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Title: **Validation of the Automatic image analyzer to assess retinal vessel caliber (*ALTAIR*).Prospective study protocol.**

Short title: **Altair study**

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ABSTRACT

Introduction: The fundus examination is a non-invasive evaluation of the microcirculation of the retina. The aim of the present study is to develop and validate (reliability and validity) the *Altair* software platform (Automatic image analyser to assess retinal vessel calibre) in order to analyse its utility in different clinical environments.

Methods and analysis:A cross-sectional study in the first phase and a prospective observational study in the second with 4-year of follow-up. The study will be performed in a primary care including 386 subjects. The main measurements will include carotid intima-media thickness, pulse wave velocity by Sphygmocor , cardio-ankle vascular index through the VASERA VS-1500®, cardiac evaluation by a digital ECG and renal injury by microalbuminuria and glomerular filtration. The retinal vascular evaluation will be performed using a TOPCON TRCNW200 non-mydriatic retinal camera to obtained digital images of the retina, and the developed software (*Altair*) will be used to automatically calculate the caliber of the retinal vessels, the vascularised area, and the branching pattern. For software validation, the intra and inter-observer reliability, the concurrent validity of the vascular structure and function, as well as the association between the estimated retinal parameters and the evolution or onset of new lesions in the target organs or cardiovascular diseases.

Ethics and dissemination: The study has been approved by the clinical research ethics committee of the healthcare area of Salamanca. All study participants will sign an informed consent to agree to participate in the study in compliance with the Declaration of Helsinki and the WHO standards for observational studies. Validation of this tool will provide greater reliability to the analysis of retinal vessels by decreasing the intervention of the observer and will result in increased validity through the use of additional information, especially in the areas of vascularisation and vessel branching patterns.

Trial registration number: Clinical Trials.gov Identifier: NCT02087605

Key words: Retinal vessel. Vascular stiffness. Cardiovascular diseases. Risk Assessment.

Strengths and limitations of this study

1.-A new software to retinal vassels evaluation will be developed, the *Altair* platform.

2.-A complete evaluation of retinal vascularization, including an automatically calculate the caliber of the retinal vessels, the vascularised area, and the branching pattern will be performed.

3.-The inter and intra-observer reliability in determining the caliber of arterial and venous vessels, the vascularised surface, and branching patterns using the Altair software platform will be performed.

4.-The concurrent validity of the Altair software platform, by analysing the relationship between retinal parameters and other parameters of vascular structure and function will be performed

5.-The design of the study during the first phase is transversal; as a result, causality relations cannot be derived, i.e., only the associations among the analysed variables can be performed.

INTRODUCTION

The fundus examination is a non-invasive evaluation of the retinal microcirculation and of the vascular damage caused by multiple cardiovascular risk factors. Different tools have been developed to evaluate the thickness of the retinal arteries and veins [1-7], yet, all of them require intervention of an observer to some extent. Moreover, the techniques and tools that are currently applied are manual or semiautomatic in nature, for which the observer has an important influence, thus providing limited information.

In population studies, an association has been found between the calibre of the retinal vessels and arterial hypertension [8], left ventricular hypertrophy [9], metabolic syndrome [10], stroke [11], and coronary heart disease [12], especially in women [13]. However, other studies disagree and show contradictory results regarding the evolution of the arteriosclerotic lesion and the calibre of the retinal vessels [14-16]. In this way, Cuspidi et al. [17] and Masaidi et al. [18] failed to detect an association between the calibre of the retinal vessels and target organ injuries (cardiac, vascular, and renal) in studies of two hypertensive populations. However, Torres et al. reported a negative association between carotid intima-media thickness (IMT) and the thickness of the retinal arteries but a positive association with the veins [19].

Recently, our group developed and validated a semiautomatic tool, the arteriovenous index calculator, to evaluate the vascular calibre of the retinal vessels [20] with reduced influence of the observer. This tool showed high reliability when measuring the calibre of the retinal vessels with an intraclass correlation coefficient (ICC) for intra- and inter-observers greater than 0.96 for veins, arteries, and the arteriovenous ratio (AVR). These measures, especially the venous calibre and the AVR, were also shown to be independent variables associated with estimated cardiovascular risk, according to the Framingham scale and the microvascular kidney lesions evaluated according to the level of microalbuminuria. This positive association between the cardiovascular risk and the venous calibre is in line with several published studies showing an association between the AVR and the risk of coronary heart disease [12 13 21]. However, longitudinal studies with a greater number of patients would help to clarify the discrepancies among previously published studies on cardiovascular risk and vascular structure and function. Moreover, it should not be forgotten that these tools provide less information than retinal imaging on the thickness of the arteries and veins, their branching patterns, and the vascularised areas, which may be relevant for

evaluating the status of the vascular tree and may be the cause of some of the discrepancies previously reported.

A new and different approach to the study of the vascular systems is the characterisation of the blood vessel patterns in the normal circulation of the human retina [22]. With this method, the distribution of the branching of the vascular system in a two-dimensional space can be analysed, and the geometrical complexity of the branching and the density of the retinal vessels can be quantified [23]. Indeed, the current scientific literature contains a number of publications on this subject [24-27].

In the present study, a novel software platform for image processing of the structural properties of the vessels is proposed using a human fundus red-free camera. The "Automatic image analyser to assess retinal vessel calibre" (*Altair*) software platform employs analytical methods and artificial intelligence (AI) algorithms to detect the retinal parameters of interest. The sequence of the algorithms consists of a new methodology that can be used to determine the properties of the veins and arteries of the retina; together, this system unifies all of the methods for automation of the measuring processes of retinal vessels.

Therefore, the **general aim** of the present study is the development and validation (reliability and validity) of the *Altair* software platform in order to analyse its utility in different clinical settings. The following specific objectives will be studied:

i) To evaluate inter- and intra-observer reliability in determining the caliber of arterial and venous vessels, the vascularised surface, and branching patterns using the *Altair* software platform.

ii) To evaluate the concurrent validity of the *Altair* software platform, in different populations, by analysing the relationship between retinal parameters and other parameters of vascular structure and function, including carotid IMT, pulse wave velocity (PWV), and the cardio-ankle vascular index (CAVI), as well as injuries in other target organs and the cardiovascular risk.

iii) To evaluate the evolution of target organ injuries and cardiovascular morbidity and mortality according to the vascularisation parameters of the retina determined using the *Altair* software platform.

METHOD AND ANALYSIS

Study design

The first phase will be a cross-sectional study aimed at validating the developed tool. Subsequently, the second phase will consist of a prospective observational study with annual follow-up evaluations over 4 years. The study will be developed in a primary healthcare setting.

Subjects

Study population

The population under study will consist of subjects from 35 to 74 years of age with a cardiovascular risk factor according to the 2013 European Society of Hypertension/European Society of Cardiology Guidelines [28]. Subjects were excluded due to the following criteria: psychic or cognitive disorders that interfere with the established requisites of the protocol; non-collaborative attitude; educational or comprehensive limitations; and severe comorbidities with a 12-month likelihood of life-threatening complications. A consecutive sampling of all patients sent to the research unit for cardiovascular risk evaluation will be performed, and those complying with the inclusion and exclusion criteria will be asked to participate until the estimated sample size is achieved.

Sample size

The sample size has been set to detect a correlation coefficient among the pulse wave velocity (PWV), the gold standard measure of arterial stiffness, and the retinal parameters of 0.15, with an alpha risk of 0.05 and a beta risk of 0.20 and a 10% estimated loss regarding the difficulty of the technique or dropout on follow-up. As a result, a total of 386 patients will be included in this study. This number of patients will be adequate to detect a difference of 1 m/sec on the PWV among the AVR tertiles, considering a standard deviation (SD) of 2.22 m/sec with an alpha risk of 0.05 and a beta risk of 0.20.

Variables and measurement instruments

The general and potentially effect-modifying variables, such as age, gender, occupation, smoking, alcohol consumption, personal history and drug use will be documented.

Laboratory determinations

Venous blood sampling will be performed between 08:00 and 09:00 hours after the individuals fasted and abstained from smoking and the consumption of alcohol and caffeinated beverages for the previous 12

hours. Fasting plasma glucose, creatinine, uric acid, serum total cholesterol, HDL-cholesterol and triglyceride concentrations will be measured using standard enzymatic automated methods. LDL cholesterol will be estimated by the Friedewald equation when the direct parameter will be not available. Glycated haemoglobin will be measured with an immune-turbidimetric assay. High sensitive C-reactive protein levels and fibrinogen concentrations will be determined by immunoturbidimetric assay. Blood samples will be collected in the health center, and will be analyzed at the University hospital of Salamanca in external quality assurance programs of the Spanish Society of Clinical Chemistry and Molecular Pathology.

Anthropometric measurements

Body weight will be determined on two occasions using a homologated electronic scale (Seca 770; Medical scale and measurement systems, Birmingham, United Kingdom) following due calibration (precision ± 0.1 kg), with the patient wearing light clothing and shoeless. These readings will be rounded to 100 g. Height in turn will be measured with a portable system (Seca 222; Medical scale and measurement systems, Birmingham, United Kingdom), recording the average of two readings, and with the patient shoeless in the standing position. The values will be rounded to the closest centimeter. Body mass index (BMI) will be calculated as weight (kg) divided by height squared (m^2). A value of > 30 kg/m^2 will be taken to define obesity. 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 will be adjusted without compressing the skin. Adiposity indices, waist-height and waist-hip, will also be calculated.

Office or clinical blood pressure

Office blood pressure measurement will involve three measurements of systolic blood pressure (SBP) and diastolic blood pressure (DBP), using the average of the last two, with a validated OMRON model M10-IT sphygmomanometer (Omron Health Care, Kyoto, Japan), by following the recommendations of the European Society of Hypertension[29]. Pulse pressure will be estimated with the mean values of the second and third measurements.

Pulse wave velocity (PWV) and central (CAIx) and peripheral augmentation index (PAIx)

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. 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 heart rate at 75 bpm. PAIx is a measurement taken directly from the late systolic shoulder of the peripheral arterial waveform, and is defined as the ratio of the difference between the 2nd peak and diastolic pressure to the difference between the 1st peak and diastolic pressure[30], it is age-dependent and could be a useful index of vascular aging [31]. PAIx will be calculated as follows: (Second peak systolic blood pressure [SBP2] - diastolic blood pressure [DBP])/(first peak SBP - DBP) × 100 (%) and it will be corrected for heart rate at 75 bpm and it will be reported as PAIx75. Carotid and femoral artery pulse waves will be analyzed, with the patient in a supine position, using the SphygmoCor System (Vx pulse wave velocity), 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 and will be multiplied by 0.8. Subclinical organ damage of PWV will be defined as a carotid–femoral PWV >10 m/s [28 32].

Assessment of vascular structure by carotid intima media thickness (IMT)

Carotid ultrasound to assess C-IMT will be performed by two investigators trained for this purpose before starting the study. A Sonosite Micromax ultrasound device paired with a 5–10 MHz multi-frequency high-resolution linear transducer with Sonocal software will be used for performing automatic measurements of carotid IMT in order to optimize reproducibility. Measurements will be made of the common carotid after the examination of a 10 mm longitudinal section at a distance of 1 cm 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. A total of 6 measurements will be obtained of the right carotid and other 6 of the left carotid, using average values (average carotid IMT) and maximum values (maximum carotid IMT) automatically calculated by the software [33]. The measurements will be obtained

with the subject lying down, with the head extended and slightly turned opposite to the examined carotid artery. The reliability was evaluated before the study began using the intraclass correlation coefficient, which showed values of 0.974 (95%CI: 0.935 to 0.990) for intra-observer agreement on repeated measurements in 20 subjects, and 0.897 (95%CI:0.740 to 0.959) for inter-observer agreement. According to the Bland-Altman analysis, the mean difference for intraobserver agreement (95% limits of agreement) was 0.022 (95%CI: -0.053 to 0.098) and intra-observer agreement was 0.012 (95%CI: -0.034 to 0.059). The average IMT will be considered abnormal if it measured > 0.90 mm, or if there will be atherosclerotic plaques with a diameter of 1.5 mm or a focal increase of 0.5 mm or 50% of the adjacent IMT [28].

Cardio Ankle Vascular Index and Ankle-brachial index

Cardio Ankle Vascular Index (CAVI) and Ankle-brachial index (ABI) will be measured using Vasera device VS-1500® (Fukuda Denshi). The pulse wave velocity (PWV) will be calculated, as well as Cardio Ankle Vascular Index (CAVI), which gives a more accurate calculation of the atherosclerosis degree. CAVI integrates cardiovascular elasticity derived from the aorta to the ankle pulse velocity through an oscillometric method and it is used as a good measure of vascular stiffness and it doesn't depend on blood pressure [34]. CAVI values will be automatically calculated by substituting the stiffness parameter ß in the following equation to detect the vascular elasticity and the cardio-ankle PWV: Stiffness parameter ß = $2\rho \times 1/ (Ps - Pd) \times \ln (Ps/Pd) \times PWV^2$, where ρ is the blood density, Ps and Pd are SBP and DBP in mmHg, and the PWV is measured between the aortic valve and ankle. The average coefficient of the variation of the CAVI is less than 5%, which is small enough for clinical use and confirm that CAVI has favorable reproducibility [35]. Cardio Ankle Vascular Index and Ankle-brachial index will be measured at rest. For the study, the lowest ABI and the highest CAVI and PWV obtained will be considered.

Renal assessment

Kidney damage will be assessed by measuring estimated glomerular filtration rate using the CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration) [36] 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 [28] Subclinical organ damage will be defined as a glomerular filtration rate below 30–60 ml/min/1.73 m^2 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 m^2 (BSA), proteinuria (>300 mg/24 h),.or albumin/creatinine ratio > 300 mg/24 h [28].

Cardiac assessment

Electrocardiographic 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 (Cornell VDP) [37]. Electrocardiographic LVH will be defined as a Sokolow–Lyon index >3.5 mV; RaVL >1.1 mV, Cornell voltage duration product >244 mV*ms or RaVL >1.1 mV; [28].

Cardiovascular risk assessment

Cardiovascular risk will be estimated using the score of the 2013 European Society of Hypertension/European Society of Cardiology Guidelines[28] and the risk equation (D'Agostino scale) based on the Framingham study [38]. Risk factors used include age, sex, total cholesterol, high-density lipoprotein cholesterol (HDL-C), and systolic blood pressure as quantitative variables, and drug treatment for hypertension, smoking, and history of diabetes mellitus as dichotomous variables.

Retinal vascular evaluation

Using a non-mydriatic retinography, TOPCON TRC NW 200, (Topcon Europe B.C., Capelle a/d Ijssel, The Netherlands) in the sitting position, a nurse trained will get nasal and temporal images centered in papilla. Then using the specific software developed (*Altair*) will be automatically calculated the retinal vessels thickness, the AVR, the area vascularized and the pattern of branching.

Development of *Altair* platform: *Automatic image analyzer to assess retinal vessel caliber*

The platform, called Altair "Automatic image analyzer to assess retinal vessel caliber", makes use of a methodology divided in different stages that are described below, to determine the characteristics of interest of the veins and arteries of the retina. This methodology uses artificial Intelligence (AI) techniques and analytical algorithms to discover retinal parameters of interest.

The methodology is separated into two phases: (i) Digitization of the retina, in which the different measures of the eye image are recognized. Here a data structure is created, which makes it possible to represent and process the retina. This phase is subdivided into the following steps as discussed below: load image and eye detection, processing, detection and segmentation. (ii) Measurements, in which we

work with retinas that have been previously identified. This phase includes extraction of knowledge and manual correction, or expert knowledge, if necessary.

Digitization of the retina: To perform this phase, the following steps are necessary:

1. Load image and eye detection: The platform will automatically try to determine which eye (left or right) is the image, based on the detection of the macula. In this step, if the automatic detection has been wrong, the supervisor can modify this value by simply clicking on the correct eye.
2. Processing: In this step, then noise is reduced, the contrast is improved, the blurriness corrected and the edges are sharpened. Some of these actions can be carried out at the hardware level, which is to say with the features included with the camera. During the testing, retinography will be performed using a Topcon TRC NW 200 nonmydriatic retinal camera, obtaining nasal and temporal images centered on the disk.
3. Detection limits: In this step, the platform is capable of locating the disk and identifying the center and edges of the retina (figure 1). The identification of the papilla is vital since it helps as the starting point for the detection and identification of the different blood vessels. The platform builds a data structure that identifies each part of the retina based on the matrices of colors representing the images obtained. In this step, image processing techniques [24 26] will be used to detect intensity based on the boundaries of the structures.
4. Segmentation: In order to detect the limits, it became necessary to carry out a process of image segmentation. Segmentation is the process that divides an image into regions or objects whose pixels have similar attributes. Each segmented region typically has a physical significance within the image. It is one of the most important processes in an automated vision system because it makes it possible to extract the objects from the image for subsequent description and recognition [39-41]. This step can be considered the heart of the methodology proposed and used in the platform and perform the following actions:
    a. Identification of vessels. Blood vessels are identified in the image by thresholding techniques. Their purpose is to remove pixels where the structuring element does not enter, in this case the blood vessels. The platform offers here a number of useful options for experts: Threshold vessels, in order to modify the threshold level automatically taken to a

new vessel detection. Recalculate vessels, recalculate the vessels taking as the threshold established with the previous parameter. Pencil/eraser thickness: Sets thickness to draw or erase lines / vessels to switch vessels. Connect: Selecting this option and interacting on the overall image of the retina, the application connects those vessels whose structure has been divided as they were not detected section.

b. Structure of vessel. At the end of this stage the entire arterio-venous tree is stored in a structured way, making it possible to know not only if a vessel passes through a point or not, but through which point each vessel passes, which one is its parent, etc.

c. Cataloging of veins and arteries (Figure 2). In this step the platform detects whether a vessel is vein or artery, main branch is taken of the vessel. In this step different classifiers based on IA as decision trees and Bayesian networks [42] are applied.

Measurements

In this second phase, the results obtained are presented. The platform can display the following measures: thickness of the veins in µm, artery thickness in µm, AVR (ratio between the thickness of the arteries and veins thickness), veins area in square µm, artery area in square µm, area of all vessels in square µm, radio papilla in millimeters.

The platform also generates internally combined parameters by quadrants and circles. These values are visible when it comes to export CSV files either individually or together with other existing images already processed database values.

Results of its validity analysis must be consistent with the findings from researches focused on both cardiovascular risk estimation and evaluation of target organ damage. The results obtained during the use of the platform will be connected and used to extract additional information by using reasoning models such as case-based reasoning (CBR) [43] [44].

As conclusion, the platform is robust to the changes in the appearance of retinal fundus images typically encountered in clinical environments, and is proposed as a unified platform to connect all the methods needed to automate all processes of measurement on the retinas.

Retinal software validation

To validate the retinal software platform, the following steps will be completed by the evaluators after previous training in imaging appreciation. 1. Intra-observer variability: To evaluate the measurement repeatability, the operator must measure the same image of an individual on at least two occasions. To this end, an operator will measure 100 images of a random subsample of 50 patients with a 1-week difference between the two measurements. In this case, the operator and the analysed images will be the same on both days, and the information from the previous measurement will be unknown. 2. Inter-observer variability: To evaluate the reproducibility of the measurement system, a different operator than who completed the assessment in phase 1 will evaluate the same 100 images previously analysed. The information from the results obtained in the previous phase will be unknown to this operator, and both operators will have the same experience in the subject and pertaining to the use of the software. Furthermore, both operators will receive the same preparatory training. 3. With the developed retinal software, the program will provide results on the AVR and the vein and artery thickness. To assess the degree of agreement between these methods, i.e., the AVR and *Altair*, the evaluation of 100 images will be performed using both tools. In this way, we will be able to demonstrate that the new method, apart from providing the same results, is more objective and faster in elaborating the results. 4. The measurement validity will be analysed in a total sample of 386 subjects and 772 retinographies, in regards to the relationship between the results of the carotid IMT, as a measurement of vascular structure, the PWV, the gold standard measure of arterial stiffness (arterial stiffness), the CAVI, kidney function, electrocardiographic parameters, and the estimated cardiovascular risk using different scales. 5. The association between different estimated parameters of the retina with the evolution or onset of new lesions in the target organs will be analysed, as well as any cardiovascular events that occur during the 4-year follow-up of the second phase of this project.

Statistical analysis

The data corresponding to quantitative variables with a normal distribution will be presented with the mean and standard deviation and with the median and an interquartile range if the distribution is asymmetric, while the qualitative variables will be presented according to the distribution of the frequencies. Normality will be evaluated with the Kolmogorov–Smirnov test. The Pearson's Chi-squared test will be used to

analyse associations between the qualitative variables. The mean comparison, in the case of two groups, will be performed using the Student's t-test for independent samples and, in the case of larger groups, the ANOVA test. Post-hoc contrasts will be performed with the least significant difference (LSD) method and an alpha value <0.05. Repeated data will be analysed with the Student's t-test for paired data. The relationship between quantitative variables will be analysed according to the Pearson's or Spearman's correlation coefficient, depending on the type of distribution being considered. Finally, a multivariate analysis with multiple lineal regressions will be performed to analyse the association of the retinal parameters generated by *Altair* with the vascular structure and function. To contrast the hypothesis, an alpha risk of 0.05 will be set as the limit of statistical significance. Statistical analysis will be performed using SPSS/PC+ software version 20.0.

For validation of the retinal software, we will evaluate the measurements of artery and vein thickness, vascularised surfaces, and branching patterns from the three phases of validation, and the intraclass correlation coefficient (ICC) will be calculated as a comparative method. Using the Bland-Altman method, the limits of agreement between the measurements of the observers will be evaluated. The kappa agreement coefficient will be analysed to categorise the variable. This coefficient will allow us to evaluate the degree of agreement between the two methods. The accordance validity will also be analysed via correlation and multiple regression analysis, by evaluating the degree of association with other parameters of vascular structure and function and target organ injury.

Project schedule

This project is on a 5-year plan, with the aim of developing and validating the *Altair* software platform during the first year. Subsequently, a 4-year follow-up study will be performed to evaluate the evolution of target organ injuries, as well as the cardiovascular risk on the analysed retinal vascularisation parameters, i.e., the artery and vein thickness, the vascularised surface, and the branching patterns. An exhaustive evaluation of the subject will be performed at baseline and during the third and fifth years. A short evaluation will be performed during the second and fourth years.

Quality control

In order to ensure data quality, the professionals in charge of assessment retinal images and data collection will receive specific training. Regular external monitoring will then be performed to verify

adequate application of methods, both in performing the different examinations and collecting the information.

Ethical and legal issues

The study has been approved by the clinical research ethics committee (CEIC) of the healthcare area of Salamanca ("CEIC of Area de Salud de Salamanca", 29 January, 2014). Subjects will be required to sign the informed consent prior to inclusion in the study, in accordance with the Declaration of Helsinki [45]. Subjects will be informed of the objectives of the project and of the risks and benefits of the examinations made. None of the examinations pose life-threatening risks for the type of subjects to be included in the study. The study includes the obtainment of biological samples; the study subjects therefore will be informed in detail. The confidentiality of the recruited subjects will be ensured at all times in accordance with the provisions of current legislation on personal data protection (15/1999 of 13 December, LOPD), and the conditions contemplated by Act 14/2007 on biomedical research.

Discussion

In a previous study performed by our group [20], we identified a positive association between the cardiovascular risk, estimated with the Framingham scale, and the retinal vessel calibre, mainly the venous calibre, and this association was maintained in the multivariate analysis. The arterial calibre, which also demonstrated a positive association, and the AVR, which demonstrated a negative association, seem to play a less relevant role than the venous calibre in regards to the cardiovascular risk.

The positive association between the cardiovascular risk and the venous calibre is in accordance with several published studies showing an association between the AVR and the coronary heart disease risk [12 13 21]. For example, McGeecham et al. [21], showed that when including the arterial and venous thickness, the Framingham model improved coronary event prediction, but only in women. This finding was repeated in several studies and was recently reported in a meta-analysis [13], which seems to support the notion that microvascular disease could be more important than coronary heart disease in women than men [46]. However, these results have been variable, as Wong et al. in the Beaver Dam Eye study [47] described the lack of an association between the AVR and the cardiovascular morbidity and mortality in the examined subgroups. In addition, Wang JJ et al.[48] identified an association between venous calibre and coronary death in men but not in women.

It is likely that some of these discrepancies are due to the different tools employed for evaluation of the retinal vessels, as all of these methods are manual or semiautomatic and, to a greater or lesser degree, are susceptible to the influence of the observer. The use of only particular information regarding the vascularisation of the retina may also influence the results. However, the *Altair* software platform resolves these problems. On the one hand, this platform is practically an automated tool with a low observer influence. In addition, we expect the inter- and intra-observer variability to be low and, as a result, the reliability to be significantly elevated. On the other hand, by using additional information on retinal vascularisation, the vascularised surface, the vascularisation patterns, and the artery and vein thickness, we expect to improve the validity of the tool and clarify the discrepancies reported in previous studies.

Study limitations

Data will be obtained from patients with a cardiovascular risk factor, who satisfy the inclusion criteria, and who were referred by a family physician to the Research Unit for vascular risk assessment. Thus, this approach uses a consecutive sampling method with inclusion criteria, i.e., a non-randomised sampling method. However, the size of the sample may buffer this limitation, and the real clinical conditions may lead us to a more real situation than that using more restrictive inclusion criteria for the study patients. The design of the study during the first phase is transversal; as a result, causality relations cannot be derived, i.e., only the associations among the analysed variables can be performed. Therefore, non-statistically detected associations between variables may be possible due to the sample size.

Abbreviations

ALTAIR: Automatic image analyzer to assess retinal vessel caliber: ABI: Ankle-brachial index; AIx: Augmentation Index; BMI: Body mass index; CAVI: Cardio Ankle Vascular index; CKD-EPI: Chronic Kidney Disease Epidemiology Collaboration; DBP: Diastolic blood pressure; ESH: European Society of Hypertension; IMT: Intima-media thickness; PWV: Pulse Wave Velocity; SBP: Systolic blood pressure.

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Collaborators. *Members of the Altair group:* Luis García-Ortiz, José I Recio-Rodríguez, Manuel A Gómez-Marcos, José A Maderuelo-Fernández, Sara Rodríguez-González, Juan F de Paz-Santana, Pablo Chamoso-Santos, Miguel Merchan-Cifuentes and Juan M Corchado-Rodríguez. María C Patino-Alonso, Emiliano Rodríguez-Sánchez, Diana Perez Arechaederra, Sara Mora Simón, Ángela de Cabo Laso, Carmela Rodriguez Martín, Luis F Valero Juan, Leticia Gómez-Sánchez, Cristina Agudo-Conde.

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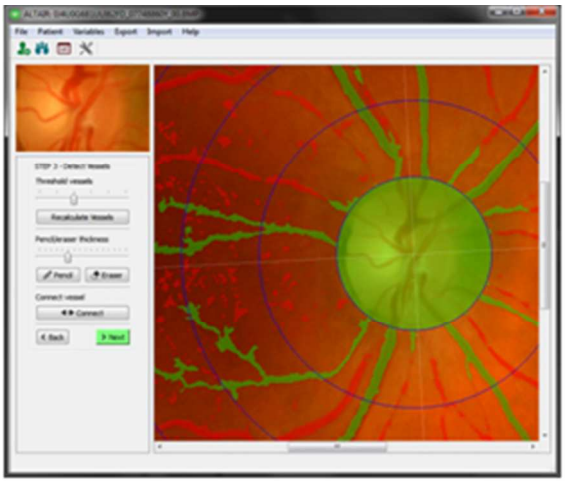
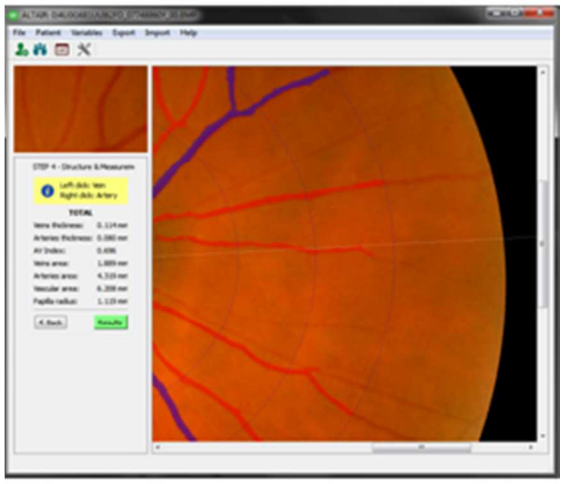
Figure 1. Detection and identification of vessels steps: locating the disk and identifying the center and edges of the retina

Figure 2. Detection and identification of vessels steps: cataloging of veins and arteries



Detection and identification of vessels steps: locating the disk and identifying the center and edges of the retina
75x62mm (96 x 96 DPI)



Detection and identification of vessels steps: cataloging of veins and arteries
73x64mm (96 x 96 DPI)

STROBE Statement—checklist of items that should be included in reports of observational studies

	Item No	Recommendation
Title and abstract	1	X (*a*) Indicate the study's design with a commonly used term in the title or the abstract
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found
Introduction		
Background/rationale	2	X Explain the scientific background and rationale for the investigation being reported
Objectives	3	X State specific objectives, including any prespecified hypotheses
Methods		
Study design	4	X Present key elements of study design early in the paper
Setting	5	X Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls *X Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case
Variables	7	X Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable
Data sources/ measurement	8*	*X* For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group
Bias	9	X Describe any efforts to address potential sources of bias
Study size	10	X Explain how the study size was arrived at
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why
Statistical methods	12	X (*a*) Describe all statistical methods, including those used to control for confounding
		(*b*) Describe any methods used to examine subgroups and interactions
		(*c*) Explain how missing data were addressed
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy
		(*e*) Describe any sensitivity analyses

Continued on next page

Results (not applicable)		
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed
		(b) Give reasons for non-participation at each stage
		(c) Consider use of a flow diagram
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders
		(b) Indicate number of participants with missing data for each variable of interest
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time
		Case-control study—Report numbers in each exposure category, or summary measures of exposure
		Cross-sectional study—Report numbers of outcome events or summary measures
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included
		(*b*) Report category boundaries when continuous variables were categorized
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses
Discussion (not applicable)		
Key results	18	Summarise key results with reference to study objectives
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence
Generalisability	21	Discuss the generalisability (external validity) of the study results
Other information		
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

BMJ Open

Validation of the Automatic image analyzer to assess retinal vessel caliber (ALTAIR).Prospective study protocol.

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Keywords:	VASCULAR MEDICINE, Medical retina < OPHTHALMOLOGY, PRIMARY CARE

Title: **Validation of the Automatic image analyzer to assess retinal vessel caliber (*ALTAIR*).Prospective study protocol.**

Short title: **Altair study**

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ABSTRACT

Introduction: The fundus examination is a non-invasive evaluation of the microcirculation of the retina. The aim of the present study is to develop and validate (reliability and validity) the *Altair* software platform (Automatic image analyser to assess retinal vessel calibre) in order to analyse its utility in different clinical environments.

Methods and analysis:A cross-sectional study in the first phase and a prospective observational study in the second with 4-year of follow-up. The study will be performed in a primary care including 386 subjects. The main measurements will include carotid intima-media thickness, pulse wave velocity by Sphygmocor , cardio-ankle vascular index through the VASERA VS-1500®, cardiac evaluation by a digital ECG and renal injury by microalbuminuria and glomerular filtration. The retinal vascular evaluation will be performed using a TOPCON TRCNW200 non-mydriatic retinal camera to obtained digital images of the retina, and the developed software (*Altair*) will be used to automatically calculate the caliber of the retinal vessels, the vascularised area, and the branching pattern. For software validation, the intra and inter-observer reliability, the concurrent validity of the vascular structure and function, as well as the association between the estimated retinal parameters and the evolution or onset of new lesions in the target organs or cardiovascular diseases.

Ethics and dissemination: The study has been approved by the clinical research ethics committee of the healthcare area of Salamanca. All study participants will sign an informed consent to agree to participate in the study in compliance with the Declaration of Helsinki and the WHO standards for observational studies. Validation of this tool will provide greater reliability to the analysis of retinal vessels by decreasing the intervention of the observer and will result in increased validity through the use of additional information, especially in the areas of vascularisation and vessel branching patterns.

Trial registration number: Clinical Trials.gov Identifier: NCT02087605

Key words: Retinal vessel. Vascular stiffness. Cardiovascular diseases. Risk Assessment.

Strengths and limitations of this study

1.-A new software to retinal vassels evaluation will be developed, the *Altair* platform.

2.-A complete evaluation of retinal vascularization, including an automatically calculate the caliber of the retinal vessels, the vascularised area, and the branching pattern will be performed.

3.-The inter and intra-observer reliability in determining the caliber of arterial and venous vessels, the vascularised surface, and branching patterns using the Altair software platform will be performed.

4.-The concurrent validity of the Altair software platform, by analysing the relationship between retinal parameters and other parameters of vascular structure and function will be performed

5.-The design of the study during the first phase is transversal; as a result, causality relations cannot be derived, i.e., only the associations among the analysed variables can be performed.

INTRODUCTION

The fundus examination is a non-invasive evaluation of the retinal microcirculation and of the vascular damage caused by multiple cardiovascular risk factors. Different tools have been developed to evaluate the thickness of the retinal arteries and veins [1-7], yet, all of them require intervention of an observer to some extent. Moreover, the techniques and tools that are currently applied are manual or semiautomatic in nature, for which the observer has an important influence, thus providing limited information.

In population studies, an association has been found between the calibre of the retinal vessels and arterial hypertension [8], left ventricular hypertrophy [9], metabolic syndrome [10], stroke [11], and coronary heart disease [12], especially in women [13]. However, other studies disagree and show contradictory results regarding the evolution of the arteriosclerotic lesion and the calibre of the retinal vessels [14-16]. In this way, Cuspidi et al. [17] and Masaidi et al. [18] failed to detect an association between the calibre of the retinal vessels and target organ injuries (cardiac, vascular, and renal) in studies of two hypertensive populations. However, Torres et al. reported a negative association between carotid intima-media thickness (IMT) and the thickness of the retinal arteries but a positive association with the veins [19].

Recently, our group developed and validated a semiautomatic tool, the arteriovenous index calculator, to evaluate the vascular calibre of the retinal vessels [20] with reduced influence of the observer. This tool showed high reliability when measuring the calibre of the retinal vessels with an intraclass correlation coefficient (ICC) for intra- and inter-observers greater than 0.96 for veins, arteries, and the arteriovenous ratio (AVR). These measures, especially the venous calibre and the AVR, were also shown to be independent variables associated with estimated cardiovascular risk, according to the Framingham scale and the microvascular kidney lesions evaluated according to the level of microalbuminuria. This positive association between the cardiovascular risk and the venous calibre is in line with several published studies showing an association between the AVR and the risk of coronary heart disease [12 13 21]. However, longitudinal studies with a greater number of patients would help to clarify the discrepancies among previously published studies on cardiovascular risk and vascular structure and function. Moreover, it should not be forgotten that these tools provide less information than retinal imaging on the thickness of the arteries and veins, their branching patterns, and the vascularised areas, which may be relevant for

evaluating the status of the vascular tree and may be the cause of some of the discrepancies previously reported.

A new and different approach to the study of the vascular systems is the characterisation of the blood vessel patterns in the normal circulation of the human retina [22]. With this method, the distribution of the branching of the vascular system in a two-dimensional space can be analysed, and the geometrical complexity of the branching and the density of the retinal vessels can be quantified [23]. Indeed, the current scientific literature contains a number of publications on this subject [24-27].

In the present study, a novel software platform for image processing of the structural properties of the vessels is proposed using a human fundus red-free camera. The "Automatic image analyser to assess retinal vessel calibre" (*Altair*) software platform employs analytical methods and artificial intelligence (AI) algorithms to detect the retinal parameters of interest. The sequence of the algorithms consists of a new methodology that can be used to determine the properties of the veins and arteries of the retina; together, this system unifies all of the methods for automation of the measuring processes of retinal vessels.

Therefore, the **general aim** of the present study is the development and validation (reliability and validity) of the *Altair* software platform in order to analyse its utility in different clinical settings. The following specific objectives will be studied:

i) To evaluate inter- and intra-observer reliability in determining the caliber of arterial and venous vessels, the vascularised surface, and branching patterns using the *Altair* software platform.

ii) To evaluate the concurrent validity of the *Altair* software platform, in different populations and ethnicities, by analysing the relationship between retinal parameters and other parameters of vascular structure and function, including carotid IMT, pulse wave velocity (PWV), and the cardio-ankle vascular index (CAVI), as well as injuries in other target organs and the cardiovascular risk.

iii) To evaluate the evolution of target organ injuries and cardiovascular morbidity and mortality according to the vascularisation parameters of the retina determined using the *Altair* software platform.

METHOD AND ANALYSIS

Study design

The first phase will be a cross-sectional study aimed at validating the developed tool. Subsequently, the second phase will consist of a prospective observational study with annual follow-up evaluations over 4 years. The study will be developed in a primary healthcare setting.

Subjects

Study population

The population under study will consist of subjects from 35 to 74 years of age with a cardiovascular risk factor according to the 2013 European Society of Hypertension/European Society of Cardiology Guidelines [28]. Subjects were excluded due to the following criteria: psychic or cognitive disorders that interfere with the established requisites of the protocol; non-collaborative attitude; educational or comprehensive limitations; and severe comorbidities with a 12-month likelihood of life-threatening complications. A consecutive sampling of all patients sent to the research unit for cardiovascular risk evaluation will be performed, and those complying with the inclusion and exclusion criteria will be asked to participate until the estimated sample size is achieved. The subjects will be mostly Caucasian, majority ethnic group among patients attended in the health center, however, at least fifty ethnic minority subjects, to give more validity to the tool, will be included.

Sample size

The sample size has been set to detect a correlation coefficient among the pulse wave velocity (PWV), the gold standard measure of arterial stiffness, and the retinal parameters of 0.15, with an alpha risk of 0.05 and a beta risk of 0.20 and a 10% estimated loss regarding the difficulty of the technique or dropout on follow-up. As a result, a total of 386 patients will be included in this study. This number of patients will be adequate to detect a difference of 1 m/sec on the PWV among the AVR tertiles, considering a standard deviation (SD) of 2.22 m/sec with an alpha risk of 0.05 and a beta risk of 0.20.

Variables and measurement instruments

The general and potentially effect-modifying variables, such as age, gender, occupation, smoking, alcohol consumption, personal history and drug use will be documented.

Laboratory determinations

Venous blood sampling will be performed between 08:00 and 09:00 hours after the individuals fasted and abstained from smoking and the consumption of alcohol and caffeinated beverages for the previous 12 hours. Fasting plasma glucose, creatinine, uric acid, serum total cholesterol, HDL-cholesterol and triglyceride concentrations will be measured using standard enzymatic automated methods. LDL cholesterol will be estimated by the Friedewald equation when the direct parameter will be not available. Glycated haemoglobin will be measured with an immune-turbidimetric assay. High sensitive C-reactive protein levels and fibrinogen concentrations will be determined by immunoturbidimetric assay. Blood samples will be collected in the health center, and will be analyzed at the University hospital of Salamanca in external quality assurance programs of the Spanish Society of Clinical Chemistry and Molecular Pathology.

Anthropometric measurements

Body weight will be determined on two occasions using a homologated electronic scale (Seca 770; Medical scale and measurement systems, Birmingham, United Kingdom) following due calibration (precision ± 0.1 kg), with the patient wearing light clothing and shoeless. These readings will be rounded to 100 g. Height in turn will be measured with a portable system (Seca 222; Medical scale and measurement systems, Birmingham, United Kingdom), recording the average of two readings, and with the patient shoeless in the standing position. The values will be rounded to the closest centimeter. Body mass index (BMI) will be calculated as weight (kg) divided by height squared (m^2). A value of > 30 kg/m^2 will be taken to define obesity. 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 will be adjusted without compressing the skin. Adiposity indices, waist-height and waist-hip, will also be calculated.

Office or clinical blood pressure

Office blood pressure measurement will involve three measurements of systolic blood pressure (SBP) and diastolic blood pressure (DBP), using the average of the last two, with a validated OMRON model M10-IT sphygmomanometer (Omron Health Care, Kyoto, Japan), by following the recommendations of the

European Society of Hypertension[29]. Pulse pressure will be estimated with the mean values of the second and third measurements.

Pulse wave velocity (PWV) and central (CAIx) and peripheral augmentation index (PAIx)

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. 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 heart rate at 75 bpm. PAIx is a measurement taken directly from the late systolic shoulder of the peripheral arterial waveform, and is defined as the ratio of the difference between the 2nd peak and diastolic pressure to the difference between the 1st peak and diastolic pressure[30], it is age-dependent and could be a useful index of vascular aging [31]. PAIx will be calculated as follows: (Second peak systolic blood pressure [SBP2] - diastolic blood pressure [DBP])/(first peak SBP - DBP) × 100 (%) and it will be corrected for heart rate at 75 bpm and it will be reported as PAIx75. Carotid and femoral artery pulse waves will be analyzed, with the patient in a supine position, using the SphygmoCor System (Vx pulse wave velocity), 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 and will be multiplied by 0.8. Subclinical organ damage of PWV will be defined as a carotid–femoral PWV >10 m/s [28 32].

Assessment of vascular structure by carotid intima media thickness (IMT)

Carotid ultrasound to assess C-IMT will be performed by two investigators trained for this purpose before starting the study. A Sonosite Micromax ultrasound device paired with a 5–10 MHz multi-frequency high-resolution linear transducer with Sonocal software will be used for performing automatic measurements of carotid IMT in order to optimize reproducibility. Measurements will be made of the common carotid after the examination of a 10 mm longitudinal section at a distance of 1 cm 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. A total of 6 measurements will be obtained of the right carotid and other 6 of the left carotid, using average values (average carotid IMT) and maximum values (maximum carotid IMT) automatically calculated by the software [33]. The measurements will be obtained with the subject lying down, with the head extended and slightly turned opposite to the examined carotid artery. The reliability was evaluated before the study began using the intraclass correlation coefficient, which showed values of 0.974 (95%CI: 0.935 to 0.990) for intra-observer agreement on repeated measurements in 20 subjects, and 0.897 (95%CI:0.740 to 0.959) for inter-observer agreement. According to the Bland-Altman analysis, the mean difference for intraobserver agreement (95% limits of agreement) was 0.022 (95%CI: -0.053 to 0.098) and intra-observer agreement was 0.012 (95%CI: -0.034 to 0.059). The average IMT will be considered abnormal if it measured > 0.90 mm, or if there will be atherosclerotic plaques with a diameter of 1.5 mm or a focal increase of 0.5 mm or 50% of the adjacent IMT [28].

Cardio Ankle Vascular Index and Ankle-brachial index

Cardio Ankle Vascular Index (CAVI) and Ankle-brachial index (ABI) will be measured using Vasera device VS-1500® (Fukuda Denshi). The pulse wave velocity (PWV) will be calculated, as well as Cardio Ankle Vascular Index (CAVI), which gives a more accurate calculation of the atherosclerosis degree. CAVI integrates cardiovascular elasticity derived from the aorta to the ankle pulse velocity through an oscillometric method and it is used as a good measure of vascular stiffness and it doesn't depend on blood pressure [34]. CAVI values will be automatically calculated by substituting the stiffness parameter ß in the following equation to detect the vascular elasticity and the cardio-ankle PWV: Stiffness parameter ß = $2\rho \times 1/ (Ps - Pd) \times \ln (Ps/Pd) \times PWV^2$, where ρ is the blood density, Ps and Pd are SBP and DBP in mmHg, and the PWV is measured between the aortic valve and ankle. The average coefficient of the variation of the CAVI is less than 5%, which is small enough for clinical use and confirm that CAVI has favorable reproducibility [35]. Cardio Ankle Vascular Index and Ankle-brachial index will be measured at rest. For the study, the lowest ABI and the highest CAVI and PWV obtained will be considered.

Renal assessment

Kidney damage will be assessed by measuring estimated glomerular filtration rate using the CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration) [36] 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 [28] Subclinical organ damage will be defined as a glomerular filtration rate below 30–60 ml/min/1.73 m^2 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 m^2 (BSA), proteinuria (>300 mg/24 h),.or albumin/creatinine ratio > 300 mg/24 h [28].

Cardiac assessment

Electrocardiographic 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 (Cornell VDP) [37]. Electrocardiographic LVH will be defined as a Sokolow–Lyon index >3.5 mV; RaVL >1.1 mV, Cornell voltage duration product >244 mV*ms or RaVL >1.1 mV; [28].

Cardiovascular risk assessment

Cardiovascular risk will be estimated using the score of the 2013 European Society of Hypertension/European Society of Cardiology Guidelines[28] and the risk equation (D'Agostino scale) based on the Framingham study [38]. Risk factors used include age, sex, total cholesterol, high-density lipoprotein cholesterol (HDL-C), and systolic blood pressure as quantitative variables, and drug treatment for hypertension, smoking, and history of diabetes mellitus as dichotomous variables.

Retinal vascular evaluation

Using a non-mydriatic retinography, TOPCON TRC NW 200, (Topcon Europe B.C., Capelle a/d Ijssel, The Netherlands) in the sitting position, a nurse trained will get nasal and temporal images centered in papilla. Then using the specific software developed (*Altair*) will be automatically calculated the retinal vessels thickness, the AVR, the area vascularized and the pattern of branching.

Development of *Altair* platform: *Automatic image analyzer to assess retinal vessel caliber*

The platform, called Altair "Automatic image analyzer to assess retinal vessel caliber", makes use of a methodology divided in different stages that are described below, to determine the characteristics of interest of the veins and arteries of the retina. This methodology uses artificial Intelligence (AI) techniques and analytical algorithms to discover retinal parameters of interest.

The methodology is separated into two phases: (i) Digitization of the retina, in which the different measures of the eye image are recognized. Here a data structure is created, which makes it possible to represent and process the retina. This phase is subdivided into the following steps as discussed below: load image and eye detection, processing, detection and segmentation. (ii) Measurements, in which we work with retinas that have been previously identified. This phase includes extraction of knowledge and manual correction, or expert knowledge, if necessary.

Digitization of the retina: To perform this phase, the following steps are necessary:

1. Load image and eye detection: The platform will automatically try to determine which eye (left or right) is the image, based on the detection of the macula. In this step, if the automatic detection has been wrong, the supervisor can modify this value by simply clicking on the correct eye.
2. Processing: In this step, then noise is reduced, the contrast is improved, the blurriness corrected and the edges are sharpened. Some of these actions can be carried out at the hardware level, which is to say with the features included with the camera. During the testing, retinography will be performed using a Topcon TRC NW 200 nonmydriatic retinal camera, obtaining nasal and temporal images centered on the disk.
3. Detection limits: In this step, the platform is capable of locating the disk and identifying the center and edges of the retina (figure 1). The identification of the papilla is vital since it helps as the starting point for the detection and identification of the different blood vessels. The platform builds a data structure that identifies each part of the retina based on the matrices of colors representing the images obtained. In this step, image processing techniques [24 26] will be used to detect intensity based on the boundaries of the structures.
4. Segmentation: In order to detect the limits, it became necessary to carry out a process of image segmentation. Segmentation is the process that divides an image into regions or objects whose pixels have similar attributes. Each segmented region typically has a physical significance within the image. It is one of the most important processes in an automated vision system because it makes it possible to extract the objects from the image for subsequent description and recognition [39-41]. This step can be considered the heart of the methodology proposed and used in the platform and perform the following actions:

a. Identification of vessels. Blood vessels are identified in the image by thresholding techniques. Their purpose is to remove pixels where the structuring element does not enter, in this case the blood vessels. The platform offers here a number of useful options for experts: Threshold vessels, in order to modify the threshold level automatically taken to a new vessel detection. Recalculate vessels, recalculate the vessels taking as the threshold established with the previous parameter. Pencil/eraser thickness: Sets thickness to draw or erase lines / vessels to switch vessels. Connect: Selecting this option and interacting on the overall image of the retina, the application connects those vessels whose structure has been divided as they were not detected section.

b. Structure of vessel. At the end of this stage the entire arterio-venous tree is stored in a structured way, making it possible to know not only if a vessel passes through a point or not, but through which point each vessel passes, which one is its parent, etc.

c. Cataloging of veins and arteries (Figure 2). In this step the platform detects whether a vessel is vein or artery, main branch is taken of the vessel. In this step different classifiers based on IA as decision trees and Bayesian networks [42] are applied.

Measurements

In this second phase, the results obtained are presented. The platform can display the following measures: thickness of the veins in µm, artery thickness in µm, AVR (ratio between the thickness of the arteries and veins thickness), veins area in square µm, artery area in square µm, area of all vessels in square µm, radio papilla in millimeters.

The platform also generates internally combined parameters by quadrants and circles. These values are visible when it comes to export CSV files either individually or together with other existing images already processed database values.

Results of its validity analysis must be consistent with the findings from researches focused on both cardiovascular risk estimation and evaluation of target organ damage. The results obtained during the use of the platform will be connected and used to extract additional information by using reasoning models such as case-based reasoning (CBR) [43] [44].

As conclusion, the platform is robust to the changes in the appearance of retinal fundus images typically encountered in clinical environments, and is proposed as a unified platform to connect all the methods needed to automate all processes of measurement on the retinas.

Retinal software validation

To validate the retinal software platform, the following steps will be completed by the evaluators after previous training in imaging appreciation.

-Evaluation of the reliability or precision

1. Intra-observer variability: To evaluate the measurement repeatability, the operator must measure the same image of an individual on at least two occasions. To this end, an operator will measure 100 images of a random subsample of 50 patients with a 1-week difference between the two measurements. In this case, the operator and the analysed images will be the same on both days, and the information from the previous measurement will be unknown.

2. Inter-observer variability: To evaluate the reproducibility of the measurement system, a different operator than who completed the assessment in phase 1 will evaluate the same 100 images previously analysed. The information from the results obtained in the previous phase will be unknown to this operator, and both operators will have the same experience in the subject and pertaining to the use of the software. Furthermore, both operators will receive the same preparatory training.

-Evaluation the validity (accuracy)

3. To assess the degree of agreement between Altair and the AV Index calculator® software (1), previously validated by us, the evaluation of 100 images will be performed using both tools. In this way, we will be able to demonstrate that the new method, apart from providing the same results, is more objective and faster in elaborating the results.

4. The measurement validity will be analysed in a total sample of 386 subjects and 772 retinographies, in regards to the relationship between the results of the carotid IMT, as a measurement of vascular structure, the PWV, the gold standard measure of arterial stiffness, the CAVI, kidney function, electrocardiographic parameters, and the estimated cardiovascular risk using different scales.

5. The association between different estimated parameters of the retina with the evolution or onset of new lesions in the target organs will be analysed, as well as any cardiovascular events that occur during the 4-year follow-up of the second phase of this project.

Statistical analysis

The data corresponding to quantitative variables with a normal distribution will be presented with the mean and standard deviation and with the median and an interquartile range if the distribution is asymmetric, while the qualitative variables will be presented according to the distribution of the frequencies. Normality will be evaluated with the Kolmogorov–Smirnov test. The Pearson's Chi-squared test will be used to analyse associations between the qualitative variables. The mean comparison, in the case of two groups, will be performed using the Student's t-test for independent samples and, in the case of larger groups, the ANOVA test. Post-hoc contrasts will be performed with the least significant difference (LSD) method and an alpha value <0.05. Repeated data will be analysed with the Student's t-test for paired data. The relationship between quantitative variables will be analysed according to the Pearson's or Spearman's correlation coefficient, depending on the type of distribution being considered. Finally, a multivariate analysis with multiple lineal regressions will be performed to analyse the association of the retinal parameters generated by *Altair* with the vascular structure and function. To contrast the hypothesis, an alpha risk of 0.05 will be set as the limit of statistical significance. Statistical analysis will be performed using SPSS/PC+ software version 20.0.

For validation of the retinal software, we will evaluate the measurements of artery and vein thickness, vascularised surfaces, and branching patterns from the three phases of validation, and the intraclass correlation coefficient (ICC) will be calculated as a comparative method. Using the Bland-Altman method, the limits of agreement between the measurements of the observers will be evaluated. The kappa agreement coefficient will be analysed to categorise the variable. This coefficient will allow us to evaluate the degree of agreement between the two methods. The accordance validity will also be analysed via correlation and multiple regression analysis, by evaluating the degree of association with other parameters of vascular structure and function and target organ injury.

Project schedule

This project is on a 5-year plan, with the aim of developing and validating the *Altair* software platform during the first year. Subsequently, a 4-year follow-up study will be performed to evaluate the evolution of target organ injuries, as well as the cardiovascular risk on the analysed retinal vascularisation parameters, i.e., the artery and vein thickness, the vascularised surface, and the branching patterns. An exhaustive evaluation of the subject will be performed at baseline and during the third and fifth years. A short evaluation will be performed during the second and fourth years.

Quality control

In order to ensure data quality, the professionals in charge of assessment retinal images and data collection will receive specific training. Regular external monitoring will then be performed to verify adequate application of methods, both in performing the different examinations and collecting the information.

Ethical and legal issues

The study has been approved by the clinical research ethics committee (CEIC) of the healthcare area of Salamanca ("CEIC of Area de Salud de Salamanca", 29 January, 2014). Subjects will be required to sign the informed consent prior to inclusion in the study, in accordance with the Declaration of Helsinki [45]. Subjects will be informed of the objectives of the project and of the risks and benefits of the examinations made. None of the examinations pose life-threatening risks for the type of subjects to be included in the study. The study includes the obtainment of biological samples; the study subjects therefore will be informed in detail. The confidentiality of the recruited subjects will be ensured at all times in accordance with the provisions of current legislation on personal data protection (15/1999 of 13 December, LOPD), and the conditions contemplated by Act 14/2007 on biomedical research.

Discussion

In a previous study performed by our group [20], we identified a positive association between the cardiovascular risk, estimated with the Framingham scale, and the retinal vessel calibre, mainly the venous calibre, and this association was maintained in the multivariate analysis. The arterial calibre, which also demonstrated a positive association, and the AVR, which demonstrated a negative association, seem to play a less relevant role than the venous calibre in regards to the cardiovascular risk.

The positive association between the cardiovascular risk and the venous calibre is in accordance with several published studies showing an association between the AVR and the coronary heart disease risk [12 13 21]. For example, McGeecham et al. [21], showed that when including the arterial and venous thickness, the Framingham model improved coronary event prediction, but only in women. This finding was repeated in several studies and was recently reported in a meta-analysis [13], which seems to support the notion that microvascular disease could be more important than coronary heart disease in women than men [46]. However, these results have been variable, as Wong et al. in the Beaver Dam Eye study [47] described the lack of an association between the AVR and the cardiovascular morbidity and mortality in the examined subgroups. In addition, Wang JJ et al.[48] identified an association between venous calibre and coronary death in men but not in women.

It is likely that some of these discrepancies are due to the different tools employed for evaluation of the retinal vessels, as all of these methods are manual or semiautomatic and, to a greater or lesser degree, are susceptible to the influence of the observer. The use of only particular information regarding the vascularisation of the retina may also influence the results. However, the *Altair* software platform resolves these problems. On the one hand, this platform is practically an automated tool with a low observer influence. In addition, we expect the inter- and intra-observer variability to be low and, as a result, the reliability to be significantly elevated. On the other hand, by using additional information on retinal vascularisation, the vascularised surface, the vascularisation patterns, and the artery and vein thickness, we expect to improve the validity of the tool and clarify the discrepancies reported in previous studies.

Study limitations

Data will be obtained from patients with a cardiovascular risk factor, who satisfy the inclusion criteria, and who were referred by a family physician to the Research Unit for vascular risk assessment. Thus, this approach uses a consecutive sampling method with inclusion criteria, i.e., a non-randomised sampling method. However, the size of the sample may buffer this limitation, and the real clinical conditions may lead us to a more real situation than that using more restrictive inclusion criteria for the study patients. The design of the study during the first phase is transversal; as a result, causality relations cannot be derived, i.e., only the associations among the analysed variables can be performed. Therefore, non-statistically detected associations between variables may be possible due to the sample size.

Abbreviations

ALTAIR: Automatic image analyzer to assess retinal vessel caliber: ABI: Ankle-brachial index; AIx: Augmentation Index; BMI: Body mass index; CAVI: Cardio Ankle Vascular index; CKD-EPI: Chronic Kidney Disease Epidemiology Collaboration; DBP: Diastolic blood pressure; ESH: European Society of Hypertension; IMT: Intima-media thickness; PWV: Pulse Wave Velocity; SBP: Systolic blood pressure.

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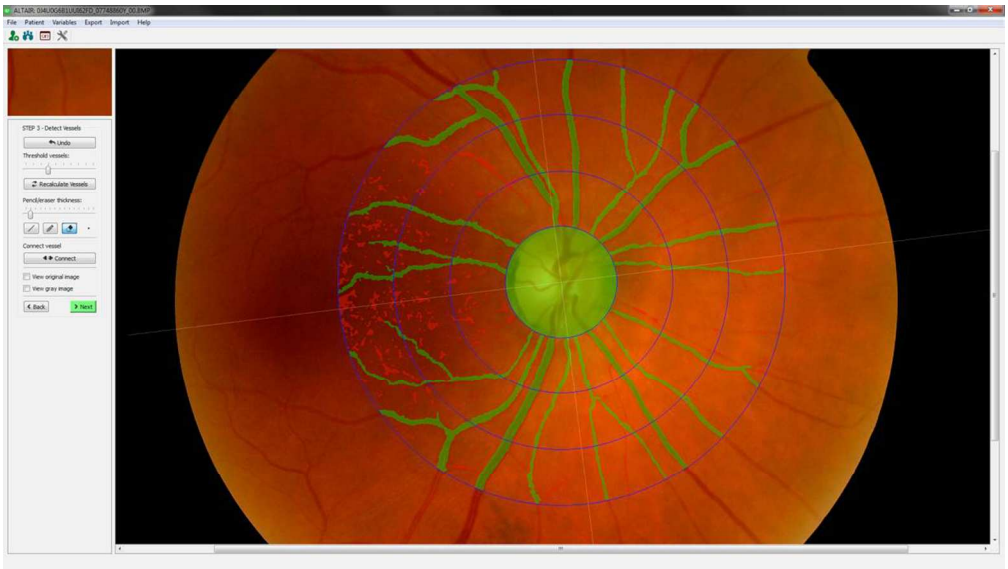
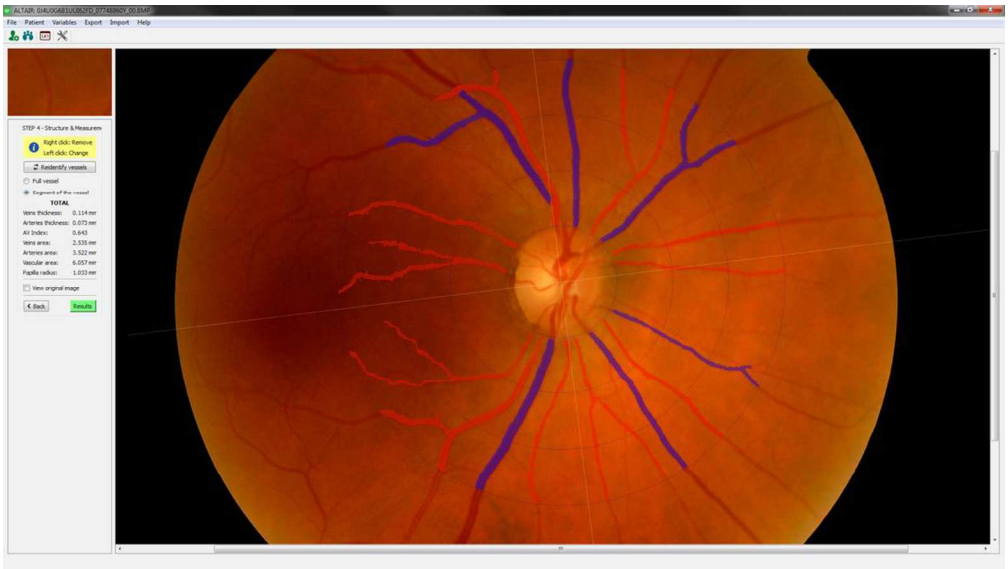
Figure 1. Detection and identification of vessels steps: locating the disk and identifying the center and edges of the retina

Figure 2. Detection and identification of vessels steps: cataloging of veins and arteries



Detection and identification of vessels steps: locating the disk and identifying the center and edges of the retina
104x59mm (300 x 300 DPI)



Detection and identification of vessels steps: cataloging of veins and arteries
103x58mm (300 x 300 DPI)

Title: **Validation of the Automatic image analyzer to assess retinal vessel caliber (*ALTAIR*).Prospective study protocol.**

Short title: **Altair study**

Authors

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ABSTRACT

Introduction: The fundus examination is a non-invasive evaluation of the microcirculation of the retina. The aim of the present study is to develop and validate (reliability and validity) the *Altair* software platform (Automatic image analyser to assess retinal vessel calibre) in order to analyse its utility in different clinical environments.

Methods and analysis:A cross-sectional study in the first phase and a prospective observational study in the second with 4-year of follow-up. The study will be performed in a primary care including 386 subjects. The main measurements will include carotid intima-media thickness, pulse wave velocity by Sphygmocor , cardio-ankle vascular index through the VASERA VS-1500®, cardiac evaluation by a digital ECG and renal injury by microalbuminuria and glomerular filtration. The retinal vascular evaluation will be performed using a TOPCON TRCNW200 non-mydriatic retinal camera to obtained digital images of the retina, and the developed software (*Altair*) will be used to automatically calculate the caliber of the retinal vessels, the vascularised area, and the branching pattern. For software validation, the intra and inter-observer reliability, the concurrent validity of the vascular structure and function, as well as the association between the estimated retinal parameters and the evolution or onset of new lesions in the target organs or cardiovascular diseases.

Ethics and dissemination: The study has been approved by the clinical research ethics committee of the healthcare area of Salamanca. All study participants will sign an informed consent to agree to participate in the study in compliance with the Declaration of Helsinki and the WHO standards for observational studies. Validation of this tool will provide greater reliability to the analysis of retinal vessels by decreasing the intervention of the observer and will result in increased validity through the use of additional information, especially in the areas of vascularisation and vessel branching patterns.

Trial registration number: Clinical Trials.gov Identifier: NCT02087605

Key words: Retinal vessel. Vascular stiffness. Cardiovascular diseases. Risk Assessment.

Strengths and limitations of this study

1.-A new software to retinal vassels evaluation will be developed, the *Altair* platform.

2.-A complete evaluation of retinal vascularization, including an automatically calculate the caliber of the retinal vessels, the vascularised area, and the branching pattern will be performed.

3.-The inter and intra-observer reliability in determining the caliber of arterial and venous vessels, the vascularised surface, and branching patterns using the Altair software platform will be performed.

4.-The concurrent validity of the Altair software platform, by analysing the relationship between retinal parameters and other parameters of vascular structure and function will be performed

5.-The design of the study during the first phase is transversal; as a result, causality relations cannot be derived, i.e., only the associations among the analysed variables can be performed.

INTRODUCTION

The fundus examination is a non-invasive evaluation of the retinal microcirculation and of the vascular damage caused by multiple cardiovascular risk factors. Different tools have been developed to evaluate the thickness of the retinal arteries and veins [1-7], yet, all of them require intervention of an observer to some extent. Moreover, the techniques and tools that are currently applied are manual or semiautomatic in nature, for which the observer has an important influence, thus providing limited information.

In population studies, an association has been found between the calibre of the retinal vessels and arterial hypertension [8], left ventricular hypertrophy [9], metabolic syndrome [10], stroke [11], and coronary heart disease [12], especially in women [13]. However, other studies disagree and show contradictory results regarding the evolution of the arteriosclerotic lesion and the calibre of the retinal vessels [14-16]. In this way, Cuspidi et al. [17] and Masaidi et al. [18] failed to detect an association between the calibre of the retinal vessels and target organ injuries (cardiac, vascular, and renal) in studies of two hypertensive populations. However, Torres et al. reported a negative association between carotid intima-media thickness (IMT) and the thickness of the retinal arteries but a positive association with the veins [19].

Recently, our group developed and validated a semiautomatic tool, the arteriovenous index calculator, to evaluate the vascular calibre of the retinal vessels [20] with reduced influence of the observer. This tool showed high reliability when measuring the calibre of the retinal vessels with an intraclass correlation coefficient (ICC) for intra- and inter-observers greater than 0.96 for veins, arteries, and the arteriovenous ratio (AVR). These measures, especially the venous calibre and the AVR, were also shown to be independent variables associated with estimated cardiovascular risk, according to the Framingham scale and the microvascular kidney lesions evaluated according to the level of microalbuminuria. This positive association between the cardiovascular risk and the venous calibre is in line with several published studies showing an association between the AVR and the risk of coronary heart disease [12 13 21]. However, longitudinal studies with a greater number of patients would help to clarify the discrepancies among previously published studies on cardiovascular risk and vascular structure and function. Moreover, it should not be forgotten that these tools provide less information than retinal imaging on the thickness of the arteries and veins, their branching patterns, and the vascularised areas, which may be relevant for

evaluating the status of the vascular tree and may be the cause of some of the discrepancies previously reported.

A new and different approach to the study of the vascular systems is the characterisation of the blood vessel patterns in the normal circulation of the human retina [22]. With this method, the distribution of the branching of the vascular system in a two-dimensional space can be analysed, and the geometrical complexity of the branching and the density of the retinal vessels can be quantified [23]. Indeed, the current scientific literature contains a number of publications on this subject [24-27].

In the present study, a novel software platform for image processing of the structural properties of the vessels is proposed using a human fundus red-free camera. The "Automatic image analyser to assess retinal vessel calibre" (*Altair*) software platform employs analytical methods and artificial intelligence (AI) algorithms to detect the retinal parameters of interest. The sequence of the algorithms consists of a new methodology that can be used to determine the properties of the veins and arteries of the retina; together, this system unifies all of the methods for automation of the measuring processes of retinal vessels.

Therefore, the **general aim** of the present study is the development and validation (reliability and validity) of the *Altair* software platform in order to analyse its utility in different clinical settings. The following specific objectives will be studied:

i) To evaluate inter- and intra-observer reliability in determining the caliber of arterial and venous vessels, the vascularised surface, and branching patterns using the *Altair* software platform.

ii) To evaluate the concurrent validity of the *Altair* software platform, in different populations **and ethnicities,** by analysing the relationship between retinal parameters and other parameters of vascular structure and function, including carotid IMT, pulse wave velocity (PWV), and the cardio-ankle vascular index (CAVI), as well as injuries in other target organs and the cardiovascular risk.

iii) To evaluate the evolution of target organ injuries and cardiovascular morbidity and mortality according to the vascularisation parameters of the retina determined using the *Altair* software platform.

METHOD AND ANALYSIS

Study design

The first phase will be a cross-sectional study aimed at validating the developed tool. Subsequently, the second phase will consist of a prospective observational study with annual follow-up evaluations over 4 years. The study will be developed in a primary healthcare setting.

Subjects

Study population

The population under study will consist of subjects from 35 to 74 years of age with a cardiovascular risk factor according to the 2013 European Society of Hypertension/European Society of Cardiology Guidelines [28]. Subjects were excluded due to the following criteria: psychic or cognitive disorders that interfere with the established requisites of the protocol; non-collaborative attitude; educational or comprehensive limitations; and severe comorbidities with a 12-month likelihood of life-threatening complications. A consecutive sampling of all patients sent to the research unit for cardiovascular risk evaluation will be performed, and those complying with the inclusion and exclusion criteria will be asked to participate until the estimated sample size is achieved. **The subjects will be mostly Caucasian, majority ethnic group among patients attended in the health center, however, at least fifty ethnic minority subjects, to give more validity to the tool, will be included.**

Sample size

The sample size has been set to detect a correlation coefficient among the pulse wave velocity (PWV), the gold standard measure of arterial stiffness, and the retinal parameters of 0.15, with an alpha risk of 0.05 and a beta risk of 0.20 and a 10% estimated loss regarding the difficulty of the technique or dropout on follow-up. As a result, a total of 386 patients will be included in this study. This number of patients will be adequate to detect a difference of 1 m/sec on the PWV among the AVR tertiles, considering a standard deviation (SD) of 2.22 m/sec with an alpha risk of 0.05 and a beta risk of 0.20.

Variables and measurement instruments

The general and potentially effect-modifying variables, such as age, gender, occupation, smoking, alcohol consumption, personal history and drug use will be documented.

Laboratory determinations

Venous blood sampling will be performed between 08:00 and 09:00 hours after the individuals fasted and abstained from smoking and the consumption of alcohol and caffeinated beverages for the previous 12 hours. Fasting plasma glucose, creatinine, uric acid, serum total cholesterol, HDL-cholesterol and triglyceride concentrations will be measured using standard enzymatic automated methods. LDL cholesterol will be estimated by the Friedewald equation when the direct parameter will be not available. Glycated haemoglobin will be measured with an immune-turbidimetric assay. High sensitive C-reactive protein levels and fibrinogen concentrations will be determined by immunoturbidimetric assay. Blood samples will be collected in the health center, and will be analyzed at the University hospital of Salamanca in external quality assurance programs of the Spanish Society of Clinical Chemistry and Molecular Pathology.

Anthropometric measurements

Body weight will be determined on two occasions using a homologated electronic scale (Seca 770; Medical scale and measurement systems, Birmingham, United Kingdom) following due calibration (precision ± 0.1 kg), with the patient wearing light clothing and shoeless. These readings will be rounded to 100 g. Height in turn will be measured with a portable system (Seca 222; Medical scale and measurement systems, Birmingham, United Kingdom), recording the average of two readings, and with the patient shoeless in the standing position. The values will be rounded to the closest centimeter. Body mass index (BMI) will be calculated as weight (kg) divided by height squared (m^2). A value of > 30 kg/m^2 will be taken to define obesity. 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 will be adjusted without compressing the skin. Adiposity indices, waist-height and waist-hip, will also be calculated.

Office or clinical blood pressure

Office blood pressure measurement will involve three measurements of systolic blood pressure (SBP) and diastolic blood pressure (DBP), using the average of the last two, with a validated OMRON model M10-IT sphygmomanometer (Omron Health Care, Kyoto, Japan), by following the recommendations of the

European Society of Hypertension[29]. Pulse pressure will be estimated with the mean values of the second and third measurements.

Pulse wave velocity (PWV) and central (CAIx) and peripheral augmentation index (PAIx)

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. 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 heart rate at 75 bpm. PAIx is a measurement taken directly from the late systolic shoulder of the peripheral arterial waveform, and is defined as the ratio of the difference between the 2nd peak and diastolic pressure to the difference between the 1st peak and diastolic pressure[30], it is age-dependent and could be a useful index of vascular aging [31]. PAIx will be calculated as follows: (Second peak systolic blood pressure [SBP2] - diastolic blood pressure [DBP])/(first peak SBP - DBP) × 100 (%) and it will be corrected for heart rate at 75 bpm and it will be reported as PAIx75. Carotid and femoral artery pulse waves will be analyzed, with the patient in a supine position, using the SphygmoCor System (Vx pulse wave velocity), 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 and will be multiplied by 0.8. Subclinical organ damage of PWV will be defined as a carotid–femoral PWV >10 m/s [28 32].

Assessment of vascular structure by carotid intima media thickness (IMT)

Carotid ultrasound to assess C-IMT will be performed by two investigators trained for this purpose before starting the study. A Sonosite Micromax ultrasound device paired with a 5–10 MHz multi-frequency high-resolution linear transducer with Sonocal software will be used for performing automatic measurements of carotid IMT in order to optimize reproducibility. Measurements will be made of the common carotid after the examination of a 10 mm longitudinal section at a distance of 1 cm 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. A total of 6 measurements will be obtained of the right carotid and other 6 of the left carotid, using average values (average carotid IMT) and maximum values (maximum carotid IMT) automatically calculated by the software [33]. The measurements will be obtained with the subject lying down, with the head extended and slightly turned opposite to the examined carotid artery. The reliability was evaluated before the study began using the intraclass correlation coefficient, which showed values of 0.974 (95%CI: 0.935 to 0.990) for intra-observer agreement on repeated measurements in 20 subjects, and 0.897 (95%CI:0.740 to 0.959) for inter-observer agreement. According to the Bland-Altman analysis, the mean difference for intraobserver agreement (95% limits of agreement) was 0.022 (95%CI: -0.053 to 0.098) and intra-observer agreement was 0.012 (95%CI: -0.034 to 0.059). The average IMT will be considered abnormal if it measured > 0.90 mm, or if there will be atherosclerotic plaques with a diameter of 1.5 mm or a focal increase of 0.5 mm or 50% of the adjacent IMT [28].

Cardio Ankle Vascular Index and Ankle-brachial index

Cardio Ankle Vascular Index (CAVI) and Ankle-brachial index (ABI) will be measured using Vasera device VS-1500® (Fukuda Denshi). The pulse wave velocity (PWV) will be calculated, as well as Cardio Ankle Vascular Index (CAVI), which gives a more accurate calculation of the atherosclerosis degree. CAVI integrates cardiovascular elasticity derived from the aorta to the ankle pulse velocity through an oscillometric method and it is used as a good measure of vascular stiffness and it doesn't depend on blood pressure [34]. CAVI values will be automatically calculated by substituting the stiffness parameter ß in the following equation to detect the vascular elasticity and the cardio-ankle PWV: Stiffness parameter ß = $2\rho \times 1/ (Ps - Pd) \times \ln (Ps/Pd) \times PWV^2$, where ρ is the blood density, Ps and Pd are SBP and DBP in mmHg, and the PWV is measured between the aortic valve and ankle. The average coefficient of the variation of the CAVI is less than 5%, which is small enough for clinical use and confirm that CAVI has favorable reproducibility [35]. Cardio Ankle Vascular Index and Ankle-brachial index will be measured at rest. For the study, the lowest ABI and the highest CAVI and PWV obtained will be considered.

Renal assessment

Kidney damage will be assessed by measuring estimated glomerular filtration rate using the CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration) [36] 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 [28] Subclinical organ damage will be defined as a glomerular filtration rate below 30–60 ml/min/1.73 m^2 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 m^2 (BSA), proteinuria (>300 mg/24 h),.or albumin/creatinine ratio > 300 mg/24 h [28].

Cardiac assessment

Electrocardiographic 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 (Cornell VDP) [37]. Electrocardiographic LVH will be defined as a Sokolow–Lyon index >3.5 mV; RaVL >1.1 mV, Cornell voltage duration product >244 mV*ms or RaVL >1.1 mV; [28].

Cardiovascular risk assessment

Cardiovascular risk will be estimated using the score of the 2013 European Society of Hypertension/European Society of Cardiology Guidelines[28] and the risk equation (D'Agostino scale) based on the Framingham study [38]. Risk factors used include age, sex, total cholesterol, high-density lipoprotein cholesterol (HDL-C), and systolic blood pressure as quantitative variables, and drug treatment for hypertension, smoking, and history of diabetes mellitus as dichotomous variables.

Retinal vascular evaluation

Using a non-mydriatic retinography, TOPCON TRC NW 200, (Topcon Europe B.C., Capelle a/d Ijssel, The Netherlands) in the sitting position, a nurse trained will get nasal and temporal images centered in papilla. Then using the specific software developed (*Altair*) will be automatically calculated the retinal vessels thickness, the AVR, the area vascularized and the pattern of branching.

Development of *Altair* platform: *Automatic image analyzer to assess retinal vessel caliber*

The platform, called Altair "Automatic image analyzer to assess retinal vessel caliber", makes use of a methodology divided in different stages that are described below, to determine the characteristics of interest of the veins and arteries of the retina. This methodology uses artificial Intelligence (AI) techniques and analytical algorithms to discover retinal parameters of interest.

The methodology is separated into two phases: (i) Digitization of the retina, in which the different measures of the eye image are recognized. Here a data structure is created, which makes it possible to represent and process the retina. This phase is subdivided into the following steps as discussed below: load image and eye detection, processing, detection and segmentation. (ii) Measurements, in which we work with retinas that have been previously identified. This phase includes extraction of knowledge and manual correction, or expert knowledge, if necessary.

Digitization of the retina: To perform this phase, the following steps are necessary:

1. Load image and eye detection: The platform will automatically try to determine which eye (left or right) is the image, based on the detection of the macula. In this step, if the automatic detection has been wrong, the supervisor can modify this value by simply clicking on the correct eye.
2. Processing: In this step, then noise is reduced, the contrast is improved, the blurriness corrected and the edges are sharpened. Some of these actions can be carried out at the hardware level, which is to say with the features included with the camera. During the testing, retinography will be performed using a Topcon TRC NW 200 nonmydriatic retinal camera, obtaining nasal and temporal images centered on the disk.
3. Detection limits: In this step, the platform is capable of locating the disk and identifying the center and edges of the retina (figure 1). The identification of the papilla is vital since it helps as the starting point for the detection and identification of the different blood vessels. The platform builds a data structure that identifies each part of the retina based on the matrices of colors representing the images obtained. In this step, image processing techniques [24 26] will be used to detect intensity based on the boundaries of the structures.
4. Segmentation: In order to detect the limits, it became necessary to carry out a process of image segmentation. Segmentation is the process that divides an image into regions or objects whose pixels have similar attributes. Each segmented region typically has a physical significance within the image. It is one of the most important processes in an automated vision system because it makes it possible to extract the objects from the image for subsequent description and recognition [39-41]. This step can be considered the heart of the methodology proposed and used in the platform and perform the following actions:

a. Identification of vessels. Blood vessels are identified in the image by thresholding techniques. Their purpose is to remove pixels where the structuring element does not enter, in this case the blood vessels. The platform offers here a number of useful options for experts: Threshold vessels, in order to modify the threshold level automatically taken to a new vessel detection. Recalculate vessels, recalculate the vessels taking as the threshold established with the previous parameter. Pencil/eraser thickness: Sets thickness to draw or erase lines / vessels to switch vessels. Connect: Selecting this option and interacting on the overall image of the retina, the application connects those vessels whose structure has been divided as they were not detected section.

b. Structure of vessel. At the end of this stage the entire arterio-venous tree is stored in a structured way, making it possible to know not only if a vessel passes through a point or not, but through which point each vessel passes, which one is its parent, etc.

c. Cataloging of veins and arteries (Figure 2). In this step the platform detects whether a vessel is vein or artery, main branch is taken of the vessel. In this step different classifiers based on IA as decision trees and Bayesian networks [42] are applied.

Measurements

In this second phase, the results obtained are presented. The platform can display the following measures: thickness of the veins in µm, artery thickness in µm, AVR (ratio between the thickness of the arteries and veins thickness), veins area in square µm, artery area in square µm, area of all vessels in square µm, radio papilla in millimeters.

The platform also generates internally combined parameters by quadrants and circles. These values are visible when it comes to export CSV files either individually or together with other existing images already processed database values.

Results of its validity analysis must be consistent with the findings from researches focused on both cardiovascular risk estimation and evaluation of target organ damage. The results obtained during the use of the platform will be connected and used to extract additional information by using reasoning models such as case-based reasoning (CBR) [43] [44].

As conclusion, the platform is robust to the changes in the appearance of retinal fundus images typically encountered in clinical environments, and is proposed as a unified platform to connect all the methods needed to automate all processes of measurement on the retinas.

Retinal software validation

To validate the retinal software platform, the following steps will be completed by the evaluators after previous training in imaging appreciation.

-Evaluation of the reliability or precision

1. Intra-observer variability: To evaluate the measurement repeatability, the operator must measure the same image of an individual on at least two occasions. To this end, an operator will measure 100 images of a random subsample of 50 patients with a 1-week difference between the two measurements. In this case, the operator and the analysed images will be the same on both days, and the information from the previous measurement will be unknown.

2. Inter-observer variability: To evaluate the reproducibility of the measurement system, a different operator than who completed the assessment in phase 1 will evaluate the same 100 images previously analysed. The information from the results obtained in the previous phase will be unknown to this operator, and both operators will have the same experience in the subject and pertaining to the use of the software. Furthermore, both operators will receive the same preparatory training.

-Evaluation the validity (accuracy)

3. To assess the degree of agreement between Altair *and* the *AV Index calculator*® software (1), previously validated by us, the evaluation of 100 images will be performed using both tools. In this way, we will be able to demonstrate that the new method, apart from providing the same results, is more objective and faster in elaborating the results.

4. The measurement validity will be analysed in a total sample of 386 subjects and 772 retinographies, in regards to the relationship between the results of the carotid IMT, as a measurement of vascular structure, the PWV, the gold standard measure of arterial stiffness, the CAVI, kidney function, electrocardiographic parameters, and the estimated cardiovascular risk using different scales.

5. The association between different estimated parameters of the retina with the evolution or onset of new lesions in the target organs will be analysed, as well as any cardiovascular events that occur during the 4-year follow-up of the second phase of this project.

Statistical analysis

The data corresponding to quantitative variables with a normal distribution will be presented with the mean and standard deviation and with the median and an interquartile range if the distribution is asymmetric, while the qualitative variables will be presented according to the distribution of the frequencies. Normality will be evaluated with the Kolmogorov–Smirnov test. The Pearson's Chi-squared test will be used to analyse associations between the qualitative variables. The mean comparison, in the case of two groups, will be performed using the Student's t-test for independent samples and, in the case of larger groups, the ANOVA test. Post-hoc contrasts will be performed with the least significant difference (LSD) method and an alpha value <0.05. Repeated data will be analysed with the Student's t-test for paired data. The relationship between quantitative variables will be analysed according to the Pearson's or Spearman's correlation coefficient, depending on the type of distribution being considered. Finally, a multivariate analysis with multiple lineal regressions will be performed to analyse the association of the retinal parameters generated by *Altair* with the vascular structure and function. To contrast the hypothesis, an alpha risk of 0.05 will be set as the limit of statistical significance. Statistical analysis will be performed using SPSS/PC+ software version 20.0.

For validation of the retinal software, we will evaluate the measurements of artery and vein thickness, vascularised surfaces, and branching patterns from the three phases of validation, and the intraclass correlation coefficient (ICC) will be calculated as a comparative method. Using the Bland-Altman method, the limits of agreement between the measurements of the observers will be evaluated. The kappa agreement coefficient will be analysed to categorise the variable. This coefficient will allow us to evaluate the degree of agreement between the two methods. The accordance validity will also be analysed via correlation and multiple regression analysis, by evaluating the degree of association with other parameters of vascular structure and function and target organ injury.

Project schedule

This project is on a 5-year plan, with the aim of developing and validating the *Altair* software platform during the first year. Subsequently, a 4-year follow-up study will be performed to evaluate the evolution of target organ injuries, as well as the cardiovascular risk on the analysed retinal vascularisation parameters, i.e., the artery and vein thickness, the vascularised surface, and the branching patterns. An exhaustive evaluation of the subject will be performed at baseline and during the third and fifth years. A short evaluation will be performed during the second and fourth years.

Quality control

In order to ensure data quality, the professionals in charge of assessment retinal images and data collection will receive specific training. Regular external monitoring will then be performed to verify adequate application of methods, both in performing the different examinations and collecting the information.

Ethical and legal issues

The study has been approved by the clinical research ethics committee (CEIC) of the healthcare area of Salamanca ("CEIC of Area de Salud de Salamanca", 29 January, 2014). Subjects will be required to sign the informed consent prior to inclusion in the study, in accordance with the Declaration of Helsinki [45]. Subjects will be informed of the objectives of the project and of the risks and benefits of the examinations made. None of the examinations pose life-threatening risks for the type of subjects to be included in the study. The study includes the obtainment of biological samples; the study subjects therefore will be informed in detail. The confidentiality of the recruited subjects will be ensured at all times in accordance with the provisions of current legislation on personal data protection (15/1999 of 13 December, LOPD), and the conditions contemplated by Act 14/2007 on biomedical research.

Discussion

In a previous study performed by our group [20], we identified a positive association between the cardiovascular risk, estimated with the Framingham scale, and the retinal vessel calibre, mainly the venous calibre, and this association was maintained in the multivariate analysis. The arterial calibre, which also demonstrated a positive association, and the AVR, which demonstrated a negative association, seem to play a less relevant role than the venous calibre in regards to the cardiovascular risk.

The positive association between the cardiovascular risk and the venous calibre is in accordance with several published studies showing an association between the AVR and the coronary heart disease risk [12 13 21]. For example, McGeecham et al. [21], showed that when including the arterial and venous thickness, the Framingham model improved coronary event prediction, but only in women. This finding was repeated in several studies and was recently reported in a meta-analysis [13], which seems to support the notion that microvascular disease could be more important than coronary heart disease in women than men [46]. However, these results have been variable, as Wong et al. in the Beaver Dam Eye study [47] described the lack of an association between the AVR and the cardiovascular morbidity and mortality in the examined subgroups. In addition, Wang JJ et al.[48] identified an association between venous calibre and coronary death in men but not in women.

It is likely that some of these discrepancies are due to the different tools employed for evaluation of the retinal vessels, as all of these methods are manual or semiautomatic and, to a greater or lesser degree, are susceptible to the influence of the observer. The use of only particular information regarding the vascularisation of the retina may also influence the results. However, the *Altair* software platform resolves these problems. On the one hand, this platform is practically an automated tool with a low observer influence. In addition, we expect the inter- and intra-observer variability to be low and, as a result, the reliability to be significantly elevated. On the other hand, by using additional information on retinal vascularisation, the vascularised surface, the vascularisation patterns, and the artery and vein thickness, we expect to improve the validity of the tool and clarify the discrepancies reported in previous studies.

Study limitations

Data will be obtained from patients with a cardiovascular risk factor, who satisfy the inclusion criteria, and who were referred by a family physician to the Research Unit for vascular risk assessment. Thus, this approach uses a consecutive sampling method with inclusion criteria, i.e., a non-randomised sampling method. However, the size of the sample may buffer this limitation, and the real clinical conditions may lead us to a more real situation than that using more restrictive inclusion criteria for the study patients. The design of the study during the first phase is transversal; as a result, causality relations cannot be derived, i.e., only the associations among the analysed variables can be performed. Therefore, non-statistically detected associations between variables may be possible due to the sample size.

Abbreviations

ALTAIR: Automatic image analyzer to assess retinal vessel caliber: ABI: Ankle-brachial index; AIx: Augmentation Index; BMI: Body mass index; CAVI: Cardio Ankle Vascular index; CKD-EPI: Chronic Kidney Disease Epidemiology Collaboration; DBP: Diastolic blood pressure; ESH: European Society of Hypertension; IMT: Intima-media thickness; PWV: Pulse Wave Velocity; SBP: Systolic blood pressure.

Contributors Conception of the idea for the study: L. Garcia-Ortiz and JM Corchado-Rodríguez. Development of the protocol, organization and funding: L. García-Ortiz, JM. Corchado-Rodríguez, J.I. Recio-Rodríguez, MÁ. Gómez-Marcos, JA Maderuelo, S. Rodríguez-González, JF. de Paz-Santana, and P. Chamoso-Santos. Writing of the manuscript: L. Garcia-Ortiz, JM Corchado-Rodríguez and MA. Gómez-Marcos. All the authors have read the draft critically, to make contributions, and approved the final text.

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Competing interests None

Patient consent Obtained.

Ethics approval The study has been approved by the clinical research ethics committee of the healthcare area of Salamanca.

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Figure 1. Detection and identification of vessels steps: locating the disk and identifying the center and edges of the retina

Figure 2. Detection and identification of vessels steps: cataloging of veins and arteries

STROBE Statement—checklist of items that should be included in reports of observational studies

	Item No	Recommendation
Title and abstract	1	X (*a*) Indicate the study's design with a commonly used term in the title or the abstract
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found
Introduction		
Background/rationale	2	X Explain the scientific background and rationale for the investigation being reported
Objectives	3	X State specific objectives, including any prespecified hypotheses
Methods		
Study design	4	X Present key elements of study design early in the paper
Setting	5	X Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls *X Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case
Variables	7	X Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable
Data sources/ measurement	8*	*X* For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group
Bias	9	X Describe any efforts to address potential sources of bias
Study size	10	X Explain how the study size was arrived at
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why
Statistical methods	12	X (*a*) Describe all statistical methods, including those used to control for confounding
		(*b*) Describe any methods used to examine subgroups and interactions
		(*c*) Explain how missing data were addressed
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy
		(*e*) Describe any sensitivity analyses

Continued on next page

Results (not applicable)		
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed
		(b) Give reasons for non-participation at each stage
		(c) Consider use of a flow diagram
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders
		(b) Indicate number of participants with missing data for each variable of interest
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time
		Case-control study—Report numbers in each exposure category, or summary measures of exposure
		Cross-sectional study—Report numbers of outcome events or summary measures
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included
		(*b*) Report category boundaries when continuous variables were categorized
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses
Discussion (not applicable)		
Key results	18	Summarise key results with reference to study objectives
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence
Generalisability	21	Discuss the generalisability (external validity) of the study results
Other information		
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.