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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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1 **ABSTRACT**

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

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

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

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

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

14 **Results:** 279,802 arterioles, and 285,791 venules from 5947 participants (mean age 67.6
15 years, SD 7.6, 57% female) were analysed. Increased venular tortuosity was associated with
16 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 %),
17 and with prevalent type 2 diabetes (6.5%, 95%CI 2.8,10.4%); wider venules were associated
18 with older age (2.6µm, 95%CI 2.2,2.9µm per decade), higher triglycerides (0.6µm, 95%CI
19 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 -
20 0.1,0.9 per %) and being a current smoker (3.0µm, 95%CI 1.7,4.3µm); similarly smoking was
21 also associated with wider arterioles (2.1µm, 95%CI 1.3,2.9µm). Thinner venules were
22 associated with HDL (1.4µm, 95%CI 0.7,2.2 per mmol/L). Arteriolar tortuosity increased
23 with age (5.4%, 95%CI 3.8,7.1% per decade), higher systolic blood pressure (1.2%, 95%CI
24 0.5,1.9% per 10mmHg), in females (3.8, 95%CI 1.4,6.4%) and with prevalent stroke (8.3%,

25 95%CI -0.6,18%); no association was observed with prevalent myocardial infarction.

26 Narrower arterioles were associated with age (0.8 μ m, 95%CI 0.6,1.0 μ m per decade), higher

27 systolic blood pressure (0.5 μ m, 95%CI 0.4,0.6 μ m per 10mmHg), total cholesterol (0.2 μ m,

28 95%CI 0.0,0.3 μ m per mmol/L) and HDL (1.2 μ m, 95%CI 0.7,1.6 μ m per mmol/L).

29 **Conclusions:** Metabolic risk factors show a graded association with both tortuosity and

30 width of retinal venules, even among people without clinical diabetes, whereas

31 atherosclerotic risk factors correlate more closely with arteriolar width, even excluding

32 those with hypertension and cardiovascular disease. These non-invasive microvasculature

33 measures should be evaluated further as predictors of future cardiometabolic disease

34 among apparently healthy individuals.

35 **Keywords:** Retinal vessels, morphology, cardiometabolic risk factors

36 Cardiovascular disease (CVD), including coronary heart disease (CHD), heart failure and
37 stroke, is responsible for a substantial burden of morbidity and disability.¹ Type 2 diabetes
38 is an increasing public health problem, affecting 1 in 10 adults globally, and a major cause of
39 premature death and morbidities, especially CVD.² Early detection and prevention both of
40 CVD and Type 2 diabetes is key to limiting future morbidity and mortality.^{3;4} While disease
41 risk factors for Type 2 diabetes, such as blood glucose levels and HbA1c, are yet to show
42 good screening performance,⁵ established markers of early vascular disease are used in risk
43 prediction models to estimate future risk of CVD, providing indications for medical / lifestyle
44 interventions to alter disease trajectory.^{6;7} There have been a number of attempts to
45 improve the performance of these risk prediction models, by adding other risk factors.^{6;7}
46 However, the addition of novel risk factors have added little to CHD prediction.⁸ Recent
47 evidence suggests that early markers for the presence of vascular disease (as opposed to
48 additional risk factors) are needed to improve risk prediction for population screening.^{5;9}
49
50 Detailed retinal vasculometry may offer such a marker. Growing evidence suggests that
51 morphological features in retinal vessels, in particular vessel width, are early physiological
52 markers of cardiometabolic risk and disease (as well as other disease processes).¹⁰⁻¹³ While
53 strong evidence has accrued for some of these associations, particularly associations with
54 Type 2 diabetes and CVD (and their related risk factors), other associations have remained
55 inconsistent. For instance, wider arterioles have been associated with higher levels of blood
56 glucose, total cholesterol, triglycerides and inflammatory markers, but not in all studies.^{10;12}
57 Similarly associations of venular width with blood pressure have also been inconclusive,¹⁰
58 although recent evidence suggests increased width associated with hypertension.¹⁴ Wider

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

77 **RESEARCH DESIGN AND METHODS**

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

91
92 **Third Follow-Up:** Between 2004 and 2011, 8623 participants provided updated information
93 on medical history and lifestyle behaviour.²² Weight and height, were measured with
94 participants in light clothing without shoes. Weight was measured to the last 0.1 kg using
95 regularly calibrated digital scales (Tanita TBF-300, Tanita UK Ltd, Middlesex, UK), and height
96 to the last complete 0.1 cm using a stadiometer (Chasmors, UK). Body mass index (BMI) was
97 calculated as weight / height squared in kg/m². Seated blood pressure was measured twice
98 using an automated blood pressure monitor (Accutorr PlusTM, Datascope Patinet
99 Monitoring, Huntington, UK); the mean of both measures was used. A non-fasting venous
100 blood sample was collected; details of the analytic measures have been published

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

105

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

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

137

138 **Ethics, Governance and Consent:** The EPIC-Norfolk Eye Study was carried out following the
139 principles of the Declaration of Helsinki and the Research Governance Framework for Health
140 and Social Care. The study was approved by the Norfolk Local Research Ethics Committee
141 (05/Q0101/191) and East Norfolk and Waveney NHS Research Governance Committee
142 (2005EC07L). All participants gave written, informed consent.

143

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

149 adjusting for age and sex were used to examine associations of cardiometabolic risk factors
150 and prevalent disease status to retinal vessel morphometry outcomes, allowing for repeated
151 measures of vessel indices within the same person. Regression models provided mean
152 differences in width and percentage differences in tortuosity for venules and arterioles
153 separately, per decade in age, females versus males, current smokers and former smokers
154 versus never-smokers, or per unit increase in cardiometabolic risk factor (per 5 kg/m²
155 increase in BMI, per 10 mmHg in systolic and diastolic blood pressure, per mmol/L increase
156 in total cholesterol, high density lipoprotein, triglyceride, and per percentage rise in HbA1c).
157 For disease outcomes, differences in vessel indices were obtained comparing those with
158 prevalent disease present (including type 2 diabetes, MI, stroke, and known / treated
159 hypertension) versus absent. Differences in associations between men and women were
160 formally examined by inclusion of an interaction term between the risk factor and sex into
161 the regression model. Risk factors found to be statistically significantly related to vascular
162 measures at the 5% level were subsequently included in mutually adjusted models. We also
163 examined associations after exclusion of participants with prevalent disease outcomes.

164 **RESULTS**

165 Of 18,380 individuals invited to participate in this phase of the study, 8,623 (47%) took part
166 (mean age 67.6 years, 57% female). Supplemental Figure 1 shows a flow diagram of the
167 numbers participating in the study. Fundus imaging and refractive assessment were carried
168 out in 7411 individuals, of whom 5,957 participants (80%) had at least one image of
169 sufficient quality and classified vessels as arterioles or venules with a probability set at 80%
170 detection. It was not possible to obtain useful data from the remainder as images were
171 miscentred, defocussed, or were obstructed by lashes and/or media opacities. A small
172 number had missing data for height, weight or blood pressure (n=10), leaving 5947
173 participants with measures of vessel width and tortuosity for 565,593 vessel segments
174 (279,802,arterioles, 285,791 venules) from 10,474 images; blood sample data were available
175 for 5514. Participant characteristics of EPIC participants at baseline, and those who took
176 part in the third health examination with and without useable fundus images have been
177 described previously.²⁶ Those attending the 3rd Health Check (3HC) were younger at
178 baseline, of higher BMI and socioeconomic status, and were less likely to be a current
179 smoker compared to participants not followed-up.²⁶ Participant characteristics of EPIC
180 participants who took part in the third health examination, and who were included in the
181 analyses compared with those who were not (5,947 versus 2,676 participants) are
182 summarised in Table 1. Other than those included being slightly younger (mean age 68
183 years vs 71 years), there was no clear evidence of a systematic difference in 3HC participant
184 characteristics. Retinal vessel morphometry in those with useable fundus images are also
185 summarised for arterioles and venules separately. Histograms of arteriolar and venular
186 width and tortuosity measures (with and without log transformation) are shown in

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

189

190 Differences in retinal vessel width in microns, and percentage differences in tortuosity by

191 Type 2 diabetes and CVD risk factors and outcomes are shown by vessel type in Table 2.

192 Arterioles were inversely associated and more tortuous with older age (0.8 μ m, 95%CI 0.6,

193 1.0 μ m and 5.4%, 95%CI 3.8, 7.1% per decade respectively). Wider venules were observed

194 with older age (mean difference 2.6 μ m, 95%CI 2.2,2.9 μ m per decade), and amongst current

195 smokers compared to never smokers (3.0 μ m, 95%CI 1.7, 4.3 μ m). Narrower arterioles

196 (0.5 μ m, 95%CI 0.2,0.8) and more tortuous arterioles and venules were strongly associated

197 with being female compared to male (3.8%, 95% CI 1.4, 6.4%; 2.2%, 95% CI 0.7, 3.6%

198 respectively).

199 *Retinal vasculometry associations with metabolic risk factors:-*

200 Venular width was positively associated with Type 2 diabetes risk factors, including higher

201 BMI (0.7 μ m, 95%CI 0.4, 1.0 μ m per 5 kg/m²), and HbA1c (0.4 μ m, 95%CI -0.1, 0.9 μ m per %).

202 Wider venules were also positively associated with elevated levels of triglycerides (0.6 μ m,

203 95%CI 0.3, 0.9 μ m per mmol/L). Venular tortuosity was also positively associated with Type

204 2 diabetes risk factors, as well as prevalent Type 2 diabetes. Venules were 2.5% more

205 tortuous (95% CI 1.7, 3.3%) per 5 kg/m² increase in BMI, 2.2% more tortuous (95% CI 1.0,

206 3.5%) per percentage rise in HbA1c, and 6.5% more tortuous (95% CI 2.8, 10.4%) amongst

207 those with Type 2 diabetes compared to those without.

208 *Retinal vasculometry associations with cardiovascular risk factors:-*

209 Arteriolar widths were inversely associated with age, systolic (0.5 μ m 95%CI 0.4, 0.6 μ m per

210 10mmHg rise) and diastolic blood pressure (1.0 μ m, 95%CI 0.9, 1.2 μ m per 10mmHg rise).

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

224 *Sensitivity and multiple variable analyses:-*

225 Sensitivity analyses examined the differences in vessel width and tortuosity associated with
226 cardiometabolic risk factors, excluding those with clinical diabetes / cardiovascular disease,
227 and those with known / treated hypertension (data available on request). Metabolic
228 associations with venular width and tortuosity persist after exclusion of those with clinical
229 diabetes, and arteriolar width associations with vascular risk factors (particularly blood
230 pressure) remain after excluding those with cardiovascular disease and hypertension.

231 Retinal vessel associations were similar in males and females (tests for interaction $P>0.05$),
232 except for HDL, for which opposing associations with arteriolar tortuosity were apparent.

233 Per mmol/L higher HDL, arteriolar tortuosity was 5.8% (95% CI 0.1, 11.8%) higher in men,
234 but 4.0% (95% CI 0.0, 7.8%) lower in women (test for interaction $p=0.006$).

235 The mutual independence of these risk factor associations was also examined. Mutually
236 adjusted risk factor associations are presented in Supplemental Table 1. Risk factors that
237 were statistically significantly associated with retinal vasculometry in Table 2 were included
238 in multiple variable regression models. Associations with both arteriolar morphometry
239 measures and cardiometabolic risk factors remained remarkably stable. Consistent
240 associations were observed between arteriolar width and age, current smoking status,
241 blood pressure and HDL cholesterol, but there was no evidence of an independent
242 association with total cholesterol. Similarly strong associations remained for arteriolar
243 tortuosity with age, sex and blood pressure. Associations from mutually adjusted models
244 for venular measures were also remarkably similar to the associations presented in Table 2.
245 Venular width associations with age, current smoking, BMI and diastolic blood pressure
246 were relatively unchanged, but associations with HDL cholesterol and triglycerides were
247 attenuated towards the null. Further investigation showed that associations with lipids
248 were primarily confounded by BMI. Venular tortuosity associations with sex and BMI were
249 relatively unchanged. However, the association with HbA1c was attenuated (1.3%, 95%CI
250 0.0,2.6%, increase in venular tortuosity per % increase in HbA1c), and the association with
251 systolic blood pressure was weakened by adjustment for BMI. Multilevel regression models
252 adjusting for age, sex and blood pressure showed a stronger association with prevalent
253 stroke than in Table 2, with 9.0% more tortuous arterioles amongst those who had suffered
254 a stroke compare to those who had not (95%CI 0.1,18.8%, $p < 0.001$), suggesting that the
255 effect on arteriolar tortuosity is independent of systolic blood pressure. Increased venular
256 tortuosity among those with prevalent diabetes was independent of sex, BMI and blood
257 pressure (5.5%, 95%CI 1.4%,8.9%).

258 **DISCUSSION**

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

269

270 While retinal signs of diabetic eye disease are well described,²⁷ there have been some
271 uncertainties about the association between diabetes, particularly risk factors for Type 2
272 diabetes, and retinal vessel morphometry, with inconsistencies between cross-sectional and
273 longitudinal findings.²⁸ However, a recent meta-analysis showed that wider venules, but not
274 arterioles, were associated with diabetes;²⁹ consistent with cross-sectional observations
275 suggesting that wider venules are associated with increasing levels of fasting glucose and
276 HbA1c levels.²⁸ Findings from the present study are consistent with these risk factor
277 observations, not only replicating the associations between increased venular width and
278 glycosylated haemoglobin (although not formally statistically significant), but also showing
279 coherent associations with other metabolic risk factors, including BMI, as well as novel
280 associations with levels of triglyceride; associations which were absent with arteriolar width.
281 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

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

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

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

330

331 By far the strongest associations observed were those with age and smoking, where per
332 decade rise in age there was arteriolar narrowing and increased tortuosity, and with current
333 smoking appreciable arteriolar and venular dilation. There was also the suggestion of
334 smaller arterioles and markedly greater tortuosity (both arteriolar and venular) in females
335 compared to males. However, sex differences in width were largely explained and
336 differences in tortuosity partially explained by height (data not presented). While
337 differences in CVD risk between males and females may have contributed to these
338 associations, explanations for potential sex differences in retinal vessel morphometry
339 remain uncertain. The effect of age was independent of blood pressure, as well as other
340 cardiometabolic risk factors, but smaller compared to a body of literature suggesting a 2 to
341 5µm decrease in arteriolar width per decade in age (although these later effect sizes were
342 seen in relation to central retinal vessel equivalent sizes, which are 2-3 times larger as they
343 are scaled-up from retinal measures taken within 0.5 to 1.5 disc diameters from the
344 disc).^{10;38} Nevertheless, these observations demonstrate the well-known association
345 between narrower more tortuous arterioles and older age.³⁹ The vasodilatory effects of
346 smoking have also been widely reported in venules, less so in arterioles.¹⁰ Increased carbon
347 monoxide levels amongst smokers may well provide a biological explanation for these
348 findings.⁴⁰

349

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

368

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

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

394

395 We have condensed these measures to provide an overall summary of mean width, but it is
396 possible that relative changes in vessel indices over time and perhaps variations in measures
397 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 cardiometabolic 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) dioptries	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)

Units of tortuosity are arbitrary

For participants included in the analyses extent of missing data is as follows:-

Total Cholesterol (TC) 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/m ² 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

Total Cholesterol (TC) 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

ACCEPTED MANUSCRIPT

Metabolic risk factors show novel graded associations with venular tortuosity, but also with width, even among those without diabetes. Vascular risk factors relate more with arteriolar morphometry, even among those without hypertension or cardiovascular disease.

ACCEPTED MANUSCRIPT