

BMJ Open Relationship between target organ damage and blood pressure, retinal vessel calibre, oxidative stress and polymorphisms in VAV-2 and VAV-3 genes in patients with hypertension: a case-control study protocol (LOD-Hipertensión)

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ABSTRACT

Introduction: Target organ damage (TOD) is associated with increased cardiovascular risk. The study objectives were to analyse the relationship of TOD to blood pressure, size of retinal arteries and veins, oxidative stress and different polymorphisms in the VAV-2 and VAV-3 genes in participants with hypertension.

Methods and analysis: A case-control study to analyse the relationship between clinical, biochemical and genetic parameters and presence of cardiac, vascular and renal TOD in 486 patients with hypertension. Participants with TOD will be considered as cases, and those without TOD will be enrolled as controls. This will be a collaborative study conducted by the groups of Primary Care, Cardiovascular and Metabolic and Degenerative Diseases of the Instituto de Investigación Biomédica de Salamanca (IBSAL). Assessment of cardiac, renal and vascular TOD. Measurement of peripheral and central blood pressure, size of eye fundus arteries and veins, and oxidative stress, and polymorphisms in the VAV-2 and VAV-3 genes.

Ethics and dissemination: The study will be conducted after approval is obtained from the Ethics Committee of Hospital Clínico Universitario de Salamanca. All study participants will sign an informed consent to agree to participate in the study, and another consent to agree on the genetic study, in compliance with the Declaration of Helsinki and the WHO standards for observational studies. The results of this study will allow for an understanding of the relationship of the different TODs with blood pressure, retinal artery and vein diameters, oxidative stress and polymorphisms in VAV-2 and VAV-3 genes.

Trial registration number: Clinical Trials. gov Identifier: NCT02022618.

INTRODUCTION

The relationship of blood pressure (BP) of morbidity and mortality from coronary artery

diseases, heart failure and renal failure is increasing and continuous.¹ Cardiovascular (CV) risk in participants with hypertension is influenced by BP levels, presence of CV risk factors, target organ damage (TOD) and clinically established CV disease (CVD), according to the 2013 guidelines of the European Society of Hypertension and the European Society of Cardiology (ESG-ESC).²

Presence of cardiac,^{3 4} renal^{5 6} and vascular⁷⁻¹⁰ TOD is considered as a surrogate variable and is associated with increased CV risk.

Ambulatory BP (ambulatory blood pressure monitoring (ABPM) and home blood pressure (HABP)) predict CV morbidity and mortality better than does office BP¹¹⁻¹⁴ and are associated with the presence of TOD.¹⁵⁻¹⁷ ABPM parameters allow for estimating the ambulatory arterial stiffness index (AASI), which is related to morbidity and mortality for CVD^{18 19} and the presence of TOD in patients with hypertension.³⁻⁸ AASI may be helpful to assess arterial stiffness^{20 21} and correlates to other classical measurements such as pulse wave velocity (PWV).²¹

ABPM also allows for assessing 24 h variability in BP.²² Thus, participants with a lower nocturnal decrease in BP have a poorer course²³ and a higher prevalence of TOD.²⁰

Central BP is a determinant factor in myocardial perfusion and cardiac work. This parameter is associated with CV morbidity and mortality^{9 24} and with intermediate markers such as left ventricular hypertrophy (LVH),²⁵ carotid intima-media thickness (IMT),²⁶ coronary atherosclerosis²⁷ or decreased glomerular filtration rate.²⁶ Central haemodynamic

indices are independent predictors of future CV events and all-cause mortality.²⁸ There is sufficient evidence to show that increased pulse-wave reflection predicts CV mortality independently of arterial stiffness in men and women.²⁹

An eye fundus examination allows for non-invasive assessment of retinal microcirculation. The classical Keith-Wagener classification has limitations for analysis of retinal vascular lesions, particularly for adequate assessment of early vascular lesions. Different tools to quantitatively assess artery size have been developed,^{30–33} but all of them require the intervention of an observer. Population studies have found that retinal vessel calibre is related to risk of hypertension,³⁴ LVH,³⁵ cerebrovascular accidents³⁶ and coronary artery disease.³⁷ A meta-analysis of six population studies showed that retinal vessel calibre was associated with increased risk of coronary disease in women, but not in men.³⁸ However, conflicting results have been reported with regard to the course of atherosclerotic lesions and retinal vessel calibre.^{39–41} Cuspidi *et al*⁴² and Masaidi *et al*⁴³ found no association between retinal vessel calibre and TOD (cardiac, vascular, renal) in a population with hypertension. This study will use a semiautomated tool developed by our AV group *Index calculator* (Ciclorisk SL, Salamanca, Spain, registry n^o. 00/2011/589), recently validated⁴⁴ and highly reliable, to analyse the association of retinal artery and vein calibre (arteriole and venule diameter and arteriovenous ratio) with different TODs.

The oxidised form of xanthine dehydrogenase promotes early endothelial dysfunction, perpetuating oxidative damage in the vascular wall and myocardium through the superoxide anion.⁴⁵ Patients with hypertension experience biochemical changes showing oxidative stress.⁴⁶ When oxidative stress exists, peroxynitrite (ONOO⁻), one of the reactive oxygen species with a greater effect on the CV system, is formed. ONOO⁻ inhibits key enzymes located in the myocardium such as ATPase Ca²⁺⁴⁷ and creatine kinase,⁴⁸ activates metalloproteinases⁴⁹ and modulates signalling systems of mitogen-activated protein kinases⁵⁰ and nuclear transcription factor EB,⁵¹ as well as activating the enzyme poly (ADP-ribose) polymerase.⁵² According to Levrard *et al*,⁵² ONOO⁻ may be involved in apoptotic events contributing to cell loss in the myocardium.

Essential hypertension is a highly heterogeneous disorder with a multifactorial aetiology.² Several genome-wide association studies and their meta-analyses point to a total of 29 single nucleotide polymorphisms, which are associated with systolic BP (SBP) and/or diastolic BP (DBP).⁵³ These findings may be useful contributors to risk scores for TOD.² The VAV gene is very widely expressed, and its distribution is virtually universal.⁵⁴ In the VAV family, a direct relationship exists between receptors with intrinsic activity or with activity associated with tyrosine kinase and pathways regulating mitogenesis and the cytoskeleton through nucleotide exchange in Rho/Rac proteins.⁵⁴ Sauzeau *et al*⁵⁵ assessed in knockout mice

the consequences of VAV-2-/- at the vascular and cardiac level and on other elements of CV homeostasis. At the vascular level, they found extensive remodelling in the whole CV system, except in pulmonary circulation, and changes in the media layer of the aortic wall. In the heart, they found LVH with fibrosis, with no right ventricle involvement. In the kidney, these authors found interstitial fibrosis, decreases in glomerular filtration, sodium excretion and creatinine clearance and increased aldosterone and vasopressin levels associated with changes in the renin-angiotensin-aldosterone system and endothelin. Elevated epinephrine and norepinephrine levels, and normal dopamine levels, were also found. All these findings allow for stating that VAV-2-/- mice have CV impairment, and for defining part of the pathophysiological substrate underlying such impairment. The same research team conducted a study⁵⁶ in modified VAV-2-/- mice and showed a new BP control pathway in which type 5 phosphodiesterase is involved at vascular smooth muscle cells, causing an increased vascular tone which was corrected with administration of phosphodiesterase inhibitors. Genetically modified mice with VAV-3-/-⁵⁷ had elevated SBP and DBP values and tachycardia. Administration of propranolol achieved control of BP and tachycardia, and decreased angiotensin II levels and cardiac remodelling. These results show a direct association between both systems (sympathetic nervous system and renin-angiotensin-aldosterone axis) in the development of arterial hypertension in VAV-3-/- mice. Renal effects were similar to those caused by VAV-2. To sum up, experimental studies conducted in animals deficient in VAV-2 and VAV-3 show that sympathetic activation plays a basic role in cardiac, vascular and renal changes.^{55 57} The potential role of polymorphisms in these genes in BP in humans and their association with the different TODs have not been studied.

The study objective was to analyse the relationship of renal, cardiac and vascular TOD with the haemodynamic parameters of BP, retinal artery and vein calibre, urinary excretion of oxidative stress molecules and different polymorphisms in the VAV-2 and VAV-3 genes in hypertensive participants.

METHODS AND ANALYSIS

Study design

A case-control study to analyse the association between clinical, biochemical and genetic parameters and the presence of cardiac, vascular and renal TOD in participants with hypertension. Participants with TOD will be considered as cases, and those without TOD will be enrolled as controls.

Setting

This will be a collaborative study conducted by the groups of Primary Care, CV and Metabolic and Degenerative Diseases of the Instituto de Investigación Biomédica de Salamanca (IBSAL). Clinical assessment

and all examinations needed for complete evaluation of the presence of TOD will be performed at the primary care research unit.² Oxidative stress testing will be performed at the pathophysiology unit, and genetic polymorphisms will be analysed at the Unit of Molecular Medicine of Salamanca University.

Study subjects

Consecutive participants aged 20–80 years referred to the La Alamedilla research unit for TOD evaluation and diagnosed with essential hypertension will be enrolled into the study. All participants who do not sign the written informed consent according to general recommendations in the Declaration of Helsinki will be excluded from the study.⁵⁸ Sample size has been estimated for detecting a 3.25% difference in the Central Augmentation Index (CAIx), half the difference used in the CAFEE study (6.5%), between participants with and without TOD. A 1:3 ratio between participants with and without TOD has been considered. Considering a common SD of 10.8 units and assuming an α risk of 0.05 and an 80% power for a two-sided test, a total of 468 participants are required, 117 in the first group and 351 in the second group.

Variables and measurement instruments

CV risk factors and CVD

Risk factors

Male sex—age (men ≥ 55 years; women ≥ 65 years). Smoking—dyslipidaemia: total cholesterol >4.9 mmol/L (190 mg/dL) or low-density lipoprotein cholesterol >3 mmol/L (115 mg/dL) or high-density lipoprotein cholesterol: men <1.0 mmol/L (40 mg/dL), women <1.2 mmol/L (46 mg/dL) or triglycerides >1.7 mmol/L (150 mg/dL). Fasting plasma glucose 5.6–6.9 mmol/L (102–125 mg/dL). Abnormal glucose tolerance test. Obesity (body mass index (BMI) ≥ 30 kg/m² (height²)). Abdominal obesity (waist circumference: men ≥ 102 cm; women ≥ 88 cm; in Caucasians). Family history of premature CVD (men aged <55 years; women aged <65 years).²

Established CV or renal disease

Cerebrovascular disease—ischaeic stroke; cerebral haemorrhage; transient ischaemic attack coronary heart disease: myocardial infarction; angina; myocardial revascularisation. Heart failure, including heart failure with preserved ejection fraction. Symptomatic peripheral artery disease in lower limbs. Chronic kidney disease (CKD) with estimated glomerular filtration rate <30 mL/min/1.73 m² or proteinuria (>300 mg/24 h). Advanced retinopathy: haemorrhage or exudate, papilloedema.²

Drug therapy

Antihypertensive, antidiabetic and lipid-lowering drugs and antiplatelet treatment.

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 ± 0.1 kg), with the patient wearing light clothing and was shoeless. These readings will be rounded to 100 g. Height will be measured using a portable system (Seca 222; Medical scale and measurement systems, Birmingham, UK). The mean of two readings taken with the patient shoeless in the standing position will be recorded. Values will be rounded to the nearest centimetre. BMI will be calculated as weight (kg) divided by (height)² (m²). Waist circumference will be measured using a flexible graduated measuring tape with the patient in the standing position without clothing. The upper border of the iliac crests will be located, and the tape will be wrapped around above this point, parallel to the floor, ensuring that it is adjusted but without compressing the skin.

Blood pressure

Office or clinical BP—office BP will be determined by three measurements of SBP and DBP, using the mean of the last two values, with a validated OMRON model M10-IT sphygmomanometer (Omron Health Care, Kyoto, Japan), according to the recommendations of the European Society of Hypertension.⁵⁹ Pulse pressure will be estimated using the mean values of the second and third measurements.

Home BP—This parameter will be measured using an OMRON model M10-IT sphygmomanometer (Omron Health Care, Kyoto, Japan). Three measurements will be made in the morning (between 6:00 and 9:00), and three in the afternoon/evening (between 18:00 and 21:00), over a period of 7 days, with a minimum interval of 1 min between measurements, and excluding the first measurement in each case and the values corresponding to the first day of measurement.⁶⁰ The investigator will train patients on BP measurement at home. Written instructions and a self-recording sheet will be provided to ensure adequate pressure monitoring.

Ambulatory BP monitoring

The monitoring will be performed on a day of standard activity using an adequate cuff for the size of the patient's arm. A control system, SpaceLabs 90207 model (Spacelabs Healthcare, Issaquah, Washington, USA), validated according to the protocol of the British Hypertension Society, will be used.⁶¹ Valid registries will be required to meet a number of pre-established criteria, including $\geq 80\%$ successful SBP and DBP recordings during the daytime and night-time periods, 24 h duration and ≥ 1 BP measurement/h. The monitor will be scheduled to obtain BP measurements every 15 min during the daytime and night-time periods. Patients will be categorised based on the circadian pattern, which will be estimated by the sleep to wake ratio of SBP, as dipper, <0.9 ; non-dipper, 0.9 – 1 ; and riser, >1 . The nocturnal dip will be defined as the difference between the mean waking BP and the mean sleeping BP expressed as

a percentage (systolic dip $\% = 100 \times (\text{mean SBP day} - \text{mean SBP night}) / \text{mean SBP day}$).

AASI and (HASI)

Arterial stiffness will be evaluated based on the AASI and home arterial stiffness index (HASI). To estimate AASI and HASI, the regression slope of diastolic on SBP will be computed for each participant based on 24 h ABPM (AASI) and on HASI readings over 6 days. Both AASI and HASI were defined as one minus the respective regression slope of DBP on SBP.⁶² AASI will also be computed from waking or sleeping BP.

Evaluation of retinal vessels

Retinography will be performed using a Topcon TRC NW 200 non-mydratic retinal camera (Topcon Europe B.C., Capelle a/d Ijssel, The Netherlands), obtaining nasal and temporal images centred on the disk. The nasal image with the centred disk will be loaded into the developed software, AV Index calculator (Ciclorisk SL, Salamanca, Spain, registry no. 00/2011/589). This software automatically recognises the disk and draws two external concentric circles which delimit area A, 0–0.5 disk diameters from the optic disk margin, and area B, 0.5–1 disk diameters from the margin. The software first identifies the limits of the different vessels, after which it automatically recognises arteries and veins, and makes multiple measurements of the diameter of the section of vessels circulating through area B. It finally estimates the mean calibre of veins and arteries in millimetres, and these measurements are summarised as an arteriole-venule ratio, arteriovenous index (AVIx). An AVIx of 1.0 suggests that arteriolar diameters are on average the same as venular diameters in that eye, whereas a smaller AVR suggests narrower arterioles. The pairs of main vessels in the upper and lower temporal quadrants will be used, rejecting all other vessels, to improve reliability and increase efficiency of the process, analysing measures for each quadrant separately and together to estimate the mean measure in each eye.

Target organ damage

Renal assessment

Kidney damage will be assessed by measuring the estimated glomerular filtration rate using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI)⁶³ equation and proteinuria, as assessed by the albumin/creatinine ratio following the criteria of the 2013 European Society of Hypertension/European Society of Cardiology Guidelines.² Subclinical organ damage will be defined as a glomerular filtration rate below 30–60 mL/min/1.73 m² or microalbuminuria (30–300 mg/24 h) or albumin-creatinine ratio (30–300 mg/g; 3.4–34 mg/mmol) (preferably on morning spot urine). Renal disease will be defined as a glomerular filtration rate <30 mL/min/1.73 m² (BSA), proteinuria (>300 mg/24 h) or albumin/creatinine ratio >300 mg/24 h.²

Cardiac assessment

ECG examination will be performed using a General Electric MAC 3.500 ECG System (Niskayuna, New York, USA) that automatically measures wave voltage and duration and estimates the criteria of the Cornell voltage-duration product (VDP).⁶⁴ ECG LVH will be defined as a Sokolow-Lyon index >3.5 mV; RaVL >1.1 mV, Cornell VDP >244 mV*ms or RaVL >1.1 mV.²

PWV and 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 mathematical transformation to estimate the aortic pulse wave and CAIx. CAIx will be estimated from aortic wave morphology using the following formula: increase in central pressure $\times 100 / \text{pulse pressure}$. Carotid and femoral artery pulse waves will be analysed, with the patient in a supine position, using the SphygmoCor System (Vx PWV), estimating the delay as compared to the ECG wave and calculating PWV. Distance measurements will be taken with a measuring tape from the sternal notch to the carotid and femoral arteries at the sensor location. Subclinical organ damage will be defined as a carotid-femoral PWV >10 m/s.^{2 65}

Assessment of vascular structure using carotid IMT

Carotid ultrasound to assess C-IMT will be performed by two investigators trained for this purpose before study start. A Sonosite Micromax ultrasound device paired with a 5–10 MHz multifrequency high-resolution linear transducer with Sonocal software will be used for performing automated measurements of carotid IMT in order to optimise reproducibility. Measurements will be made of the common carotid after examination of a 10 mm longitudinal section at a distance of 1 cm from the bifurcation. Measurements will be taken in the proximal wall, 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. A total of six measurements will be taken of both the right carotid and left carotid arteries, using average values (average carotid IMT) and maximum values (maximum carotid IMT) automatically calculated by the software.⁶⁶ Measurements will be taken with the participant lying down, with the head extended and slightly turned opposite to the examined carotid artery. Average IMT will be considered abnormal if >0.90 mm or if there are atherosclerotic plaques with a diameter of 1.5 mm or a focal increase of 0.5 mm or 50% of the adjacent IMT.²

Evaluation of peripheral artery involvement

This will be assessed using the ankle-brachial index, calculated in the morning in patients who have not drunk coffee or smoked tobacco for at least 8 h before

measurement and at a room temperature of 22–24°C. With the patient lying in a supine position and with the feet uncovered, pressure in the lower limbs will be measured after resting for 20 min using a portable WatchBP Office for assessing the ankle–brachial index (Microlife AG Swiss Corporation Espenstrasse 139, CH-9443 Widnau/Switzerland). The ankle–brachial index will be calculated automatically for each foot by dividing the higher of the two systolic pressures in the ankle by the higher of the two systolic pressures in the arm. An ankle–brachial index <0.9 will be considered abnormal.²

Laboratory measurements

Venous blood sampling will be performed between 8:00 and 9:00 after participants have fasted and abstained from smoking and consumption of alcohol and caffeinated beverages for 12 h. Fasting plasma glucose, creatinine, uric acid, serum total cholesterol, high-density lipoprotein cholesterol and triglyceride levels will be measured using standard enzymatic automated methods. Low-density lipoprotein cholesterol will be estimated by the Friedewald equation when the direct parameter is not available. Glycated haemoglobin will be measured using an immune turbidimetric assay. High-sensitive C reactive protein and fibrinogen levels will be measured by an immune turbidimetric assay. Insulin blood levels will be measured using a chemiluminescent microparticle immunoassay. Insulin sensitivity will be determined with the Homeostasis Model Assessment Insulin Resistance (HOMA-IR) index⁶⁷ using the following formula: fasting glucose (mmol/L)×fasting insulin (mU/mL)/22.5.

Superoxide anion and catecholamine (epinephrine and norepinephrine) levels in plasma and urine will be measured using ELISA (CatCombi ELISA, IBL, Deventer, The Netherlands) following the manufacturer's instructions. Superoxide dismutase activity in plasma will be assessed using ELISA kits (Cu-Zn Superoxid-dismutase ELISA, IBL), and lipid peroxide plasma levels and urinary excretion by measuring thiobarbituric acid reactive substances (TBARS; OxiSelect TBARS Assay Kit, Cell Biolabs, Inc, San Diego, California, USA).

Polymorphisms in the VAV-2 and VAV-3 genes will be tested in all study participants in a DNA sample from peripheral blood erythrocytes. For this purpose, 10 mL of venous blood anticoagulated with EDTA will be taken.

People who perform the different tests will be blinded to the clinical patient data. All organ damage assessments will be made within 10 days.

Statistical analysis

Normal distribution of variables will be verified using a Kolmogorov-Smirnov test. Quantitative variables will be displayed as the mean±SD if normally distributed or as the 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's t test or a Mann-Whitney U test, as appropriate, while qualitative

variables will be analysed using a χ^2 test. To analyse 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. The relationship of quantitative variables to each other will be tested using Pearson's or Spearman's correlation as appropriate. A multivariate analysis of variance will be performed with analysis of covariance and multivariate analysis of variance to adjust results for potentially confounding variables. Logistic regression will be used to analyse clinical, biochemical and genetic variables associated with the presence of cardiac, vascular and renal TOD. IBM SPSS/PC + statistical software V.20.0 will be used. Data will be computed using a Teleform system in a questionnaire previously designed for the project and exporting data to SPSS software for subsequent analysis.

Quality control

Different processes will be carried out to guarantee study data quality and thus maximise the validity and reliability of measurements of the results. To this effect, field work operation manuals have been prepared which specify the adequate procedure for performing each test. Educational leaflets will be developed to ensure adequate pressure measurement by patients at home. All of these actions will confirm adequate performance of each procedure. Monthly meetings will be held with the principal investigator of the study to analyse the entire process, and an annual report on study progress will be prepared.

Ethical and legal issues

All study participants will sign an informed consent to agree to participate in the study, and another consent to agree on the genetic study, in compliance with the Declaration of Helsinki⁵⁸ and the WHO standards for observational studies. The study will involve collection and storage of biological samples, and study participants will therefore be thoroughly informed. Sample traceability will be maintained until study completion, after which samples will be irreversibly dissociated from personal data or destroyed, as decided by the study participant. Owing to the foregoing, confidentiality of participants enrolled into the study will be guaranteed at all times in accordance with provisions in the Spanish Organic Act on Personal Data Protection (15/1999, of 13 December, LOPD) and under the conditions set down in Act 14/2007 on Biomedical Research.

DISCUSSION

BP measurement, both at the clinic and on an out-patient, is a widespread clinical practice, and its direct correlation to mortality and morbidity has been extensively studied.¹ However, association of 24 h ambulatory BP, central haemodynamic parameters and AVI of retinal vessels with presence and occurrence of TOD (renal, cardiac and vascular) has not been collectively analysed

in the same group of patients. Similarly, we do not know the relationship of TOD and all other parameters assessing vascular function with oxidative stress and catecholamine excretion in urine. Also, it is not known if there are certain genetic polymorphisms in the VAV-2 and VAV-3 genes predisposing to the occurrence of TOD in patients with hypertension.

The impact of ABPM and HABP parameters on TOD occurrence has not been fully established, but there are studies relating BP variability and night-time elevation to the presence of TOD. However, we must still analyse all other parameters and quantify the association in order to be able to predict with greater precision the occurrence of TOD as a function of the BP parameter evaluated.

Measurement of central BP and augmentation index is of great interest for mechanistic analyses in pathophysiology, pharmacology and therapeutics. Measurement of central BP, as compared to brachial BP, in patients with hypertension raises increasing interest because of its predictive value for CV events and the differential effect of antihypertensive drugs. Arterial pressure waveform is a composite of the forward pressure wave created by ventricular contraction and a reflected wave.² It should be analysed at the central level, that is, in the ascending aorta, because it represents the true load imposed on the heart, brain, kidney and large arteries. Early epidemiological studies in the 2000s showed that CAIx and pulse pressure, directly measured by carotid tonometry, were independent predictors of all-cause and CV mortality in patients with end-stage renal disease.² A recent meta-analysis confirmed these findings in several populations.²⁸ However, the additive predictive value of central BP beyond brachial BP was either marginal or not statistically significant in most studies.²⁸

Although there is some evidence of an association between oxidative stress and CVD, the relationship between molecules analysing oxidative stress and TOD occurrence is currently not known. Our group has also shown that sympathetic activation and oxidative stress have been found to be the basis for CV and renal damage in other hypertension models.^{68–69} Similarly, there are no studies analysing the relationship between the presence of TOD and polymorphisms in the VAV-2 and VAV-3 genes.

To sum up, we hope that the results of this study will contribute to a clarification of the association between occurrence of TOD and the above discussed variables.

Study limitations

The cross-sectional design of this study may lead us to observe an association, but not its direction over time. There may also be confounding factors which have not been considered. On the other hand, some patients with hypertension are on a non-homogeneous antihypertensive therapeutic regimen. Although these drugs may have an impact on BP measurements and certain TODs, they cannot be discontinued for ethical reasons. They will, however, be controlled in the analysis phase. Finally, assignment by consecutive sampling prevents generalisation of results.

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Contributors MAG-M and LG-O participated in the conception of the idea for the study. MAG-M, LG-O, JIR-R and CA-C participated in the development of the protocol, organisation and funding. MAG-M participated in the writing of the manuscript. RGS and CM-S participated in the design of the study and contributed to the discussion, writing and review of the manuscript. All authors have read the draft critically to make contributions and also approved the final text. The project will be conducted by the LOD-Hipertensión group.

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Competing interests None.

Patient consent Obtained.

Ethics approval The study will be conducted after approval is obtained from the Ethics Committee of Hospital Clínico Universitario of Salamanca.

Provenance and peer review Not commissioned; peer reviewed for ethical and funding approval prior to submission.

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