Novel locus influencing retinal venular tortuosity is also associated with risk of coronary artery disease

Abirami Veluchamy[1], Lucia Ballerini[2, 6], Veronique Vitart[3], Katharina E Schraut[4, 5], Mirna Kirin[4,9], Harry Campbell[4], Peter K Joshi[4], Devanjali Relan[6], Sarah Harris[8,10], Ellie Brown[12], Suraj K Vaidya[12], Bal Dhillon[6], Kaixin Zhou[1], Ewan R Pearson[1], Caroline Hayward[3], Ozren Polasek[4,9], Ian J Deary[7,8], Thomas MacGillivray[6,11,12], James F Wilson[3,4], Emanuele Trucco[2], Colin NA Palmer[1*], Alexander S F Doney[1*]

[1] Division of Molecular & Clinical Medicine, University of Dundee, Ninewells Hospital and Medical School, Dundee DD1 9SY - Scotland, United Kingdom.

[2] VAMPIRE project, Computer Vision and Image Processing Group, School of Science and Engineering (Computing), University of Dundee, Dundee, DD1 4HN - Scotland, United Kingdom.

[3] MRC Human Genetics Unit, Institute of Genetics and Molecular Medicine, University of Edinburgh, Western General Hospital, Edinburgh, EH4 2XU - Scotland, United Kingdom.

[4] Centre for Global Health Research, Usher Institute of Population Health Sciences and Informatics, University of Edinburgh, Teviot Place, Edinburgh, EH8 9AG - Scotland, United Kingdom.

[5] Centre for Cardiovascular Science, Queen's Medical Research Institute, University of Edinburgh, Royal Infirmary of Edinburgh, 47 Little France Crescent, Edinburgh, EH16 4TJ - Scotland, United Kingdom.

[6] VAMPIRE project, Centre for Clinical Brain Sciences, Chancellor's Building, Royal Infirmary of Edinburgh, 49, Little France Crescent, Edinburgh, EH16 4SB – Scotland, United Kingdom.

[7] Psychology, University of Edinburgh, 7, George Square, EH8 9JZ - Edinburgh, United Kingdom.

[8] Centre for Cognitive Ageing and Cognitive Epidemiology, University of Edinburgh, 7, George Square, EH8 9JZ - Edinburgh, United Kingdom.

[9] Department of Public Health, University of Split , School of Medicine, Soltanska 2, 21000 Split ,Croatia.

[10] Medical Genetics Section, Centre for Genomic and Experimental Medicine and MRC Institute of Genetics and Molecular Medicine, University of Edinburgh ,Western General Hospital, Crewe Road, Edinburgh EH4 2XU, United Kingdom.

[11] Edinburgh Clinical Research Facility, University of Edinburgh, Western General Hospital, Crewe Road South, Edinburgh, EH4 2XU – Scotland, United Kingdom.

[12] Clinical Research Imaging Centre, Queen's Medical Research Institute, University of Edinburgh, Royal Infirmary of Edinburgh, 47 Little France Crescent, Edinburgh, EH16 4TJ – Scotland, United Kingdom.

* These authors contributed equally to the study.

Correspondence to Prof Colin Palmer, c.n.a.palmer@dundee.ac.uk or Dr Alex Doney, a.doney@dundee.ac.uk

Structural variation in retinal blood vessels is associated with global vascular health in humans and may provide a readily accessible indicator of several diseases of vascular origin. We report a meta-analysis of genome-wide association studies (GWAS) for quantitative retinal vascular traits derived using semi-automatic image analysis of digital retinal photographs from the GoDARTS (n=1736) and ORCADES (n=1358) cohorts. We identified a novel genome-wide significant locus at 19q13 (*ACTN4/CAPN12*) for retinal venular tortuosity, and one at 13q34 (*COL4A2*) for retinal arteriolar tortuosity; these two loci were subsequently confirmed in three independent cohorts. In the combined analysis, the lead SNP at each locus was rs1808382 in *ACTN4/CAPN12* ($P=2.39\times10^{-13}$) and rs7991229 in *COL4A2* ($P=4.66\times10^{-12}$). Notably, the ACTN4/CAPN12 locus associated with retinal venular tortuosity traits is also associated with coronary artery disease and heart rate. Our findings demonstrate the contribution of genetics in retinal vascular traits, and provide new insights into vascular diseases.

Retinal vascular traits can be readily measured non-invasively from fundus images and have been linked to a number of clinical conditions associated with vascular health[1] including diabetes mellitus[2], stroke[3], cardiovascular disease[4,5], hypertension[6], and neurodegenerative disease[7,8]. Understanding the genetic determinants of retinal vascular traits may contribute to greater understanding of molecular mechanisms involved in determining disease risks and progression. Recent genome-wide association studies (GWAS) reported loci for widely investigated retinal traits including the central retinal vein equivalent (*CRVE*)[9–11] and the retinal arteriolar equivalent (*CRAE*)[10], and optic disc morphology[12–15]. While retinal vascular tortuosity has to be associated with a range of cardiovascular risk factors[16–19], to our knowledge no studies have performed GWAS on this trait.

We carried out a GWAS to examine the underlying genetic factors influencing the retinal tortuosity traits (arteriolar tortuosity (*TortA*), maximum *TortA* (*TortAmax*), venular tortuosity (*TortV*), and maximum *TortV* (*TortVmax*)) and other retinal vascular traits including *CRAE*, *CRVE*, Arteriole-to-Venule ratio (*AVR*), as well as Optic Disc radius (*ODradius*). Two independent discovery cohorts were included; patients with type 2 diabetes from the Genetics of Diabetes Audit and Research in Tayside Study (GoDARTS, n=1736) and a population-based sample comprising the Orkney Complex Disease Study (ORCADES, n=1358). In both cohorts, traits were measured from retinal fundus images (**Figure 1**) using VAMPIRE 3.1[20,21] (Vascular Assessment and Measurement Platform for Images of Retina), which enables efficient, semi-automatic measurement of the retinal vasculature from large numbers of images. The VAMPIRE methodology used in the discovery stage has been reported in detail[21–23]. The study design and characteristics of the discovery cohorts are shown in **Supplementary Figure 1**, and **Supplementary Table 1**.

In the discovery stage, we performed a GWAS using the GoDARTS cohort for each retinal trait separately and tested the additive effect of each variant, adjusted for age, gender and the first three principal components. Similarly, GWAS was performed for the same traits in the ORCADES cohort, using a mixed model to account for kinship. We combined the summary results from these two cohorts for each trait using a fixed effect meta-analysis. **Supplementary Table 2** summarize the single nucleotide polymorphisms (SNP) associated with retinal traits from meta-analysis as well as independent cohort results. Manhattan plots, QQ plots and regional plots are shown in **Supplementary Figure 2, 3**, and **4**, respectively.

This analysis revealed one genome-wide significant SNP associated with *TortA* at 13q34 (lead SNP rs56399312 near *COL4A2*; Beta=0.182, SE= 0.032, P= 2.70×10^{-8}, and another SNP rs9515212 near *COL4A2* that was just below the threshold for genome-wide significance; Beta=0.151, SE= 0.028, P= 8.59×10^{-8}). Conditional analysis on the lead SNP indicated that these are not independent signals (**Supplementary Table 3**). Two novel genome-wide significant loci ($p<5\times10^{-8}$) were associated with *TortV*, including SNPs at 19q13 near *ACTN4* (lead SNP rs1808382; Beta=-0.123, SE= 0.022, P= 1.55×10^{-8}), and a SNP at 12q24.33 near *TMEM132D* (lead SNP rs73157566; Beta= -0.294, SE= 0.054, P= 4.07×10^{-8}); these associations have not been reported previously with any retinal vascular parameters.

Although we replicated previously reported loci for *CRVE*, we did not find any novel genome-wide significant loci for this trait[9,11]. Furthermore, we did not replicate any of the previously reported SNPs associated with *CRAE*[10]. Finally, we replicated a previously reported significant locus for *ODradius* at 10q21.3 near *PBLD* **(**lead SNP rs61854835; Beta= -3.840, SE= 0.575, P= 4.06×10^{-11}**)** and confirmed a number of other loci for this trait [12–15](**Supplementary Table 4**).

We selected three lead SNPs near *ACTN4*, *TMEM132*D, and *COL4A2*, that all reached significance P $\leq 1.07\times10^{-07}$ as well as their effect size and direction were similar across the studies, as candidates to carry forward for replication, and confirmed these in three independent cohorts comprised of up to 1398 individuals of European ancestry (**Supplementary Table 5**); the Lothian Birth Cohort 1936[24] (LBC1936), Croatia-Korcula, and Croatia-Split. Retinal images from these cohorts had been analyzed by SIVA 3.1[25,26] (Singapore I Vessels Assessment) software to quantify the tortuosity traits. In overall meta-analysis (discovery and replication stage), only SNPs at 13q34 (*TortA*) and 19q13 (*TortV*) were confirmed at genome-wide significance. Although *TortA* associated SNPs rs7991229 and rs9515212 were not genome-wide significant in the discovery meta-analysis, they did in the overall meta-analysis; $P_{overall}=4.66\times10^{-12}$ and $P_{overall}=6.52\times10^{-10}$, respectively. Whereas lead SNP in COL4A2 for *TortA* (rs56399312) at discovery stage, did not reach genome-wide significance in overall meta-analysis

($P_{overall}=1.95\times10^{-07}$). For *TortV* the lead SNPs maintained genome-wide significance rs1808382 ($P_{overall}=2.39\times10^{-13}$), rs3786835 ($P_{overall}=3.31\times10^{-13}$) near *ACTN4/CAPN12* (**Table 1**). These SNPs are in tight LD and therefore do not represent independent signals. Regional plots and Forest plots for the genome-wide significant SNPs in the combined analysis are shown in **Figure 2** and **Supplementary Figure 5**, respectively.

COL4A2 encodes collagen type IV alpha 2, one of the six subunits of type IV collagens which are major structural components of basement membranes, forming a thin sheet of fibers under the endothelium controlling passage of vasoactive substances. These are conserved across species and C-terminal non-collagenous domains play a role in angiogenesis[27]. Several studies suggest that the mutations in *COL4A2* and (or) *COL4A1* (a paralogue immediately proximal to *COL4A2*, with which it shares a promoter and is co-expressed) cause a broad spectrum of diseases including retinopathy[28], glaucoma[28], familial cerebrovascular, small vessel diseases[29], and retinal hemorrhages[30]. In addition, *COL4A2* mutations in mice are associated with small vessel disease, intracerebral hemorrhage and retinal changes[31]. Mendelian variants in *COL4A1* underlie syndromes which include tortuous retinal vessels[32]. Recent GWAS report that common variants around *COL4A1* and *COL4A2*, are associated with coronary artery calcification[33], arterial stiffness[34], and coronary artery disease[35–38] (CAD). Interestingly, gene expression data from GeneAtlas[39], a human protein-coding transcriptome study validated the high expression of *COL4A2* in retinal micro-vessel endothelial cells (**Supplementary Figure 6**) whereas *COL4A1* is weakly expressed in retina and this supports the specific role of *COL4A2* in the retinal vasculature. Genome-wide significant *TortA*-associated variants near *COL4A2* significantly alter the transcription factor binding motifs, overlap enhancer histone marks and have putative effects on transcription as annotated by the ENCODE (**Supplementary Table 6**). Additionally, expression data from the GTEx database[40] confirmed that these significant SNPs, are associated with the expression of *COL4A2* in heart left ventricle and artery aorta, shown in **Supplementary Table 7, Supplementary Figure 7,** and these SNPs are in linkage disequilibrium (LD; $r^2=0.99$, D'=1). Lead SNPs associated with *TortA* were still significant after conditioning on the previously reported cardiovascular risk variants (rs11617955[36], rs4773144[37], rs9515203[38]) (**Supplementary Figure 8**, **Supplementary Table 8**). Conversely the lead SNPs for *TortA* are not associated with CAD and myocardial infarction (MI) risk in the CARDIoGRAMplus C4D consortium meta-analysis[36] (**Supplementary Table 9**). Finally, the CAD associated variants in *COL4A1* from CARDIOGRAMplusC4D are not associated with *TortA*, whereas CAD associated *COL4A2* variants are only weakly associated with *TortA* (**Supplementary Table 10**). Together, these data demonstrate that variants in this gene complex may independently influence micro and macrovascular disease.

ACTN4 encodes alpha-actinin 4, a cross-linking protein belonging to the spectrin superfamily; mutations in this gene cause focal segmental glomerulosclerosis in humans[41]. *ACTN2*, a homolog of *ACTN4*, interacts with *ACTN4* and missense mutations in *ACTN2* are linked to a range of cardiac diseases[42]. Annotation by ENCODE[43] indicates that the two genome-wide significant variants (rs1808382, rs3786835) associated with *TortV* near *ACTN4* may have direct regulatory effects as they are located within a DNase I hypersensitivity site in multiple cell types and are located in genomic regions enriched for promoter/enhancer histone marks especially in heart tissues (**Supplementary Table 6**). *ACTN4* overlaps with *CAPN12* (calcium-activated neural proteases) by 339 bases at their 3' ends and multi-tissue expression quantitative trait loci (eQTL) analysis confirms that these SNPs in *ACTN4* are associated with mRNA expression of both *ACTN4* and *CAPN12* in aorta, tibial artery, atrial appendage and left ventricle of the heart (**Supplementary Table 7, Supplementary Figure 9**). Additionally, this analysis indicates that the T allele at rs1808382 is correlated with lower *ACTN4* (artery aorta; $P=2.1\times10^{-03}$) and this correlation is even stronger with *CAPN12* (artery aorta; $P=2.0\times10^{-07}$). However, while gene expression data using GENEINVESTIGATOR validated the high expression of *ACTN4* in arterial tissue, the highest expression of *CAPN12* appears to be in the hematopoietic system. Furthermore, lead SNPs in *ACTN4* are significantly associated with coronary artery disease in the CARDIoGRAMplus C4D consortium meta-analysis[36] (**Supplementary Table 9**) and are associated with CAD risk factors; HDL cholesterol and triglycerides in the Global Lipid Genetics Consortium analysis[44] (**Supplementary Table 11**). Finally, recent meta-analysis of 35 GWAS studies reported the association of SNP (rs11083475) in the *ACTN4* locus with increased resting heart rate[45] which may increase cardiovascular disease risk. This signal is the same as that for *TortV* with strong LD being observed between the lead SNPs for *TortV* and the index SNP for heart rate. Furthermore we found that these SNPs are associated with heart rate in UK Biobank (**Supplementary Table 12, Supplementary Figure 10**).

In summary, this first GWAS for *TortA* and *TortV* reveals SNPs influencing expression of *COL4A2* and *ACTN4*/*CAPN12* respectively. Our results demonstrate that the *TortA*-associated variants in *COL4A2* are not associated with CAD and MI, and point to a selective role of *COL4A2* rather than *COL4A1* in the retinal vessels. Strikingly, we found *TortV*-associated *ACTN4/CAPN12* SNPs are associated with CAD and heart rate. Detailed investigation of this new finding is essential to elucidate the causal roles of *ACTN4* and/or *CAPN12* in the observed cardiovascular pathophysiology. These findings highlight the potential genetic impacts of retinal vasculature to provide new insights into wide-range of vascular disease.

URLs

SNPTEST V 2.5.2, https://mathgen.stats.ox.ac.uk/genetics_software/snptest/snptest.html; **SHAPEIT v2**, https://mathgen.stats.ox.ac.uk/genetics_software/shapeit/shapeit.html; **IMPUTE v2** https://mathgen.stats.ox.ac.uk/impute/impute_v2.html; **1000 Genomes Project**, http://www.1000genomes.org/; **Vampire,** http://vampire.computing.dundee.ac.uk/index.html; **GWAMA** http://www.well.ox.ac.uk/gwama/; **R statistical program package**, http://www.r-project.org/; **BEDTools**, http://bedtools.readthedocs.org/en/latest/; **LocusZoom**, http://csg.sph.umich.edu/locuszoom/; **UCSC Genome Browser**, https://genome.ucsc.edu/; **HaploReg**, http://www.broadinstitute.org/mammals/haploreg/haploreg.php; **PLINK,** https://www.cog-genomics.org/plink2/; **Genotype-Tissue Expression (GTEx) project**, http://www.gtexportal.org/home/; **ENCODE**, http://www.genome.gov/encode/ and http://genome.ucsc.edu/ENCODE/; **RegulomeDB**, http://www.regulomedb.org/ ; **EIGENSTRAT**, http://genetics.med.harvard.edu/reich/Reich_Lab/Software.html; **Type2Diabetes Knowledge portal**, http://www.type2diabetesgenetics.org/ ; **Genevisible**, https://genevisible.com/search; **GoDARTS**, http://diabetesgenetics.dundee.ac.uk/ ; **ORCADES**, http://www.orcades.ed.ac.uk/orcades/index.html ; **LBC1936**, http://www.lothianbirthcohort.ed.ac.uk/; **UK Biobank**, http://www.ukbiobank.ac.uk/.

Supplementary information

Supplementary Figures and Tables (separate pdf file)

ACKNOWLEDGEMENTS

We are grateful to all the participants in the GoDARTS study, the general practitioners, the Scottish School of Primary Care for their help in recruiting the participants, and to the whole team, which includes interviewers, computer and laboratory technicians, clerical workers, research scientists, volunteers, managers, receptionists, and nurses. The study complies with the Declaration of Helsinki. We acknowledge the support of the Health Informatics Centre, University of Dundee for managing and supplying the anonymized data and NHS Tayside, the original data owner. The Wellcome Trust United Kingdom Type 2 Diabetes Case Control Collection (GoDARTS) was funded by The Wellcome Trust (072960/Z/03/Z, 084726/Z/08/Z, 084727/Z/08/Z, 085475/Z/08/Z, 085475/B/08/Z) and as part of the EU IMI-SUMMIT program.

ORCADES was supported by the Chief Scientist Office of the Scottish Government (CZB/4/276, CZB/4/710), the Royal Society, the MRC Human Genetics Unit, Arthritis Research UK and the European Union framework program 6 EUROSPAN project (contract no. LSHG-CT-2006-018947). DNA extractions were performed at the Wellcome Trust Clinical Research Facility in Edinburgh. We would like to acknowledge the invaluable contributions of the research nurses in Orkney, the administrative team in Edinburgh and the people of Orkney.

We thank the Lothian Birth Cohort 1936 (LBC1936) participants and team members who contributed to these studies. Phenotype collection was supported by Age UK (The Disconnected Mind project). Genotyping was funded by the BBSRC (BB/F019394/1). The work was undertaken by The University of Edinburgh Centre for Cognitive Ageing and Cognitive Epidemiology, part of the cross council Lifelong Health and Wellbeing Initiative (MR/K026992/1). Funding from the BBSRC and Medical Research Council (MRC) is gratefully acknowledged.

The Croatia- Korčula and Croatia-Split study were funded by grants from the Medical Research Council (UK), European Commission Framework 6 project EUROSPAN (Contract No. LSHG-CT-2006-018947), European Commission Framework 7 project BBMRI-LPC (FP7 313010), the Republic of Croatia Ministry of Science, Education and Sports research grant (216-1080315-0302) and the Croatian Science Foundation (grant 8875). We would like to acknowledge the staff of several institutions in Croatia that supported the field work, including but not limited to the University of Split and Zagreb Medical Schools and Croatian Institute for Public Health. The SNP genotyping for the Korčula cohort was performed in Helmholtz Zentrum München, Neuherberg, Germany.

VAMPIRE team: Parts of the VAMPIRE software and its use for measuring the image set described here was funded by the Leverhulme Trust project RPG-419 "Discovery of retinal biomarkers for genetics with large cross-linked data sets". VAMPIRE 3.1 has been developed under funding from EPSRC (EPSRC EP/M005976/1), the EU (REVAMMAD ITN).

For the analysis of the association of the identified genetic variants with heart rate, this research has been conducted using the UK Biobank Resource under Application Number 20405.

AUTHOR CONTRIBUTIONS

The study was designed by C.NA.P, A.SF.D, and E.T for GoDARTS cohort, J.F.W for ORCADES cohort, I.J.D for LBC1936 cohort, C.H, O.P for Croatia-Split, and Croatia- Korčula cohort. VAMPIRE software was designed and developed by E.T, T.M, D.R, L.B, S.K.V, E.B and B.D. Retinal images were collected and analysis was performed by E.T, T.M, J.F.W, L.B, M.K, D.R, and V.V. Genotype data processing and statistical analysis was conducted by A.V, K.E.S, P.K.J, H.C, L.B, M.K, S.H, V.V and K.Z. Bioinformatics analysis was performed by A.V. The manuscript was drafted by A.V, C.NA.P A.SF.D, and revised by E.T, J.F.W, T.M, I.J.D, S.H, L.B, O.P, E.R.P and K.Z. All the authors reviewed the manuscript.

Competing interest: None

References

1. MacGillivray, T. J. *et al.* Retinal imaging as a source of biomarkers for diagnosis, characterization and prognosis of chronic illness or long-term conditions. *Br. J. Radiol.* **87,** (2014).
2. Ikram, M. K. *et al.* Retinal vascular caliber as a biomarker for diabetes microvascular complications. *Diabetes Care* **36,** 750–759 (2013).
3. Baker, M. L., Hand, P. J., Wang, J. J. & Wong, T. Y. Retinal signs and stroke: Revisiting the link between the eye and brain. *Stroke* **39,** 1371–1379 (2008).
4. Schuster, A. K. G., Fischer, J. E. & Vossmerbaeumer, U. A retinal snap shot may indicate individual risk for cardiovascular disease - The MIPH Eye&Health Study. *Int. J. Cardiol.* **180,** 30–33 (2015).
5. Roy, M. S., Klein, R. & Janal, M. N. Relationship of retinal vessel caliber to cardiovascular disease and mortality in African Americans with type 1 diabetes mellitus. *Arch. Ophthalmol. (Chicago, Ill. 1960)* **130,** 561–7 (2012).
6. Ding, J. *et al.* Retinal vascular caliber and the development of hypertension: a meta-analysis of individual participant data. *J. Hypertens.* **32,** 207–15 (2014).
7. MacCormick, I. J., Czanner, G. & Faragher, B. Developing retinal biomarkers of neurological disease: an analytical perspective. *Biomark. Med.* **9,** 691–701 (2015).
8. Williams, M. A. *et al.* Retinal microvascular network attenuation in Alzheimer's disease. *Alzheimer's Dement. Diagnosis, Assess. Dis. Monit.* **1,** 229–235 (2015).

9. Kamran Ikram, M. *et al.* Four novel loci (19q13, 6q24, 12q24, and 5q14) influence the microcirculation In vivo. *PLoS Genet.* **6,** 1–12 (2010).
10. Sim, X. *et al.* Genetic Loci for Retinal Arteriolar Microcirculation. *PLoS One* **8,** 1–12 (2013).
11. Jensen, R. A. *et al.* Novel Genetic Loci Associated with Retinal Microvascular Diameter. *Circ Cardiovasc Genet.* **9,** 45–54 (2016).
12. Ramdas, W. D. *et al.* A genome-wide association study of optic disc parameters. *PLoS Genet.* **6,** 1–12 (2010).
13. Macgregor, S. *et al.* Genome-wide association identifies ATOH7 as a major gene determining human optic disc size. *Hum. Mol. Genet.* **19,** 2716–2724 (2010).
14. Khor, C. C. *et al.* Genome-wide association studies in Asians confirm the involvement of ATOH7 and TGFBR3, and further identify CARD10 as a novel locus influencing optic discarea. *Hum. Mol. Genet.* **20,** 1864–1872 (2011).
15. Henriët Springelkamp, Aniket Mishra, Pirro G. Hysi, Puya Gharahkhani, René Höhn, Chiea-Chuen Khor, Jessica N. Cooke Bailey, Xiaoyan Luo, Wishal D. Ramdas, Eranga Vithana, Victor Koh, Seyhan Yazar, Liang Xu, and C. J. H. Meta-analysis of Genome-Wide Association Studies Idnetifies Novel Loci Associated With Optic Disc Morphology. *Genet. Epidemiol.* **39,** 207–216 (2015).
16. Owen, C. G. *et al.* Retinal arteriolar tortuosity and cardiovascular risk factors in a multi-ethnic population study of 10-year-old children; The child heart and health study in England (CHASE). *Arterioscler. Thromb. Vasc. Biol.* **31,** 1933–1938 (2011).
17. Taarnhøj, N. C. B. B. *et al.* Straight versus tortuous retinal arteries in relation to blood pressure and genetics. *Br. J. Ophthalmol.* **92,** 1055–1060 (2008).
18. Kirin, M. et al. Determinants of retinal microvascular features and their relationships in two European populations. *Journal of Hypertension.* Manuscript accepted, 1–39 (2017).
19. Cheung, C. Y.-L. *et al.* Retinal vascular tortuosity, blood pressure, and cardiovascular risk factors. *Ophthalmology* **118,** 812–8 (2011).
20. Trucco, E. *et al.* Morphometric measurements of the retinal vasculature in fundus images with VAMPIRE. *Biomed. Image Underst. Methods Appl.* 91–111 (2015).
21. MacGillivray, T. J. *et al.* Suitability of UK Biobank retinal images for automatic analysis of morphometric properties of the vasculature. *PLoS One* **10,** 1–10 (2015).
22. Giachetti, A. & Trucco, E. Accurate and reliable segmentation of the optic disc in digital fundus images the optic disc in digital fundus images. *J. Med. Imaging* **1,** (2014).
23. Lupaşcu, C. A., Tegolo, D. & Trucco, E. Accurate estimation of retinal vessel width using bagged decision trees and an extended multiresolution Hermite model. *Med. Image Anal.* **17,** 1164–1180 (2013).
24. Deary, I. J., Gow, A. J., Pattie, A. & Starr, J. M. Cohort profile: The lothian birth cohorts of 1921 and 1936. *Int. J. Epidemiol.* **41,** 1576–1584 (2012).
25. Sasongko, M. B. *et al.* Alterations in retinal microvascular geometry in young type 1 diabetes. *Diabetes Care* **33,** 1331–1336 (2010).
26. Koh, V. *et al.* Relationship of retinal vascular tortuosity with the neuroretinal rim: The singapore malay eye study. *Investig. Ophthalmol. Vis. Sci.* **51,** 3736–3741 (2010).
27. Kuo, D. S., Labelle-Dumais, C. & Gould, D. B. Col4a1 and col4a2 mutations and disease: Insights into pathogenic mechanisms and potential therapeutic targets. *Hum. Mol. Genet.* **21,** (2012).
28. Alavi, M. V *et al.* Col4a1 mutations cause progressive retinal neovascular defects and retinopathy. *Sci. Rep.* **6,** 18602 (2016).
29. Verbeek, E. *et al.* COL4A2 mutation associated with familial porencephaly and small-vessel disease. *Eur. J. Hum. Genet.* **20,** 844–851 (2012).
30. Jeanne, M. *et al.* COL4A2 mutations impair COL4A1 and COL4A2 secretion and cause hemorrhagic stroke. *Am. J. Hum. Genet.* **90,** 91–101 (2012).

31. Favor, J. *et al.* Type IV procollagen missense mutations associated with defects of the eye, vascular stability, the brain, kidney function and embryonic or postnatal viability in the mouse, Mus musculus: An extension of the Col4a1 allelic series and the identification of the first two Col4a2 mutant alleles. *Genetics* **175,** 725–736 (2007).
32. Plaisier, E. *et al.* Novel COL4A1 mutations associated with HANAC syndrome: A role for the triple helical CB3[IV] domain. *Am. J. Med. Genet. Part A* **152 A,** 2550–2555 (2010).
33. Odonnell, C. J. *et al.* Genome-wide association study for coronary artery calcification with follow-up in myocardial infarction. *Circulation* **124,** 2855–2864 (2011).
34. Tarasov, K. V. COL4A1 Is Associated With Arterial Stiffness By Genome Wide Association Scan. **2,** 151–158 (2009).
35. Schunkert, H. *et al.* Large-scale association analysis identifies 13 new susceptibility loci for coronary artery disease. *Nat Genet* **43,** 333–338 (2011).
36. Nikpay et al. A comprehensive 1000 Genomes-based genome-wide association meta-analysis of coronary artery disease. **47,** 1121–1130 (2016).
37. Yang, W. *et al.* Coronary-Heart-Disease-Associated Genetic Variant at the COL4A1/COL4A2 Locus Affects COL4A1/COL4A2 Expression, Vascular Cell Survival, Atherosclerotic Plaque Stability and Risk of Myocardial Infarction. *PLoS Genet.* **12,** 1–15 (2016).
38. Deloukas, P. *et al.* Large-scale association analysis identifies new risk loci for coronary artery disease. *Nat Genet* **45,** 25–33 (2013).
39. Su, A. I. *et al.* A gene atlas of the mouse and human protein-encoding transcriptomes. *Proc. Natl. Acad. Sci. U. S. A.* **101,** 6062–7 (2004).
40. Ardlie, K. G. *et al.* The Genotype-Tissue Expression (GTEx) pilot analysis: Multitissue gene regulation in humans. *Science (80-.).* **348,** 648–660 (2015).
41. Feng, D., DuMontier, C. & Pollak, M. R. The role of alpha-actinin-4 in human kidney disease. *Cell Biosci.* **5,** 44 (2015).
42. Chiu, C. *et al.* Mutations in Alpha-Actinin-2 Cause Hypertrophic Cardiomyopathy. A Genome-Wide Analysis. *J. Am. Coll. Cardiol.* **55,** 1127–1135 (2010).
43. Myers, R. M. *et al.* A user's guide to the Encyclopedia of DNA elements (ENCODE). *PLoS Biol.* **9,** (2011).
44. Willer, C. J. *et al.* Discovery and refinement of loci associated with lipid levels. *Nat. Genet.* **45,** 1274–83 (2013).
45. den Hoed, M. *et al.* Identification of heart rate-associated loci and their effects on cardiac conduction and rhythm disorders. *Nat. Genet.* **45,** 621–31 (2013).
46. McQuillan, R. *et al.* Runs of Homozygosity in European Populations. *Am. J. Hum. Genet.* **83,** 359–372 (2008).
47. Marchini, J. & Howie, B. Genotype imputation for genome-wide association studies. *Nat. Rev. Genet.* **11,** 499–511 (2010).
48. Howie, B., Marchini, J. & Stephens, M. Genotype Imputation with Thousands of Genomes. *G3* **1,** 457–470 (2011).
49. Price, a. L. *et al.* Principal components analysis corrects for stratification in genome-wide association studies. *Nat. Genet.* **38,** 904–909 (2006).
50. O'Connell, J. *et al.* A General Approach for Haplotype Phasing across the Full Spectrum of Relatedness. *PLoS Genet.* **10,** (2014).
51. Joshi, P. K. *et al.* Local Exome Sequences Facilitate Imputation of Less Common Variants and Increase Power of Genome Wide Association Studies. *PLoS One* **8,** (2013).
52. Magi, R. & Morris, A. P. GWAMA: software for genome-wide association meta-analysis. *BMC Bioinformatics* **11,** 288 (2010).
53. Karssen, L., Kooyman, M., Aulchenko, Y. & Struchalin, M. ProbABEL. 1–24 (2016).
54. Chang, C. C. *et al.* Second-generation PLINK: rising to the challenge of larger and richer datasets. *Gigascience* **4,** 7 (2015).

55. Team, R. C. R: A language and environment for statistical computing. *R Found. Stat. Comput. Vienna, Austria.* (2014).
56. Pruim, R. J. *et al.* LocusZoom: Regional visualization of genome-wide association scan results. *Bioinformatics* **27,** 2336–2337 (2011).
57. Aulchenko, Y. S., Ripke, S., Isaacs, A. & van Duijn, C. M. GenABEL: An R library for genome-wide association analysis. *Bioinformatics* **23,** 1294–1296 (2007).
58. Sudlow, C. *et al.* UK Biobank: An Open Access Resource for Identifying the Causes of a Wide Range of Complex Diseases of Middle and Old Age. *PLoS Med.* **12,** 1–10 (2015).
59. Quinlan, A. R. *BEDTools: The Swiss-Army tool for genome feature analysis*. *Current Protocols in Bioinformatics* **2014,** (2014).
60. Speir, M. L. *et al.* The UCSC Genome Browser database: 2016 update. *Nucleic Acids Res.* **44,** D717–D725 (2015).
61. Ward, L. D. & Kellis, M. HaploReg: A resource for exploring chromatin states, conservation, and regulatory motif alterations within sets of genetically linked variants. *Nucleic Acids Res.* **40,** 1–5 (2012).
62. Boyle, A. P. *et al.* Annotation of functional variation in personal genomes using RegulomeDB. *Genome Res.* **22,** 1790–1797 (2012).
63. Welter, D. *et al.* The NHGRI GWAS Catalog, a curated resource of SNP-trait associations. *Nucleic Acids Res.* **42,** 1001–1006 (2014).

ONLINE METHODS

Study participants.

Discovery Cohorts. Participants in the discovery phase of this study were obtained from the two independent cohorts, the Genetics of Diabetes Audit and Research in Tayside (GoDARTS) and the Orkney Complex Disease Study (ORCADES). GoDARTS comprises individuals of European-heritage from Tayside, Scotland who provided a sample of blood for genetic analysis and consent to link their genetic information to the anonymized electronic health records. Approval for recruitment to GoDARTS was obtained from the Tayside Committee on Medical Research Ethics. 18,190 individuals were recruited with approximately half having type 2 diabetes at the time of recruitment with the other half being diabetes free. 7,290 individuals currently have genome-wide data for analysis. ORCADES is a family-based study of 2078 individuals aged 16-100 years recruited between 2005 and 2011 in the isolated Scottish archipelago of Orkney[46]. Genetic diversity in this population is decreased compared to Mainland Scotland, consistent with the high levels of endogamy historically. Fasting blood samples were collected and over 300 health-related phenotypes and environmental exposures were measured in each individual. All participants provided written informed consent and the study was approved by Research Ethics Committees in Orkney and Aberdeen.

Replication Cohorts. Lothian Birth Cohort 1936 (LBC1936) comprised of 1091 participants who were born in 1936, most of whom took part in the Scottish Mental Survey of 1947. At a mean age of 69.5 years (SD 0.8), between 2004 and 2007, they were recruited to a study to determine influences

on cognitive aging[24]. The CROATIA- Korčula study sampled from the Adriatic island of Korčula, between the ages of 18 and 88. The fieldwork was performed in 2007 in the eastern part of the island, targeting healthy volunteers who underwent complete eye examination and provided their blood sample for genetic analysis from the town of Korčula and the villages of Lumbarda, Žrnovo and Račišće. The Croatia-Split study included inhabitants of the Croatian coastal city of Split, aged 18 to 93. The sampling scheme was similar to Croatia- Korčula, and it took place during 2008 and 2009.

Retinal Vascular Parameters Measurement.

Retinal image analysis. Standard digital retinal photographs used for routine diabetic retinopathy screening were obtained from the clinical record in 2,104 participants in GoDARTS. Images of the right eye of usable quality, defined using criteria reported in[21,22], were selected and categorized into two datasets based on the image pixel resolution: GoDARTS dataset 1 (n=788) and GoDARTS dataset 2 (n=1288). Finally, 661 images from the GoDARTS dataset 1, and 1083 images from GoDARTS dataset 2 were included after quality control (QC). 28 individual's images from the GoDARTS were excluded due to inadequate resolution. Standard fundal retinal photographs centred between the macula and optic disc were obtained using digital fundus camera from 1,743 participants in ORCADES. After image processing and QC, 1595 individual's retinal images were used for this study.

VAMPIRE 3.0, was used to measure retinal vascular traits in fundus images (see **Figure 1**) from both GoDARTS and ORCADES. The measurement process is organized as a sequence of automatic and manual stages. Manual stages allowed correction of errors made by the automatic software (e.g. vessel labeling as artery or vein) and to minimize their impact on statistical analysis. The standard protocols were followed to measure the retinal vessel parameters. Briefly, after automatic detection of the optic disc and its radius (*ODradius*), the 6 thickest arterioles and 6 thickest venules appearing in a zone extending from the optic disc boundary to 2 optic disc diameters out were sampled to calculate the median (*TortA*) and maximum (*TortAmax*) arteriolar tortuosity and the median (*TortV*) and maximum (*TortVmax*) venular tortuosity. *CRAE, CRVE* and the Arteriole-to-Venule ratio (*AVR*; CRAE/CRVE) qualify vessel calibers and were measured in a zone 2 to 3 optic disc radii from the center of the optic disc. Among the eight parameters, *TortA, TortAmax, TortV* and *TortVmax* mean values were natural log transformed for association analysis. **Supplementary Table 1** shows descriptive statistics for retinal parameters and population characteristics for both discovery cohorts.

Replication Cohorts. Standard retinal fundus images using digital fundus camera from 1091 individuals from LBC1936 were collected at the recruitment stage and three years later, retinal traits were measured at a subsequent wave of testing using SIVA v3.1, at a mean age of 72.5 years (SD 0.7).

A total of 897 and 976 individual's retinal fundus images centred between the macula and optic disc from Croatia- Korčula and Croatia-Split cohorts were collected using digital fundus camera and retinal traits were quantified using SIVA v3.1[25,26]. SIVA is a semi-automated software which can be used to measure the retinal vascular parameters including retinal vascular tortuosity and vascular caliber from retinal images. After automatic detection of the optic disc, it placed a grid with reference to the center of the optic disc. Then the tortuous vessels were identified and tortuosity traits including *TortA*, and *TortV* were measured using the standard grading protocol by the software; this process was monitored by trained graders and adjusted manually if necessary. **Supplementary Table 2** shows descriptive statistics of variables.

Genotyping, quality control and imputation. GoDARTS samples were genotyped using the Affymetrix 6.0 (n=927) and Illumina Human Omni Express (n=809) platforms. The poor quality variants, samples were excluded based on the quality control (QC) criteria included the following: SNPs call rate < 95%, Hardy–Weinberg equilibrium (HWE) P value < 10^{-6}, sample call rate < 95%, sample relatedness (IBD >0.8), and mismatch between reported and genotypic gender information. QC'd genotype data was imputed using IMPUTE2[47,48] on the basis of 1000 Genome Projects reference panel for all population. Finally, ancestry information of the individuals were derived using EIGENSTRAT[49] and first three principal components (PCs) were used for the association analyses to adjust the population stratification. ORCADES samples were genotyped with either the Illumina HumanHap300 bead chip (n=890) or the Illumina Omni1 (n=304) or Illumina Omni Express bead chips (n=1073). Alleles were called in Bead Studio/Genome Studio (Hap300/Omni) using Illumina cluster files. Subjects were excluded if they fulfilled any of the following criteria: genotypic call rate <98%, mismatch between reported and genotypic sex, unexpectedly low genomic sharing with first or second degree relatives, excess autosomal heterozygosity, or outliers identified by IBS clustering analysis. We excluded SNPs on the basis of minor allele frequency (<0.01/monomorphism), HWE (P<10^-6), call rate (<97%). Given the very high overlap in SNPs between the two Omni chips, the intersection of QC'd SNPs was used to impute and phase individuals' genotyped on the Omni arrays together, whilst the Hap300 individuals were phased and imputed, separately. Samples were phased using Shapeit v2[50]. Imputation was carried out using IMPUTE2 and the 1,000 genomes reference panel. All ancestries phase1 integrated v3 reference panel, with a secondary reference panel of local exome sequences, sequenced using the Agilent Sure Select All Exon Kit v2.0 and Illumina 100 bp paired end reads (average 30x depth), derived from 90 ORCADES subjects chosen to optimally represent the haplotypes present. Imputations for the Hap300 and Omni subjects were then combined to form a combined panel of 37.5m SNPs for 2222 subjects[51]. Imputed genotypes for 658, 1078, 1358

individuals from the GoDARTS dataset 1, GoDARTS dataset 2 and ORCADES cohorts, respectively, were used for the three independent GWAS analysis. Additional information on the characteristics of both study cohorts and study design can be found in **Supplementary Figure 1 and Table 1**.

Replication Cohorts. LBC1936 samples were genotyped at the Wellcome Trust Clinical Research Facility, Edinburgh, using the Illumina Human 610Quad BeadChip. Individuals were excluded based on unresolved gender discrepancy, relatedness, call rate (≤0.95), and evidence of non-Caucasian descent. SNPs were included if they met the following conditions: call rate ≥ 0.98, minor allele frequency ≥ 0.01, and Hardy-Weinberg equilibrium test with P ≥ 0.001. Imputation to the 1000 Genomes (March 2012 release) reference set was performed using minimac software. A total of 1398 participants from the two independent Croatian replication cohorts were available for the analysis and subjects were genotyped on different genotyping platforms including Illumina CNV370v1 and CNV370-Quadv3 for Croatia-Korčula (n=378), and Illumina CNV370-Quadv3 and IlluminaOmniExpressExome-8v1_A for Croatia-Split (n=376). Samples and markers were excluded based on the following QC metrics; SNPs call rate < 98%, Hardy–Weinberg equilibrium (HWE) P value < 10^{-6}, sample call rate < 97%, MAF < 1%, outliers identified by IBS clustering analysis and unresolved gender discrepancy. Imputation was carried out using IMPUTE2 software and 1000G Phase I v3 (March 14, 2012) reference panel.

Statistical analyses. We performed association analyses with each data sets from GoDARTS separately for each of the eight retinal traits using SNPTEST V2.5[47], linear regression assuming an additive genetic model, adjusting for 3 ancestry PCs, age at eye examination and gender. Subsequently, markers with low imputation quality scores (< 0.4) and minor allele frequency cutoffs (< 0.03) were filtered from each GWAS summary output data separately. Then we performed the meta-analysis using a fixed-effects model in GWAMA[52] with the QC filtered data sets. Association analysis in ORCADES was performed for each of the eight retinal traits, using linear mixed modelling to account for relatedness and assuming an additive genetic model, adjusting for 3 ancestry PCs, age at eye examination and gender, using MMscore in ProbABEL[53]. As in GoDARTS, markers with low imputation quality scores (< 0.4) and minor allele frequency cutoffs (< 0.03) were filtered and meta-analysis was performed with the GoDARTS and ORCADES results using GWAMA. The strand alignment and build check between studies were performed prior to meta-analysis. Also, the genomic inflation factor (λ) was estimated by GWAMA (λ=0.99). All statistical analyses and QCs were performed using SNPTEST v2.5[47], ProbABEL[53], GWAMA[52], PLINK v1.09[54], EIGENSTRAT[49], custom shell scripts, and R scripts. Manhattan plots, Quantile-Quantile plots and forest plots were generated using in-build R scripts, and metafor - R package[55]. Regional plots were generated using the

Locus Zoom tool[56] and other data processing was performed using R scripts. Conditional analyses were performed in SNPTEST v2.5 using the genome-wide associated loci in the *COL4A2* region, conditioned on lead SNPs (rs56399312). Also, this new locus was conditioned on previously reported genome-wide significant SNPs (rs4773144, rs11617955, rs9515203) associated with coronary artery disease (CAD).

Replication-analyses. Top three SNPs ($P \leq 1.07\times10^{-07}$) near *ACTN4*, *TMEM132*D, and *COL4A2* from the discovery stage for the tortuosity traits were taken forward for examination in three replication cohorts of European ancestry. In LBC1936 cohort, association analysis was performed for arterial and venular tortuosity traits using linear regression model adjusting for age at eye examination, sex, and 3 ancestry PCs, using mach2qtl. Similarly, in the Croatia – Split, - Korčula cohorts, association analysis were performed for each traits separately using the mixed model in R - hglm package to account for kinship derived using gkin function of the GenABEL package[57].

Then we combined the summary association statistics for lead SNPs associated with *TortA* and *TortV* from the two discovery and three replication cohorts and effect estimates from each study cohorts were presented in the forest plots using metafor - R package. Due to the difference in the units of the beta and standard errors between the discovery and replication studies arising from different approaches to measurement we standardized the effect estimates (using Cohen's d) from each individual's study results.

***In-silico* look-ups of the novel variants for clinical outcomes.** We performed *in-silico* look-ups of variants of interest for cardiovascular related outcomes including coronary artery disease, myocardial infarction, hypertension, HDL, and triglycerides from the CARDIoGRAMplus C4D consortium[36] and Global Lipid Genetics Consortium analysis[44]. The CARDIoGRAMplusC4D 1000 Genomes-based meta-analysis data comprised of 60,801 CAD cases and 123,504 controls from European, South Asian, and East Asian descent. In the Global Lipid Genetics Consortium, genetic data from 188,577 individuals of European, East Asian, South Asian, and African ancestry were used to examine the genetic loci associated with blood lipids levels. We retrieved summary association results for the index SNPs from these studies to investigate the association of the lead SNPs for *TortA*, and *TortV* with cardiovascular outcomes.

A recent study reported the association of *ACTN4* locus with heart rate. In order to examine whether the lead SNPs associated with *TortV* in *ACTN4* were also associated with heart rate, we checked the LD (r^2>0.8) between our SNPs and the index SNP (rs11083475) for heart rate. Furthermore, we selected these SNPs and investigated the association of these SNPs with pulse rate in UK Biobank

data. This data comprised of 112,008 participants who had a measure of pulse rate at the main interview and had genotype data. We extracted the imputed genotypes for the these SNPs from the interim release data set of the UK Biobank[58] and performed multiple linear regressions including covariates of age, gender, and the first ten principal components obtained using EIGENSTRAT.

***In-silico* functional annotation.** The sentinel genome-wide significant variants were mapped to the gene, 20 kb upstream/downstream using BEDTools[59], and UCSC Genome Browser[60]. Top SNPs were queried in the HaploReg v4.1 database[61] to catalogue the all SNPs near noncoding variants with $r^2 > 0.8$, and RegulomeDB[62]**,** and GWAS catalog databases[63] used to explore the known and predicted regulatory elements and relevant genetic association studies. Functional effects of the top genes were predicted using the Encyclopedia of DNA Elements[43] (ENCODE) project and Roadmap Epigenomics projects which aggregate the information about the transcription factor, motifs, histone modification, and chromatin states. Additionally, functional elements were investigated using HaploReg, UCSC Genome Browser, and RegulomeDB. We used the expression Quantitative Trait Loci (eQTL) browser database in Genotype-Tissue Expression[40] (GTEx) to examine the cis-eQTLs for the top retinal traits associated SNPs mapped to the gene within the genomic region. Gene Visible web database from GENEINVESTIGATOR which integrates manually curated gene expression data from microarray and RNAseq experiments, was used to find the expression level of the genes, associated with tortuosity traits, in the human tissues.

Table 1. Results of discovery, replication and overall meta-analysis for tortuosity traits.

SNP	Chr.	BP	Candidate gene	Effect allele (Freq.)	Cohort	BETA	SE	P	Het P (I^2)
TortA									
rs7991229	13	111091995	*COL4A2*	G (0.42)	GODARTS	0.104	0.024	2.30×10^{-05}	
					ORCADES	0.092	0.027	0.000728	
					Stage 1 meta-analysis	0.098	0.018	$\mathbf{1.07\times10^{-07}}$	0.75(0)
					LBC1936	0.081	0.039	0.039721	
					All Croatia	0.142	0.036	9.83×10^{-05}	
					Stage 2 meta-analysis	0.114	0.027	$\mathbf{2.08\times10^{-05}}$	0.28(0.2)
					Combined	0.102	0.015	$\mathbf{4.66\times10^{-12}}$	0.65(0)
rs9515212	13	111087563	*COL4A2*	G (0.42)	GODARTS	0.104	0.024	1.96×10^{-05}	
					ORCADES	0.092	0.027	0.000685	
					Stage 1 meta-analysis	0.099	0.018	$\mathbf{8.59\times10^{-08}}$	0.75(0)
					LBC1936	0.079	0.039	0.043685	
					All Croatia	0.143	0.068	8.40×10^{-05}	
					Stage 2 meta-analysis	0.095	0.034	**0.0052**	0.45(0)
					Combined	0.102	0.016	$\mathbf{6.52\times10^{-10}}$	0.85(0)
rs56399312	13	111121981	*COL4A2*	C (0.269)	GODARTS	0.122	0.024	6.67×10^{-07}	
					ORCADES	0.069	0.027	0.010473	
					Stage 1 meta-analysis	0.099	0.018	$\mathbf{2.70\times10^{-08}}$	0.15(0.5)
					LBC1936	0.053	0.039	0.17726	
					All Croatia	0.012	0.036	0.0386	
					Stage 2 meta-analysis	0.031	0.027	**0.24743**	0.44(0)
					Combined	0.080	0.015	$\mathbf{1.95\times10^{-07}}$	0.07(0.6)
TortV									
rs1808382	19	39151034	*ACTN4*	T (0.475)	GODARTS	-0.116	0.024	2.14×10^{-06}	
					ORCADES	-0.080	0.027	0.0031218	
					Stage 1 meta-analysis	-0.101	0.018	$\mathbf{1.55\times10^{-08}}$	0.69(0)
					LBC1936	-0.134	0.039	0.00064	
					All Croatia	-0.125	0.036	0.0006	
					Stage 2 meta-analysis	-0.129	0.027	$\mathbf{1.35\times10^{-06}}$	0.87(0)
					Combined	-0.109	0.015	$\mathbf{2.39\times10^{-13}}$	0.61(0)
rs3786835	19	111087563	*ACTN4*	A (0.471)	GODARTS	-0.116	0.024	1.95×10^{-06}	
					ORCADES	-0.076	0.027	0.004924	
					Stage 1 meta-analysis	-0.099	0.018	$\mathbf{2.26\times10^{-08}}$	0.87(0)
					LBC1936	-0.136	0.039	0.000537	
					All Croatia	-0.125	0.036	0.0006	
					Stage 2 meta-analysis	-0.130	0.027	$\mathbf{1.10\times10^{-06}}$	0.84(0)
					Combined	-0.109	0.015	$\mathbf{3.31\times10^{-13}}$	0.53(0)
rs73157566	12	129533847	*TMEM132D*	A (0.043)	GODARTS	-0.114	0.024	3.28×10^{-06}	
					ORCADES	-0.075	0.027	0.005458	
					Stage 1 meta-analysis	-0.097	0.018	$\mathbf{4.07\times10^{-08}}$	0.28(0.1)
					LBC1936	-0.054	0.039	0.17197	
					All Croatia	-0.004	0.036	0.9045	
					Stage 2 meta-analysis	-0.027	0.027	**0.30973**	0.35(0)
					Combined	-0.075	0.015	$\mathbf{2.61\times10^{-06}}$	0.08(0.6)

GoDARTS: Genetics of Diabetes Audit and Research in Tayside; ORCADES: Orkney Complex Disease Study; LBC1936: Lothian Birth Cohorts 1936; All Croatia: Croatia island of Korcula+ Croatia Split. Natural log transformed - TortA retinal arteriolar tortuosity, TortV retinal venular tortuosity. Standardized beta estimate: Change in natural log transformed retinal tortuosity traits for each copy of the effect allele; SE: standard error.

Figure Legends

Figure 1. Retinal fundus image. Solid lines (red for arterioles and dark blue for venules) represent the vessels detected automatically and measured by VAMPIRE (Vasculature Assessment and Measurement Platform for Images of the REtina) software (version 3.0, Universities of Edinburgh and Dundee, UK). Dotted lines (light blue) represent the measurement zones on a fundus image; based on optic disc (light blue circle) location and radius.

Figure 2. Regional association and recombination plots of variants that reached genome-wide significance in overall meta-analysis (discovery and replication stage). Each plot was created using LocusZoom for the lead SNP in genomic region 400 kb in either side of the significant signal. Blue spikes represents the estimated recombination rates. Colour scale (high to low r2) circles depicts the pairwise correlation (r2) between lead SNP and other SNPs in the loci, and grey colour indicates that linkage disequilibrium (LD) information was not available in the reference population. The lead SNP in that region is indicated by purple colour solid diamond and gene annotations in this region is shown in the bottom panels. Chromosome, base position and SNPID information is based on NCBI build 37 and dbSNP138.