the Declaration of Helsinki. Confidentiality of participants data will always be guaranteed, in accordance with the Regulation (EU) 2016/679 of the European Parliament and of the Council of 27 April 2016, on the protection of natural persons with regard to the processing of personal data and on the free movement of such data, and repealing Directive 95/46/EC.

A SPIRIT checklist is available for this protocol. The clinical trial has been registered at ClinicalTrials.gov, with the identifier NCT03900338.

Dissemination plan

Data will be available to members of the research group and members of the Iberian Network on Arterial Structure, Central Hemodynamics and Neurocognition, following the criteria previously defined by the management team.

The research group plans to achieve rapid and widespread dissemination of results to ensure maximum visibility of this study. To this end, results of the study will be published in open-access scientific journals with peer review. At least one publication of the main results and others with the secondary results are planned. This will be complemented by the presentation of the results of the study at relevant scientific conferences and seminars, of national and international scope. Also, a doctoral thesis based on this project will be prepared. Appropriate dissemination will likewise be carried out through social networks and other media.

Patients or the public were not involved in the design, or conduct, or reporting, or dissemination plans of our research.

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DISCUSSION

In recent years, there has been an increase in attention to gut microbiota richness and complexity. The detailed evaluation on the biochemical role of gut microbiome unveils its contribution to local and systemic inflammation and to the development of metabolic diseases, by both diet dependent and independent mechanisms.[21 23]

A relation between microbiota and arterial stiffness, an early marker of vascular lesion, is expected.[48] A recent study from London found an inverse association between gut microbiome diversity and arterial stiffness, in women.[49] Another study, from Moscow, reported a relation between metabolic dysfunctions, gut microbiota low diversity and increased representation of opportunistic pathogens.[50]

We propose to analyze microbiota in patients with documented arterial stiffness. We believe that results from this study will provide novel data that will contribute to the understanding of microbiota role in the development of cardiovascular diseases. That knowledge may help to develop non-pharmacological approaches and strategies to prevent CVD, through lifestyle modificatio**n.**

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Author Contributions LGO, PGC and RS contributed to the conception and design of the study. RS and LGO prepared the manuscript of the study protocol. CLS, CAC, ACL, MAGM, ERS,SS, RB and JMHS contributed to the development of the study protocol. LGO and JMHR provided assistance with statistical methodology and knowledge. LGO, PGC, JMHR, MAGM and ERS provided a critical review of the manuscript. All authors have read and accepted the final version of the protocol.

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Figure legends

Figure 1: Study Flow chart**. cf-PWV** - carotid-femoral pulse wave velocity; **CAVI** - cardio-ankle vascular Index; **ba PWV** - brachial-ankle pulse wave velocity.

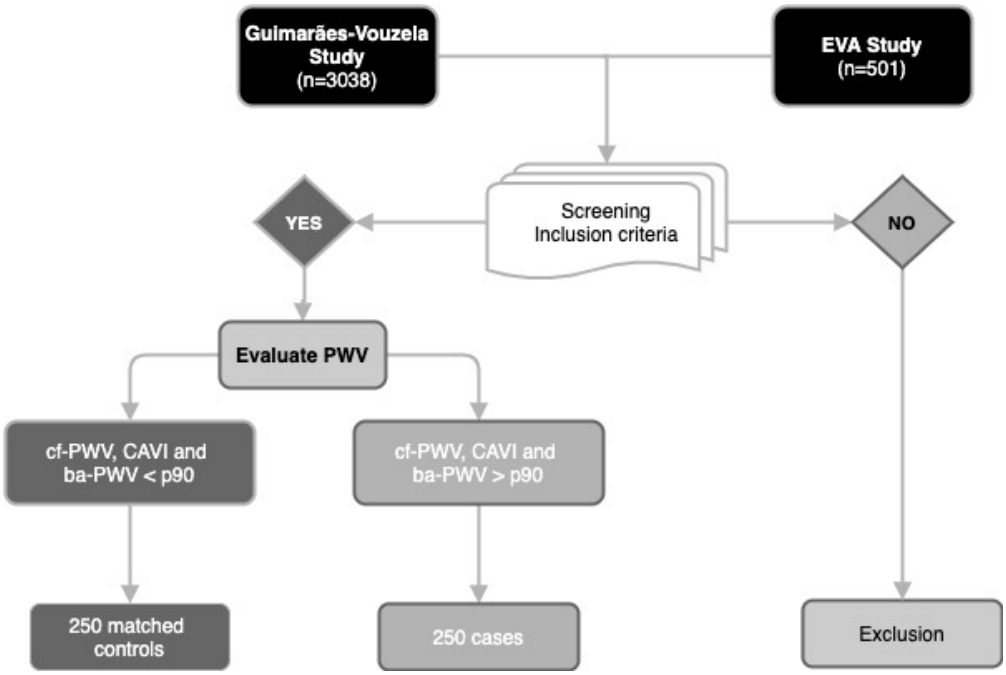

Figure 1: Study Flow chart. cf-PWV - carotid-femoral pulse wave velocity; CAVI - cardio-ankle vascular Index; ba PWV - brachial ankle pulse wave velocity

212x141mm (72 x 72 DPI)

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	Addressed on page number
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	2
	2b	All items from the World Health Organization Trial Registration Data Set	2
Protocol version	3	Date and version identifier	2
Funding	4	Sources and types of financial, material, and other support	21
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	21
	5b	Name and contact information for the trial sponsor	1+21
	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	n/a
	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)	n/a

Introduction			
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	4-6
	6b	Explanation for choice of comparators	6
Objectives	7	Specific objectives or hypotheses	6+7
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)	7+18
Methods: Participants, interventions, and outcomes			
Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	7
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)	8
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	n/a
	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)	n/a
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	n/a
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	n/a
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	8-18
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)	8-16,18

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	8
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	n/a

Methods: Assignment of interventions (for controlled trials)

Allocation:			
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	n/a
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	n/a
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	n/a
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	n/a
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	n/a

Methods: Data collection, management, and analysis

Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	8-16
	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	n/a

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	8
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	16+17
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	16-17
	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)	n/a-
Methods: Monitoring			
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	18
	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	n/a
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	n/a
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	18
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	18
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)	18

Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	18
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	n/a
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	18+19
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	21
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	21
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	n/a
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	19
	31b	Authorship eligibility guidelines and any intended use of professional writers	n/a
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	n/a
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	appendice 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	

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated.