Title: Retinal vasculometry associations with cardiometabolic risk factors in the European Prospective Investigation of Cancer Norfolk study

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SUPPLEMENTAL MATERIAL

This article contains additional online-only material. The following should appear online-only: Supplemental Figures 1, 2 and 3 and Supplementary Table 1.

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CONFLICT OF INTEREST

None.

RUNNING HEADER

Retinal vasculometry associations with cardiometabolic risk factors

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ABSTRACT

Purpose: To examine associations between retinal vessel morphometry and cardiometabolic risk factors in older British men and women.

Design: Retinal imaging examination as part of the European Prospective Investigation into Cancer-Norfolk Eye study.

Participants: 7411 participants underwent retinal imaging and clinical assessment. Retinal images were analysed using a fully automated validated computerised system, which provides novel measures of vessel morphometry.

Methods: Associations between cardiometabolic risk factors, chronic disease and retinal markers were analyzed using multi-level linear regression, adjusted for age, sex and within person clustering, to provide percentage differences in tortuosity and absolute differences in width.

Main outcomes measures: Retinal arteriolar and venular tortuosity and width.

Results: 279,802 arterioles, and 285,791 venules from 5947 participants (mean age 67.6 years, SD 7.6, 57% female) were analysed. Increased venular tortuosity was associated with higher BMI (2.5%, 95% CI 1.7,3.3% per 5 kg/m²) and HbA1c (2.2%, 95%CI 1.0,3.5% per %), and with prevalent type 2 diabetes (6.5%, 95%CI 2.8,10.4%); wider venules were associated with older age (2.6μm, 95%CI 2.2,2.9μm per decade), higher triglycerides (0.6μm, 95%CI 0.3,0.9μm per mmol/L), BMI (0.7μm, 95%CI 0.4,1.0 per 5 kg/m²) and HbA1c (0.4μm, 95%CI -0.1,0.9 per %) and being a current smoker (3.0μm, 95%CI 1.7,4.3μm); similarly smoking was also associated with wider arterioles (2.1μm, 95%CI 1.3,2.9μm). Thinner venules were associated with HDL (1.4μm, 95%CI 0.7,2.2 per mmol/L). Arteriolar tortuosity increased with age (5.4%, 95%CI 3.8,7.1% per decade), higher systolic blood pressure (1.2%, 95%CI 0.5,1.9% per 10mmHg), in females (3.8, 95%CI 1.4,6.4%) and with prevalent stroke (8.3%,

95%CI -0.6,18%); no association was observed with prevalent myocardial infarction. Narrower arterioles were associated with age (0.8μm, 95%CI 0.6,1.0μm per decade), higher systolic blood pressure (0.5μm, 95%CI 0.4,0.6μm per 10mmHg), total cholesterol (0.2μm, 95%CI 0.0,0.3μm per mmol/L) and HDL (1.2μm, 95%CI 0.7,1.6μm per mmol/L).

Conclusions: Metabolic risk factors show a graded association with both tortuosity and width of retinal venules, even among people without clinical diabetes, whereas atherosclerotic risk factors correlate more closely with arteriolar width, even excluding those with hypertension and cardiovascular disease. These non-invasive microvasculature measures should be evaluated further as predictors of future cardiometabolic disease among apparently healthy individuals.

Keywords: Retinal vessels, morphology, cardiometabolic risk factors

Cardiovascular disease (CVD), including coronary heart disease (CHD), heart failure and stroke, is responsible for a substantial burden of morbidity and disability.[1] Type 2 diabetes is an increasing public health problem, affecting 1 in 10 adults globally, and a major cause of premature death and morbidities, especially CVD.[2] Early detection and prevention both of CVD and Type 2 diabetes is key to limiting future morbidity and mortality.[3;4] While disease risk factors for Type 2 diabetes, such as blood glucose levels and HbA1c, are yet to show good screening performance,[5] established markers of early vascular disease are used in risk prediction models to estimate future risk of CVD, providing indications for medical / lifestyle interventions to alter disease trajectory.[6;7] There have been a number of attempts to improve the performance of these risk prediction models, by adding other risk factors.[6;7] However, the addition of novel risk factors have added little to CHD prediction.[8] Recent evidence suggests that early markers for the presence of vascular disease (as opposed to additional risk factors) are needed to improve risk prediction for population screening.[5;9]

Detailed retinal vasculometry may offer such a marker. Growing evidence suggests that morphological features in retinal vessels, in particular vessel width, are early physiological markers of cardiometabolic risk and disease (as well as other disease processes).[10-13] While strong evidence has accrued for some of these associations, particularly associations with Type 2 diabetes and CVD (and their related risk factors), other associations have remained inconsistent. For instance, wider arterioles have been associated with higher levels of blood glucose, total cholesterol, triglycerides and inflammatory markers, but not in all studies.[10;12] Similarly associations of venular width with blood pressure have also been inconclusive,[10] although recent evidence suggests increased width associated with hypertension.[14] Wider

venules also seem to be associated with diabetes, elevated glycosylated haemoglobin, lower levels of high density lipoprotein, inflammatory markers, smoking and obesity.[10-12] However, some inconsistencies in the presence or absence of these associations (perhaps due to uncertainty caused by sample size) remain.[11;12] Moreover, in comparison to studies examining vessel width, associations with vessel tortuosity have been little studied,[15] especially in relation to metabolic markers, and may provide further insight into vasculometry changes associated with cardiometabolic risk. Large population studies are needed to resolve these uncertainties, and to allow the comparative performance of width and tortuosity associations to be gauged. However, the assessment of retinal vessel morphometry from retinal images, even with computerized assistance, has so far been heavily reliant on subjective operator involvement, which is time consuming and open to measurement error,[16] limiting its use in large scale, preventative initiatives in a community setting. We have developed a fully automated system for examining retinal vessel size and tortuosity, which overcomes many of these difficulties.[17-19] We have used this system to examine the associations between cardiometabolic risk factors and retinal vascular characteristics in a large prospective population study of older British men and women, to confirm associations previously reported with vessel width, but to provide novel associations with measures of vessel tortuosity.

RESEARCH DESIGN AND METHODS

Study Population:- The European Prospective Investigation into Cancer (EPIC) study is a European based prospective cohort study designed to investigate the aetiology of major chronic diseases.[20] The UK component of the study, EPIC-Norfolk, recruited from general practices in and around the city of Norfolk, and examined 25,639 participants (99.7% white European) aged 40 to 79 at baseline, between 1993 and 1997 (response rate 33%).[21;22] Study participants had a detailed examination (including anthropometry, blood pressure, urine and venous blood sampling) and questionnaire assessment at entry (including information on pre-existing cardiovascular disease, type 2 diabetes and other medical conditions), and completed periodic questionnaires about their health (with a particular focus on dietary habits). Participants have been followed up over a 13-year period for morbidity and mortality. In addition to questionnaire data, participants were invited for further clinical examinations over this period, including repeat anthropometric assessment, venous blood sampling, retinal imaging, and physiological measures.[22]

Third Follow-Up: Between 2004 and 2011, 8623 participants provided updated information on medical history and lifestyle behaviour.[22] Weight and height, were measured with participants in light clothing without shoes. Weight was measured to the last 0.1 kg using regularly calibrated digital scales (Tanita TBF-300, Tanita UK Ltd, Middlesex, UK), and height to the last complete 0.1 cm using a stadiometer (Chasmors, UK). Body mass index (BMI) was calculated as weight / height squared in kg/m^2. Seated blood pressure was measured twice using an automated blood pressure monitor (Accutorr PlusTM, Datascope Patinet Monitoring, Huntington, UK); the mean of both measures was used. A non-fasting venous blood sample was collected; details of the analytic measures have been published

previously.[22] HbA1c was measured in whole blood using high performance liquid chromatography. Serum total cholesterol and HDL-cholesterol were measured using an auto-analyser (RA 1000 Technicon, Bayer Diagnostics, Basingstoke, UK). LDL-cholesterol was calculated using the Fredrickson–Friedewald equation.[23]

Ocular Examination: Ocular assessment included measurement of vision, visual acuity (LogMAR acuity) and closed field auto-refraction (Humphrey model 500, Humphrey Instruments, San Leandro, California, USA). Macular centred 45º digital fundus photographs were taken using a TRC-NW6S non-mydriatic retinal camera and IMAGEnet Telemedicine System (Topcon Corporation, Tokyo, Japan) with a 10 megapixel Nikon D80 camera (Nikon Corporation, Tokyo, Japan) without pharmacological dilation of the pupil. Image processing was carried out using an automated computerised system (QUARTZ).[17-19] The automated system distinguishes between right and left eyes (by optic disc localisation), venules and arterioles, identifies vessel segments, out-puts centreline coordinates, and measures vessel width and angular change between vessel centreline coordinates, as well as providing further measures of tortuosity.[17-19;24] An ensemble classifier of bagged decision trees (with colour information) was used to classify vessels as being either venules or arterioles. Only vessels which were classified with 80% or more probability were retained, to balance the number of venules and arterioles detected, as well as maximise the number of vessels included for analyses.[18] The performance of the Arteriole/Venule (A/V) detection program was manually verified in a sub-set of images, and had detection rates of 84% for arterioles and 77% for venules, and corresponding false positive rates of 23% and 16% respectively.[18] An automated assessment of image quality was also made based on the segmented vasculature.[18] The system obtains thousands of measures of width and tortuosity from the

whole retinal image (dependent on image quality), not just concentric areas centred on the disc.[10] These measures were summarised using mean width in microns and tortuosity with arbitrary units, weighted by segment length, for arterioles and venules separately for each image. In the case of multiple images per person, an automated algorithm developed to assess image quality allowed the best right eye and best left eye images to be selected for analyses. A previously validated tortuosity measure which shows good agreement with subjective assessment of vessel tortuosity, based on the mean change in chord length between successive divisions of the vessel was used.[24] System performance has been outlined in detail and validated previously, and allows automated batch processing of images from large population based studies.[17-19] A model eye was used to quantify the magnification characteristics of the telecentric fundus camera used (Topcon TRC-NW6S), allowing pixel dimensions of vessel width to be converted to real size.[25]

Ethics, Governance and Consent: The EPIC-Norfolk Eye Study was carried out following the principles of the Declaration of Helsinki and the Research Governance Framework for Health and Social Care. The study was approved by the Norfolk Local Research Ethics Committee (05/Q0101/191) and East Norfolk and Waveney NHS Research Governance Committee (2005EC07L). All participants gave written, informed consent.

Statistical Analysis: Statistical analyses were carried out using STATA software (version 13, StataCorp LP, College Station, TX). Segment wise weighted mean widths and tortuosity were used, to provide a measure for venules and arterioles separately, for each eye. Histograms of retinal vessel widths showed normal distributions, while measures of tortuosity were positively skewed and log-transformed. Multilevel linear regression models

adjusting for age and sex were used to examine associations of cardiometabolic risk factors and prevalent disease status to retinal vessel morphometry outcomes, allowing for repeated measures of vessel indices within the same person. Regression models provided mean differences in width and percentage differences in tortuosity for venules and arterioles separately, per decade in age, females versus males, current smokers and former smokers versus never-smokers, or per unit increase in cardiometabolic risk factor (per 5 kg/m^2 increase in BMI, per 10 mmHg in systolic and diastolic blood pressure, per mmol/L increase in total cholesterol, high density lipoprotein, triglyceride, and per percentage rise in HbA1c). For disease outcomes, differences in vessel indices were obtained comparing those with prevalent disease present (including type 2 diabetes, MI, stroke, and known / treated hypertension) versus absent. Differences in associations between men and women were formally examined by inclusion of an interaction term between the risk factor and sex into the regression model. Risk factors found to be statistically significantly related to vascular measures at the 5% level were subsequently included in mutually adjusted models. We also examined associations after exclusion of participants with prevalent disease outcomes.

RESULTS

Of 18,380 individuals invited to participate in this phase of the study, 8,623 (47%) took part (mean age 67.6 years, 57% female). Supplemental Figure 1 shows a flow diagram of the numbers participating in the study. Fundus imaging and refractive assessment were carried out in 7411 individuals, of whom 5,957 participants (80%) had at least one image of sufficient quality and classified vessels as arterioles or venules with a probability set at 80% detection. It was not possible to obtain useful data from the remainder as images were miscentred, defocussed, or were obstructed by lashes and/or media opacities. A small number had missing data for height, weight or blood pressure (n=10), leaving 5947 participants with measures of vessel width and tortuosity for 565,593 vessel segments (279,802,arterioles, 285,791 venules) from 10,474 images; blood sample data were available for 5514. Participant characteristics of EPIC participants at baseline, and those who took part in the third health examination with and without useable fundus images have been described previously.[26] Those attending the 3rd Health Check (3HC) were younger at baseline, of higher BMI and socioeconomic status, and were less likely to be a current smoker compared to participants not followed-up.[26] Participant characteristics of EPIC participants who took part in the third health examination, and who were included in the analyses compared with those who were not (5,947 versus 2,676 participants) are summarised in Table 1. Other than those included being slightly younger (mean age 68 years vs 71 years), there was no clear evidence of a systematic difference in 3HC participant characteristics. Retinal vessel morphometry in those with useable fundus images are also summarised for arterioles and venules separately. Histograms of arteriolar and venular width and tortuosity measures (with and without log transformation) are shown in

Supplemental Figure 2, and shows appreciable variation in these measures within this study population.

Differences in retinal vessel width in microns, and percentage differences in tortuosity by Type 2 diabetes and CVD risk factors and outcomes are shown by vessel type in Table 2. Arterioles were inversely associated and more tortuous with older age (0.8μm, 95%CI 0.6, 1.0μm and 5.4%, 95%CI 3.8, 7.1% per decade respectively). Wider venules were observed with older age (mean difference 2.6μm, 95%CI 2.2,2.9μm per decade), and amongst current smokers compared to never smokers (3.0μm, 95%CI 1.7, 4.3μm). Narrower arterioles (0.5μm, 95%CI 0.2,0.8) and more tortuous arterioles and venules were strongly associated with being female compared to male (3.8%, 95% CI 1.4, 6.4%; 2.2%, 95% CI 0.7, 3.6% respectively).

Retinal vasculometry associations with metabolic risk factors:-

Venular width was positively associated with Type 2 diabetes risk factors, including higher BMI (0.7μm, 95%CI 0.4, 1.0μm per 5 kg/m^2), and HbA1c (0.4μm, 95%CI -0.1, 0.9μm per %). Wider venules were also positively associated with elevated levels of triglycerides (0.6μm, 95%CI 0.3, 0.9μm per mmol/L). Venular tortuosity was also positively associated with Type 2 diabetes risk factors, as well as prevalent Type 2 diabetes. Venules were 2.5% more tortuous (95% CI 1.7, 3.3%) per 5 kg/m^2 increase in BMI, 2.2% more tortuous (95% CI 1.0, 3.5%) per percentage rise in HbA1c, and 6.5% more tortuous (95% CI 2.8, 10.4%) amongst those with Type 2 diabetes compared to those without.

Retinal vasculometry associations with cardiovascular risk factors:-

Arteriolar widths were inversely associated with age, systolic (0.5μm 95%CI 0.4, 0.6μm per 10mmHg rise) and diastolic blood pressure (1.0μm, 95%CI 0.9, 1.2μm per 10mmHg rise).

Arteriolar tortuosity was also positively associated with systolic blood pressure (1.2%, 95% CI 0.5, 1.9% per 10mmHg respectively). Arteriolar width was inversely associated with total cholesterol (0.2μm, 95%CI 0.0, 0.3μm per mmol/L) and HDL (1.2μm, 95%CI 0.7, 1.6μm per mmol/L). Narrower venules and decreased venular tortuosity were associated with HDL cholesterol (1.4μm, 95%CI 0.7, 2.1μm, 1.8%, 95%CI -0.1, 3.7% less tortuosity per mmol/L). No associations were observed with prevalent MI, but there was a suggestion of increased arteriolar tortuosity with prevalent stroke (8.3%, 95%CI -0.6, 18%). Arterioles were narrower and more tortuous with increasing age; venular width increased with age. Both vessel types were wider amongst smokers compared with lifelong never smokers. Figure 1 shows the associations between retinal vessel indices and Type 2 diabetes and CVD risk factors by quintile; statistically significant associations appeared to be graded. These associations remained after exclusion of those with prevalent disease, including MI, stroke, and diabetes (n=466).

Sensitivity and multiple variable analyses:-

Sensitivity analyses examined the differences in vessel width and tortuosity associated with cardiometabolic risk factors, excluding those with clinical diabetes / cardiovascular disease, and those with known / treated hypertension (data available on request). Metabolic associations with venular width and tortuosity persist after exclusion of those with clinical diabetes, and arteriolar width associations with vascular risk factors (particularly blood pressure) remain after excluding those with cardiovascular disease and hypertension. Retinal vessel associations were similar in males and females (tests for interaction $P>0.05$), except for HDL, for which opposing associations with arteriolar tortuosity were apparent. Per mmol/L higher HDL, arteriolar tortuosity was 5.8% (95% CI 0.1, 11.8%) higher in men, but 4.0% (95% CI 0.0, 7.8%) lower in women (test for interaction $p=0.006$).

The mutual independence of these risk factor associations was also examined. Mutually adjusted risk factor associations are presented in Supplemental Table 1. Risk factors that were statistically significantly associated with retinal vasculometry in Table 2 were included in multiple variable regression models. Associations with both arteriolar morphometry measures and cardiometabolic risk factors remained remarkably stable. Consistent associations were observed between arteriolar width and age, current smoking status, blood pressure and HDL cholesterol, but there was no evidence of an independent association with total cholesterol. Similarly strong associations remained for arteriolar tortuosity with age, sex and blood pressure. Associations from mutually adjusted models for venular measures were also remarkably similar to the associations presented in Table 2. Venular width associations with age, current smoking, BMI and diastolic blood pressure were relatively unchanged, but associations with HDL cholesterol and triglycerides were attenuated towards the null. Further investigation showed that associations with lipids were primarily confounded by BMI. Venular tortuosity associations with sex and BMI were relatively unchanged. However, the association with HbA1c was attenuated (1.3%, 95%CI 0.0,2.6%, increase in venular tortuosity per % increase in HbA1c), and the association with systolic blood pressure was weakened by adjustment for BMI. Multilevel regression models adjusting for age, sex and blood pressure showed a stronger association with prevalent stroke than in Table 2, with 9.0% more tortuous arterioles amongst those who had suffered a stroke compare to those who had not (95%CI 0.1,18.8%, $p<0.001$), suggesting that the effect on arteriolar tortuosity is independent of systolic blood pressure. Increased venular tortuosity among those with prevalent diabetes was independent of sex, BMI and blood pressure (5.5%, 95%CI 1.4%,8.9%).

DISCUSSION

Our results are consistent with previously documented retinal vasculometry associations with Type 2 diabetes and CVD risk factors and outcomes,[10-13] but provide further insight where uncertainties over the presence or absence of associations exist. Moreover, novel associations with vessel tortuosity provide further evidence of vasculometry changes. Findings suggest that Type 2 diabetes risk factors and prevalent Type 2 diabetes are associated with the morphology of retinal venules, both in terms of width and tortuosity, while coronary risk factors have a greater influence on arteriolar width. These associations remain after exclusion of those with prevalent diabetes, cardiovascular disease, and with known / treated hypertension, suggesting that these vessel changes may be indicative of preclinical phases of disease.

While retinal signs of diabetic eye disease are well described,[27] there have been some uncertainties about the association between diabetes, particularly risk factors for Type 2 diabetes, and retinal vessel morphometry, with inconsistencies between cross-sectional and longitudinal findings.[28] However, a recent meta-analysis showed that wider venules, but not arterioles, were associated with diabetes;[29] consistent with cross-sectional observations suggesting that wider venules are associated with increasing levels of fasting glucose and HbA1c levels.[28] Findings from the present study are consistent with these risk factor observations, not only replicating the associations between increased venular width and glycosylated haemoglobin (although not formally statistically significant), but also showing coherent associations with other metabolic risk factors, including BMI, as well as novel associations with levels of triglyceride; associations which were absent with arteriolar width. The present study also showed that narrow venules were associated with increased HDL,

282 which when considered in relation to levels of triglyceride, might be considered as a further
283 indicator of insulin resistance.[30] However, venular width associations with HDL and
284 triglycerides were weakened after multivariable adjustment, and HDL-tortuosity
285 associations differed in males and females. Reasons for these sex differences are unclear,
286 but may relate to sex differences observed in retinal width-CHD associations, where
287 associations are evident in women not men.[13;31] Moreover, this study was novel in showing
288 consistent metabolic associations with retinal vessel tortuosity, whereby increased venular
289 tortuosity was associated with Type 2 diabetes risk factors (including levels of BMI and
290 HbA1c), in addition to showing a strong association with prevalent Type 2 diabetes. These
291 associations persist after mutual adjustment, and exclusion of those with clinical diabetes,
292 suggesting that these associations may be independent early markers of the disease
293 process. Associations observed in this study appear to contrast with those observed with
294 overt disease, whereby arteriolar (not venular) tortuosity has been related to the duration
295 of diabetes.[32] Associations with Type 2 diabetes risk markers (including levels of BMI and
296 HbA1c), as well as other cardiovascular risk factors (systolic blood pressure and blood
297 cholesterol) were not observed amongst this diseased group.[32] This may suggest
298 differences in retinal vessel morphometry associations between disease development and
299 overt disease.

300

301 Cross sectional and longitudinal associations between retinal vasculometry and CVD
302 outcomes have been studied, including coronary heart disease (CHD), stroke and
303 cardiovascular mortality.[13;33-35] However, more recent evidence from prospective studies
304 has raised some inconsistencies. In particular, retinal vessel calibre changes are only

associated with CHD events in women not men,[13;31] and in some studies vessel width associations with stroke appear only apparent in venules, which appears to contradict the perceived disease process.[36] In the present study, we observed no association between retinal vascular width measures and prevalent CHD, although there was the suggestion of a positive association between arteriolar tortuosity and prevalent stroke, which was stronger after adjustment for age, sex and blood pressure. An association between narrower arterioles and high blood pressure has been well documented.[10;11;14;37] The present study confirms these findings, showing decreased arteriolar width associated with both increased systolic and diastolic blood pressure.

Evidence examining associations between venular width and blood pressure have been less consistent,[10] although a recent meta-analysis suggested increased width associated with hypertension.[14] Our study showed a small but statistically significant decrease in venular width with increasing diastolic blood pressure, which remained after multivariable adjustment, although the magnitude of association was less than the association observed with arterioles. This association was no longer statistically significant when those with prevalent cardiovascular disease and known / treated hypertension were excluded, but associations with systolic blood pressure remained. The observation of an association between vessel width and systolic blood pressure amongst non-hypertensives, strengthens the potential additional use of retinal vessel morphometry assessment in routine health checks. Of particular note were the different associations with vessel tortuosity, where increased arteriolar and venular tortuosity was associated with greater systolic blood pressure (but not diastolic blood pressure), while decreased venular tortuosity was associated with higher HDL. The apparent different direction of associations with these

cardiovascular risk factors are potentially consistent, and replicate findings observed in one other large population based study.[15]

By far the strongest associations observed were those with age and smoking, where per decade rise in age there was arteriolar narrowing and increased tortuosity, and with current smoking appreciable arteriolar and venular dilation. There was also the suggestion of smaller arterioles and markedly greater tortuosity (both arteriolar and venular) in females compared to males. However, sex differences in width were largely explained and differences in tortuosity partially explained by height (data not presented). While differences in CVD risk between males and females may have contributed to these associations, explanations for potential sex differences in retinal vessel morphometry remain uncertain. The effect of age was independent of blood pressure, as well as other cardiometabolic risk factors, but smaller compared to a body of literature suggesting a 2 to 5µm decrease in arteriolar width per decade in age (although these later effect sizes were seen in relation to central retinal vessel equivalent sizes, which are 2-3 times larger as they are scaled-up from retinal measures taken within 0.5 to 1.5 disc diameters from the disc).[10;38] Nevertheless, these observations demonstrate the well-known association between narrower more tortuous arterioles and older age.[39] The vasodilatory effects of smoking have also been widely reported in venules, less so in arterioles.[10] Increased carbon monoxide levels amongst smokers may well provide a biological explanation for these findings.[40]

Computerised assessment of vessels from retinal images have so far been heavily reliant on operator involvement, which is subjective, open to measurement error and time consuming,[16] limiting its use in large population based studies. The EPIC Eye study is such a study, which is richly phenotyped, allowing examination of multiple CVD risk factors within the same cohort. Our fully automated system provides a rapid, detailed quantification of retinal vasculature in this population, for both arterioles and venules separately, since they show some opposing patterns of association with risk markers and disease states.[41] The system has been extensively validated, and was successful in obtaining vessel measures in 4 out of five who underwent retinal imaging. It was not possible to obtain useful data from the remainder, as image quality was graded as insufficient (with the AV detection program unable to distinguish arterioles from venules), with images being decentred, defocussed, or obstructed by media opacities or lashes; an inevitable consequence of non-mydriasis, especially in this older age group. This did not appear to reflect a selection bias, as there was no evidence of a marked differences in other phenotypes between those with and without vessel measures. While those participating in the 3HC did appear to be select (being significantly younger, with higher BMI and of more privileged socioeconomic status compared to participants at baseline), this is unlikely to invalidate retinal vessel morphometry and cardiometabolic risk factor associations.[42]

Our image analysis system has improved performance or is similar to earlier approaches,[43-46] obtaining measures from the whole retinal image, not just concentric areas centred on the disc.[10] Earlier studies have considered effect sizes in relation to central retinal artery and central retinal vein equivalents (CRAE, CRVE).[10] It was not possible to directly compare measures with CRAE and CRVE, as the number of measures of width were considerably

more and located over the entire image. Reducing the measurement area, typically between 0.5 to 1.5 disc diameters, to provide these measures would result in a huge data reduction, which might exclude vessel changes occurring elsewhere in the retina. Moreover, poor agreement between different systems has been highlighted, making direct comparisons in retinal calibre measures between systems problematic.[47] Despite this we report similar effect sizes (e.g., the change in vessel width associated with smoking) in relation to a narrower mean width indicative of a far greater measurement area. Vessel density is not uniform across the retina.[48] Supplemental Figure 3 shows the extent of vessel measures in a typical image. While the measures are not constrained to concentric areas close to the disc, as used in comparable systems,[47] this was not perceived as a weakness given that our system is fully automated and does not allow for measurement areas to be selected. Moreover, consistent inclusivity of measures across the whole image was observed in all images that were automatically selected as being of sufficient quality for inclusion, limiting any potential selection effects.[19] Our approach is further supported by the first paper examining use of artificial intelligence (AI) in detecting cardiovascular disease, which appears to show that retinal vessels over their entire length are key areas of interest in estimating cardiovascular risk factors, such as age, blood pressure and HbA1c.[49] While it is difficult at present to get precise information on how AI algorithms arrive at decisions, these findings suggest that retinal vasculometry studies, such as ours, are key to understanding processes associated with cardiometabolic disease.

We have condensed these measures to provide an overall summary of mean width, but it is possible that relative changes in vessel indices over time and perhaps variations in measures along the length of a vessel may be stronger predictors of vascular health than absolute size,

398 although this remains to be established. The presence of differential retinal vasculometry
399 associations with cardiometabolc risk factors underline the importance of making separate
400 arteriolar and venular width and tortuosity measures, calling into question the validity of
401 arteriolar / venular ratio measures for cardiovascular risk profiling.
402
403 The modest vasculometry association with prevalent stroke and the absence of associations
404 with prevalent MI does not necessarily mean that retinal vasculometry measures are
405 unlikely to have a role in CVD risk prediction. Prevalent cases are likely to be very different
406 to premorbid incident cases, with established cases often receiving vasoactive medications,
407 which might have a modifying effect on vascular morphometry. It is also possible that there
408 was insufficient power to determine change in these dichotomous outcomes, given the
409 small number of prevalent events within this study population. However, retinal vessel
410 associations with Type 2 diabetes risk markers and diabetes mellitus were observed, even
411 after exclusion of those with prevalent outcome, suggesting that pre-clinical vasculometry
412 changes are apparent. This is commensurate with recent longitudinal evidence, raising the
413 possibility that retinal vasculometry may have a role in risk prediction),[50] as well as
414 surveillance and disease management. Power to determine change in continuous outcomes
415 was greater, replicating previous observations and yielding a number of novel associations,
416 particularly those with vessel tortuosity, as well as metabolic markers. However, given the
417 cross-sectional nature of data collection, these associations between cardiometabolic risk
418 factors and retinal vessel abnormalities do not of themselves allow the potential role of
419 retinal vessel quantification in disease risk prediction to be formally ascertained; future
420 follow-up of this and other large cohorts with high quality retinal imaging data will allow this
421 issue to be investigated.

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CONTRIBUTION STATEMENT

All Authors contributed to this manuscript. CGO,ARR,SAB,DPS,PHW,PJF designed the present study and raised funding; RL,SAH,NJW,KTK,PJF for the EPIC Eye study. RL,SAH,SAB,RAW,ARR collected data for the study and undertook data management. RAW,SAB,ARR analysed the data. CGO wrote the first draft of the report, to which all authors contributed. CGO is responsible for data integrity and will act as guarantor.

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FIGURE LEGENDS

Figure 1: Adjusted mean vessel width and tortuosity by quintiles of cardiovascular and Type 2 diabetes risk factors, for venues and arterioles. Adjusted means (solid square symbols), 95% CIs (error bars), regression lines (solid line) and associated 95% CIs (dashed lines) are from a multilevel model allowing for age, sex and repeated measure of vessel indices within person.

Supplemental Figure 1: Flow diagram of participant recruitment for different phases of the European Prospective Investigation of Cancer in Norfolk study, and in particular the third follow-up which included an eye examination.

Supplemental Figure 2: Histogram of arteriolar and venular width and tortuosity measures (including with and without log transformation for tortuosity measures).

Supplemental Figure 3: Automated arteriolar (red) and venular (blue) width measures recorded in one EPIC Eye image.

Table 1. Participant characteristics of EPIC participants who took part in the 3rd health check with and without useable fundus images (5947 versus 2676 participants)

Characteristic	Third Health Examination	
	Included in the analyses	Excluded from the analyses
Number	5947	2676
Age (SD) years	67.6 (7.6)	71.3 (8.6)
Gender n (% Female)	3,393 (57)	1,365 (51)
Current smokers n (%)	267 (4.5)	107 (4.0)
Former smoker n (%)	2,628 (44)	1284 (48)
Height (cm)	166.4 (9.1)	166.2 (9.2)
Weight (Kg)	74.4 (14.3)	74.6 (14.0)
BMI (Kg/m^2)	26.8 (4.3)	27.0 (4.2)
Systolic blood pressure (mmHg)	135.7 (16.6)	137.3 (16.8)
Diastolic blood pressure (mmHg)	78.4 (9.2)	77.9 (9.6)
Total cholesterol (mmol/L)	5.4 (1.1)	5.3 (1.1)
LDL cholesterol (mmol/L)	3.2 (1.0)	3.1 (1.0)
HDL cholesterol (mmol/L)	1.5 (0.4)	1.5 (0.4)
Triglycerides (mmol/L)	1.7 (0.9)	1.6 (0.9)
HbA1c (%)	5.8 (0.6)	5.9 (0.7)
HbA1c (mmol/mol)	40	41
Prevalent MI n (%)	187 (3.1)	106 (4.0)
Prevalent stroke n (%)	118 (2.0)	67 (2.5)
Prevalent Type 2 diabetes n (%)	237 (4.0)	156 (5.8)
Mean axial length (SD) mm	23.6 (1.2)	23.5 (1.2)
Mean best vision sphere (SD) dioptres	0.2 (2.2)	0.2 (2.3)
Mean arteriolar width (SD) microns	74.8 (6.9)	-
Mean venular width (SD) microns	88.4 (11.3)	-
Arteriolar tortuosity x 1000*	4.2 (1.6)	-
Venular tortuosity x1000*	3.3 (1.3)	-

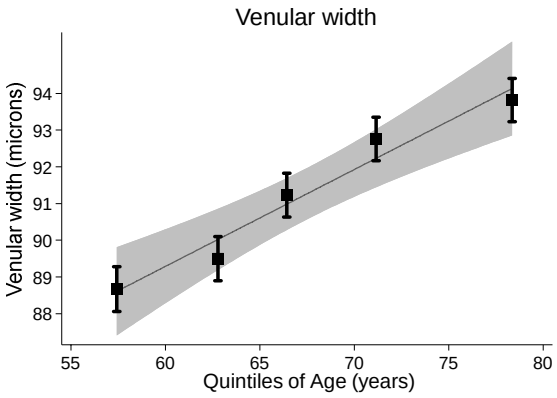
Mean (SD) or n (%) as indicated.
* Geometric mean (SD)
For participants included in the analyses extent of missing data is as follows:-
Cholesterol missing data for 429 participants
LDL Cholesterol missing data for 511 participants
HDL Cholesterol missing data for 428 participants
Triglycerides missing data for 429 participants
HbA1c missing data for 498 participants

Table 2. Difference in vessel width (μm) and tortuosity (%) associated with Type 2 diabetes and CVD risk factors and outcomes for individual factors in multivariable regression model age and sex adjusted

Risk marker	Difference in arteriolar width (95% CI) μm	P-value	Difference in venular width (95% CI) μm	P-value	Difference in arteriolar tortuosity (95% CI) %	P-value	Difference in venular tortuosity (95% CI) %	P-value
Per decade in age	-0.79 (-1.00, -0.58)	<0.001	2.56 (2.20, 2.91)	<0.001	5.44 (3.80, 7.11)	<0.001	-0.23 (-1.15, 0.69)	0.619
Female vs male	-0.51 (-0.83, -0.19)	0.002	-0.32 (-0.86, 0.22)	0.245	3.83 (1.37, 6.35)	0.002	2.16 (0.74, 3.60)	0.003
Current vs never smoked	2.13 (1.34, 2.91)	<0.001	3.03 (1.71, 4.34)	<0.001	-2.70 (-8.22, 3.16)	0.360	1.66 (-1.75, 5.18)	0.345
Former vs never smoked	0.11 (-0.23, 0.44)	0.522	0.31 (-0.25, 0.87)	0.275	-0.21 (-2.67, 2.31)	0.870	0.88 (-0.58, 2.36)	0.240
Per 5 kg/m² in BMI	0.15 (-0.03, 0.34)	0.098	0.72 (0.41, 1.03)	<0.001	-0.24 (-1.59, 1.13)	0.729	2.52 (1.71, 3.34)	<0.001
Per 10mmHg in SBP	-0.50 (-0.60, -0.41)	<0.001	-0.06 (-0.23, 0.10)	0.458	1.20 (0.47, 1.94)	0.001	0.45 (0.02, 0.88)	0.039
Per 10mmHg in DBP	-1.04 (-1.22, -0.87)	<0.001	-0.32 (-0.61, -0.02)	0.035	0.75 (-0.56, 2.07)	0.263	-0.55 (-1.30, 0.21)	0.157
Per 1mmol/L in TC	-0.18 (-0.33, -0.02)	0.024	-0.16 (-0.41, 0.10)	0.233	0.42 (-0.72, 1.58)	0.472	-0.52 (-1.18, 0.15)	0.131
Per 1mmol/L in LDL	-0.09 (-0.26, 0.08)	0.313	-0.24 (-0.53, 0.05)	0.108	0.60 (-0.69, 1.90)	0.362	-0.39 (-1.14, 0.36)	0.310
Per 1mmol/L in HDL	-1.18 (-1.62, -0.74)	<0.001	-1.42 (-2.16, -0.69)	<0.001	-0.61 (-3.82, 2.70)	0.714	-1.83 (-3.70, 0.07)	0.059
Per 1mmol/L in Triglycerides	0.06 (-0.12, 0.23)	0.524	0.57 (0.27, 0.86)	<0.001	0.29 (-1.01, 1.62)	0.661	-0.18 (-0.94, 0.59)	0.647
Per % in HbA1c per	0.22 (-0.08, 0.51)	0.148	0.41 (-0.07, 0.90)	0.097	0.95 (-1.21, 3.15)	0.393	2.24 (0.96, 3.53)	0.001
Prevalent MI vs absent	0.66 (-0.27, 1.58)	0.165	1.20 (-0.35, 2.75)	0.129	4.36 (-2.57, 11.77)	0.224	1.87 (-2.14, 6.05)	0.366
Prevalent Stroke vs absent	0.79 (-0.37, 1.95)	0.181	0.59 (-1.35, 2.53)	0.553	8.30 (-0.59, 17.99)	0.068	3.66 (-1.42, 9.01)	0.161
Prevalent DM vs absent	-0.08 (-0.90, 0.75)	0.857	0.48 (-0.90, 1.86)	0.494	1.64 (-4.38, 8.03)	0.602	6.53 (2.78, 10.41)	0.001

Number included n=5,942. Regression coefficients are from a multilevel model allowing for repeated images from the same person (random effect for person) and adjusting for age and sex as fixed effects. Prevalent MI, stroke, DM (Diabetes Mellitus); n=187, 118, 238 respectively
Cholesterol missing data for 429 participants
LDL Cholesterol missing data for 511 participants
HDL Cholesterol missing data for 428 participants
Triglycerides missing data for 429 participants
HbA1c missing data for 498 participants

AGE


Venular width
Venular width (microns)
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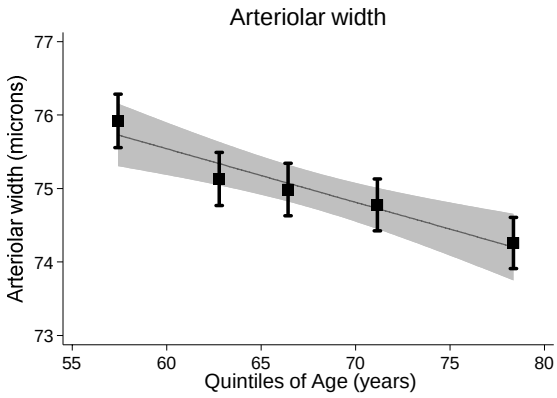
89
90
91
92
93
94
55
60
65
70
75
80
Quintiles of Age (years)


Arteriolar width
Arteriolar width (microns)
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74
75
76
77
55
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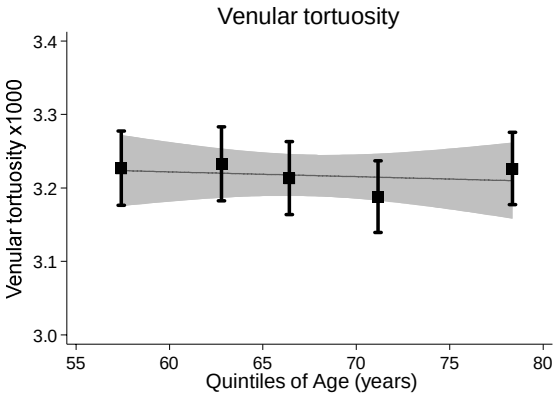
65
70
75
80
Quintiles of Age (years)


Venular tortuosity
Venular tortuosity x1000
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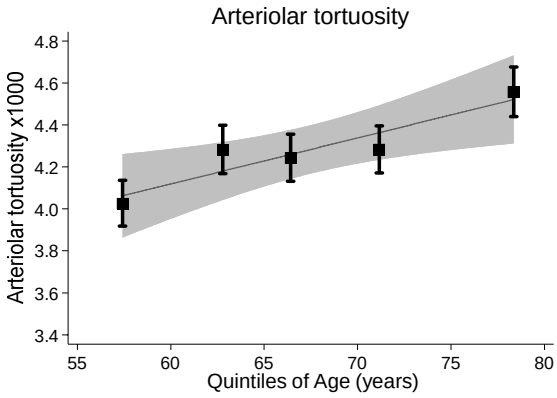
3.1
3.2
3.3
3.4
55
60
65
70
75
80
Quintiles of Age (years)


Arteriolar tortuosity
Arteriolar tortuosity x1000
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3.6
3.8
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4.4
4.6
4.8
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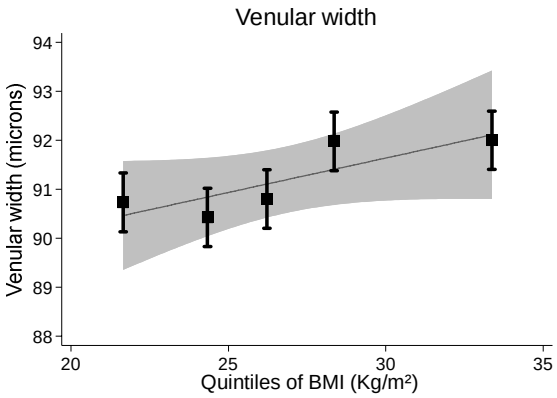
60
65
70
75
80
Quintiles of Age (years)

BMI


Venular width
Venular width (microns)
88
89
90
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93
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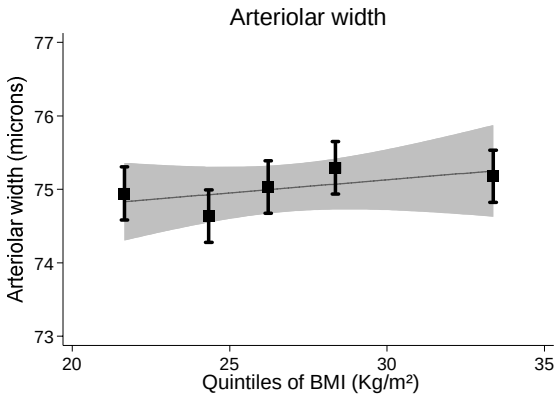
30
35
Quintiles of BMI (Kg/m²)


Arteriolar width
Arteriolar width (microns)
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77
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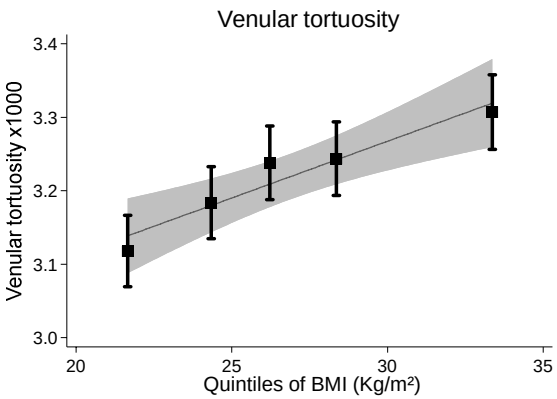
35
Quintiles of BMI (Kg/m²)


Venular tortuosity
Venular tortuosity x1000
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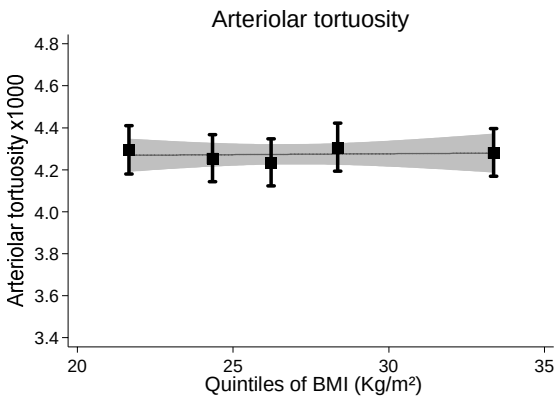
35
Quintiles of BMI (Kg/m²)


Arteriolar tortuosity
Arteriolar tortuosity x1000
3.4
3.6
3.8
4.0
4.2
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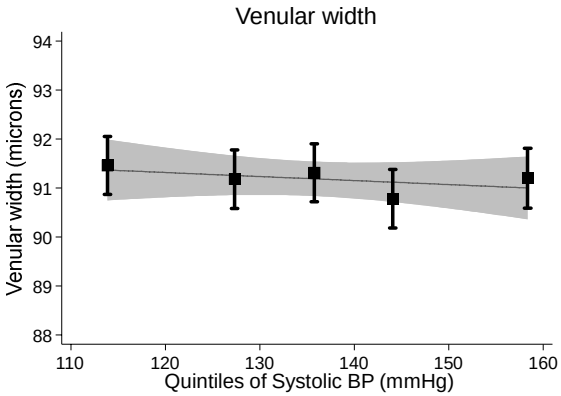
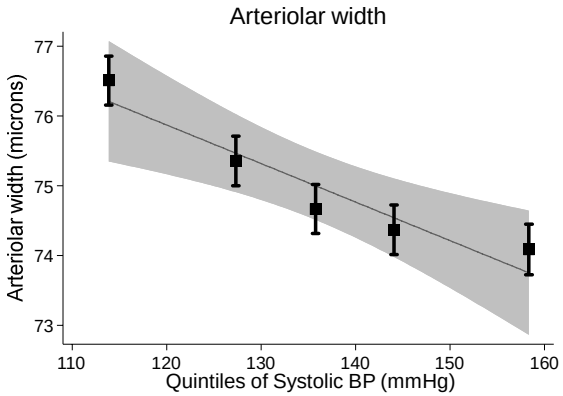
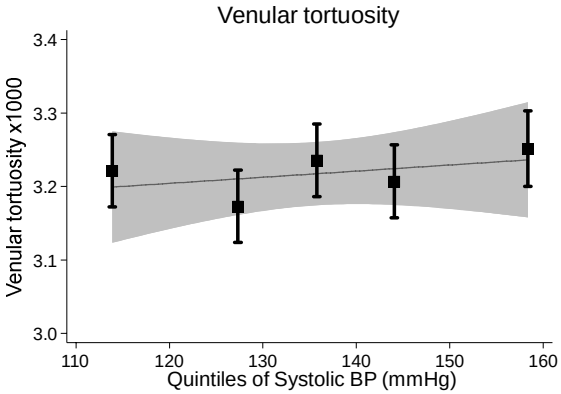
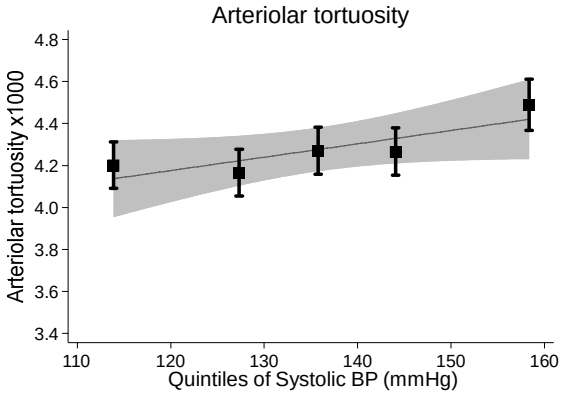
4.6
4.8
20
25
30
35
Quintiles of BMI (Kg/m²)

SYSTOLIC BLOOD PRESSURE

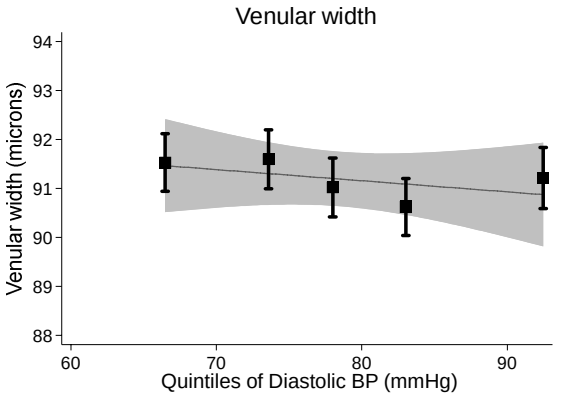


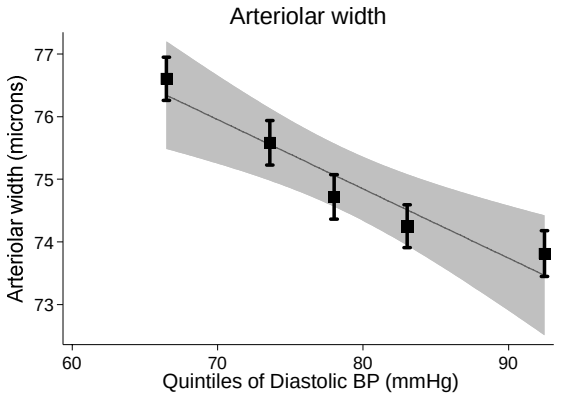


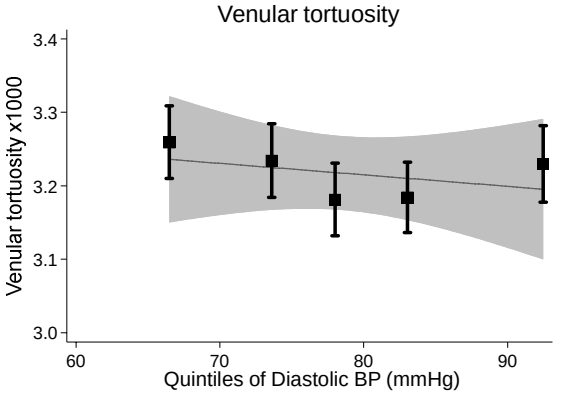


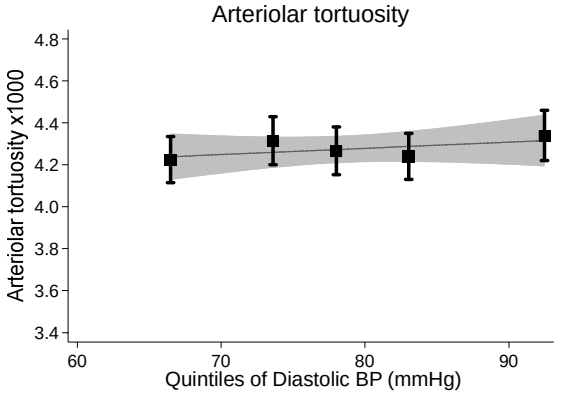


DIASTOLIC BLOOD PRESSURE









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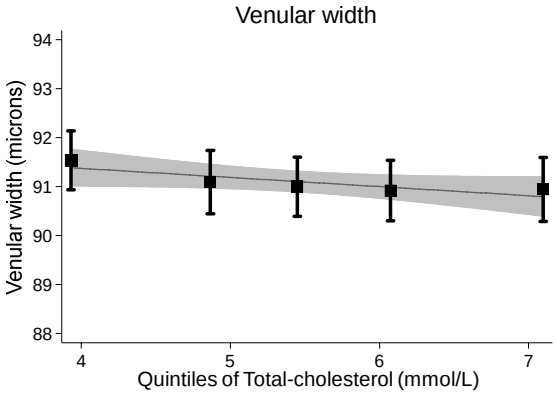

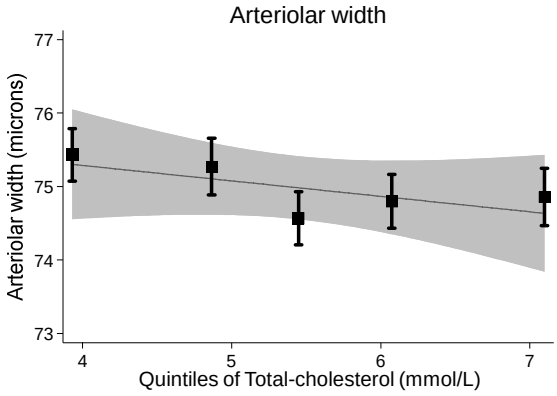

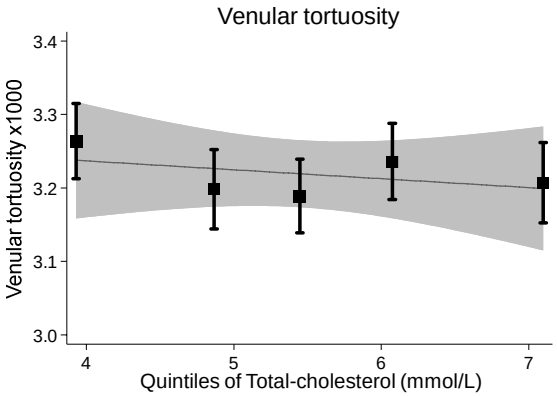

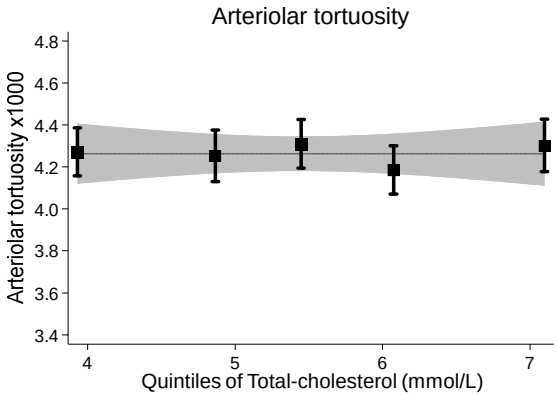

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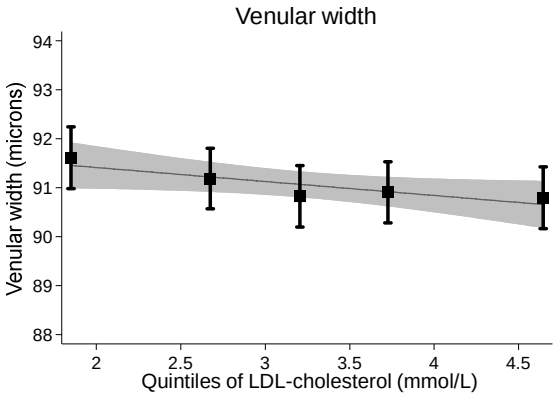

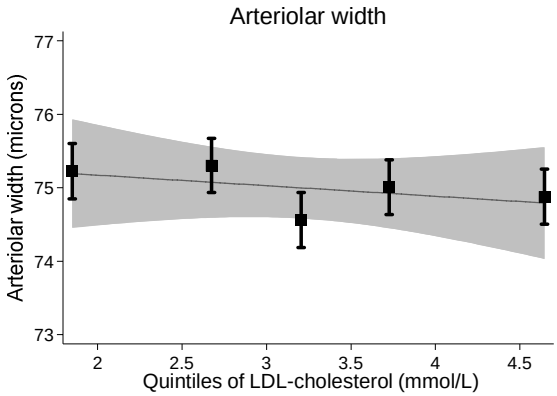

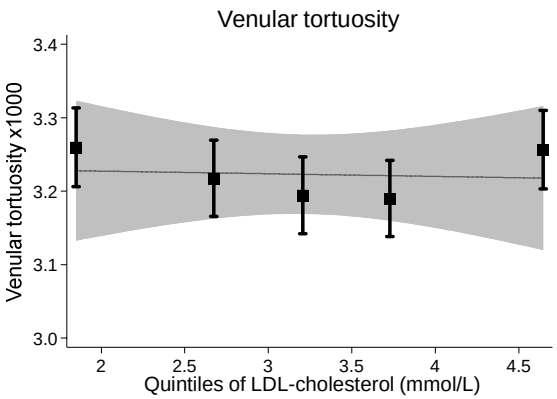

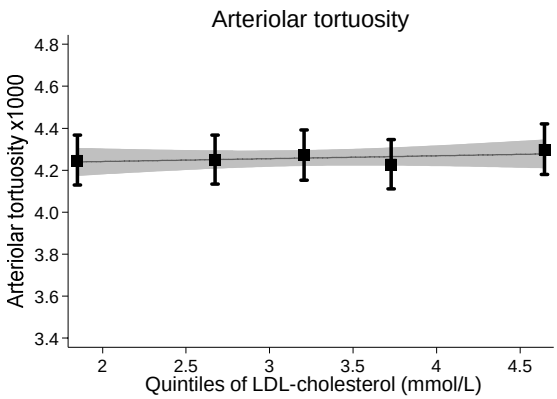

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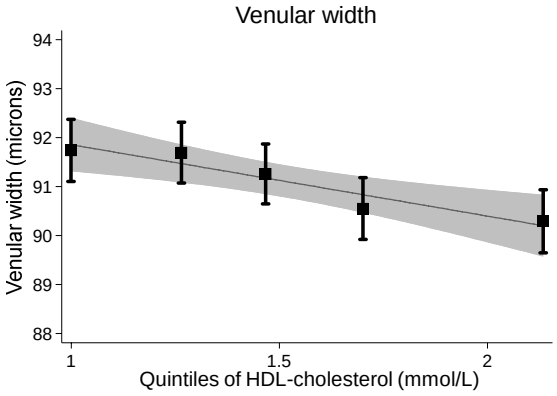

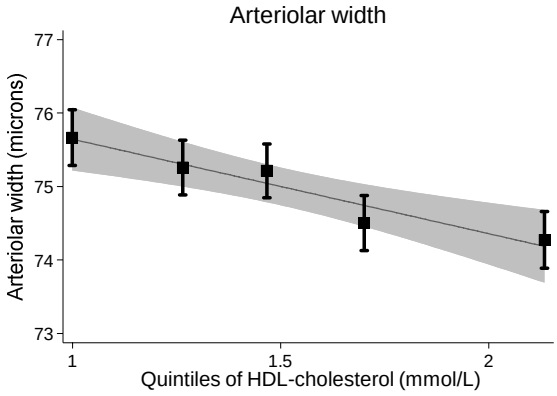

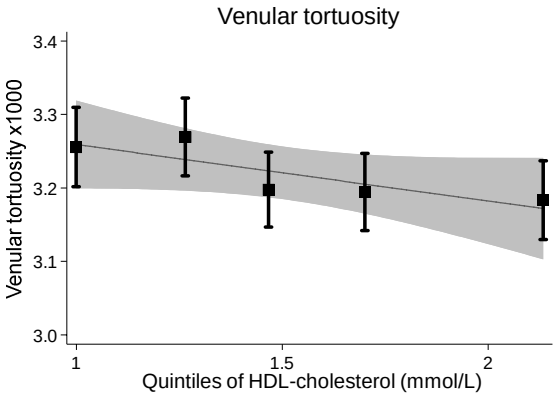

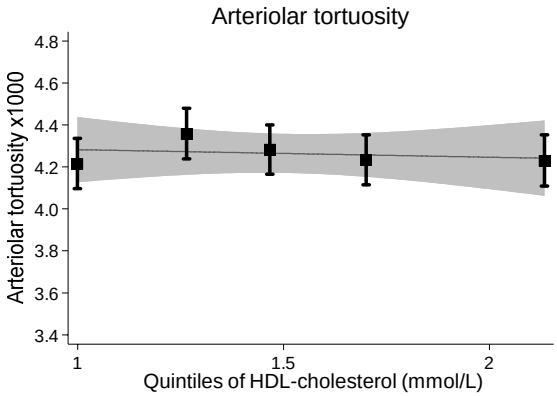

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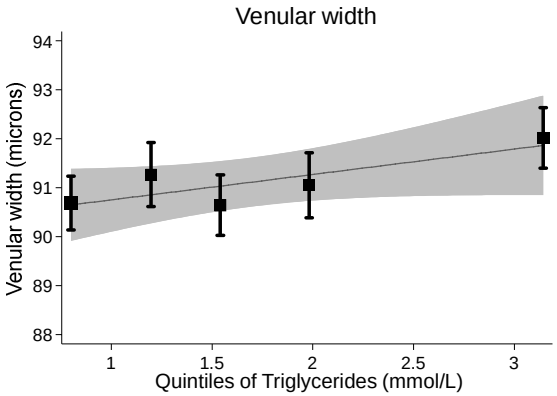

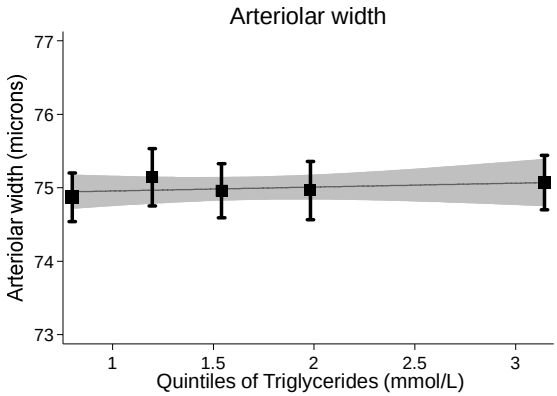

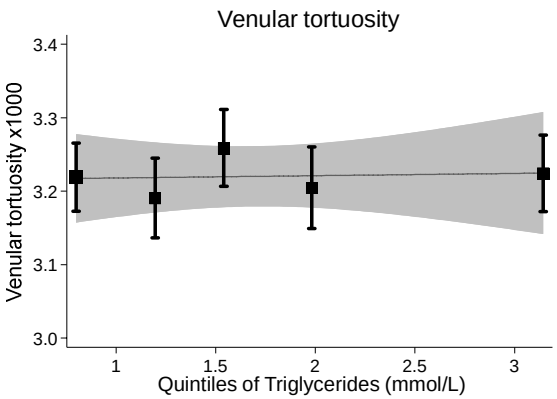

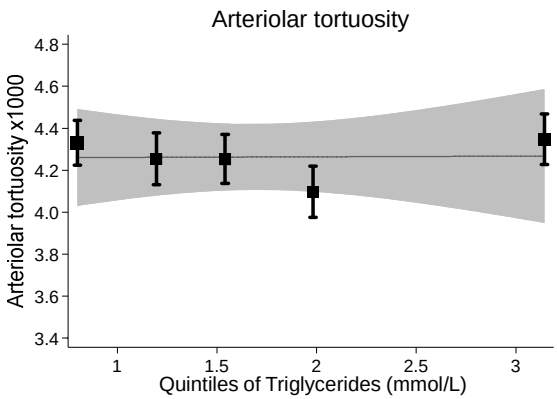

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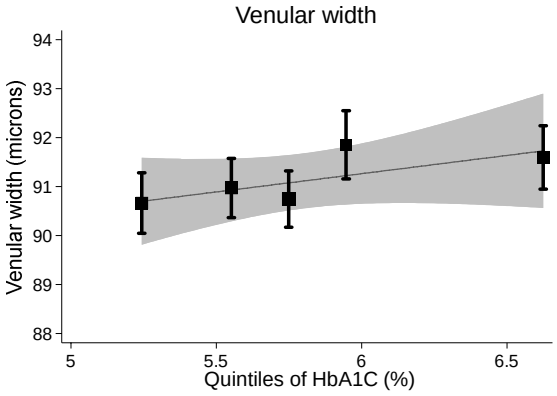

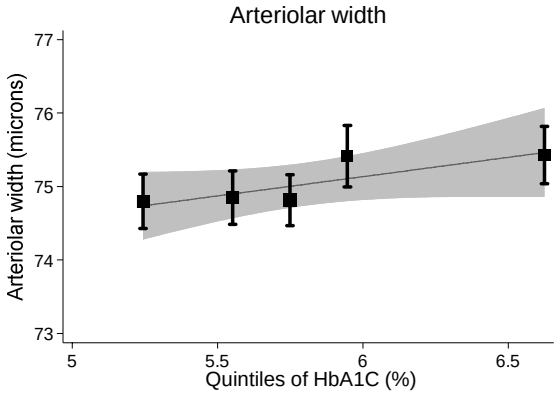

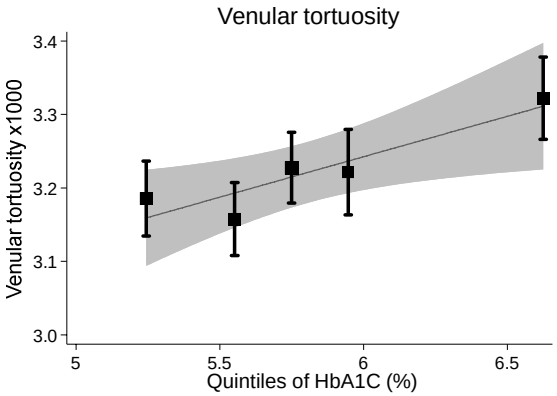

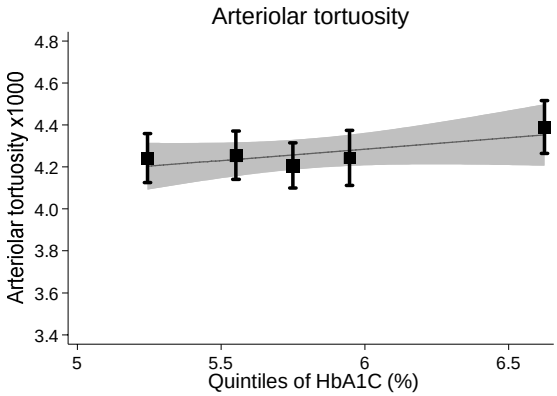

HEIGHT