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Novel Genetic Locus Influencing Retinal Venular Tortuosity Is Also Associated With Risk of Coronary Artery Disease

Abirami Veluchamy, Lucia Ballerini, Veronique Vitart, Katharina E. Schraut, Mirna Kirin, Harry Campbell, Peter K. Joshi, Devanjali Relan, Sarah Harris, Ellie Brown, Suraj S. Vaidya, Baljean Dhillon, Kaixin Zhou, Ewan R. Pearson, Caroline Hayward, Ozren Polasek, Ian J. Deary, Thomas MacGillivray, James F. Wilson, Emanuele Trucco, Colin N.A. Palmer,* Alexander S.F. Doney*

OBJECTIVE: The retina may provide readily accessible imaging biomarkers of global cardiovascular health. Increasing evidence suggests variation in retinal vascular traits is highly heritable. This study aimed to identify the genetic determinants of retinal vascular traits.

APPROACH AND RESULTS: We conducted a meta-analysis of genome-wide association studies for quantitative retinal vascular traits derived using semi-automatic image analysis of digital retinal photographs from the GoDARTS (Genetics of Diabetes Audit and Research in Tayside; N=1736) and ORCADES (Orkney Complex Disease Study; N=1358) cohorts. We identified a novel genome-wide significant locus at 19q13 (ACTN4/CAPN12) for retinal venular tortuosity (TortV) and one at 13q34 (COL4A2) for retinal arteriolar tortuosity (TortA); these 2 loci were subsequently confirmed in 3 independent cohorts (Ntotal=1413). In the combined analysis of discovery and replication cohorts, the lead single-nucleotide polymorphism in ACTN4/CAPN12 was rs1808382 (βs.d.=-0.109; SE=0.015; P=2.39×10-13) and in COL4A2 was rs7991229 (βs.d.=0.103; SE=0.015; P=4.66×10-12). Notably, the ACTN4/CAPN12 locus associated with TortV is also associated with coronary artery disease, heart rate, and atrial fibrillation.

CONCLUSIONS: Genetic determinants of retinal vascular tortuosity are also linked to cardiovascular health. These findings provide a molecular pathophysiological foundation for the use of retinal vascular traits as biomarkers for cardiovascular diseases.

VISUAL OVERVIEW: An online visual overview is available for this article.

Key Words: atrial fibrillation • biomarkers • cardiovascular diseases • genome-wide association study • heart rate • retina

Retinal vascular traits can be readily measured non-invasively from fundus images, and changes in these traits have been linked to a number of clinical conditions associated with vascular health, including cardiovascular disease, stroke, hypertension, and neurodegenerative disease. The association between retinal vascular calibers and cardiovascular disease has been reported in numerous studies, and structural variation in retinal vasculature could predict cardiovascular risk. More recently, deep learning applied to retinal images has been successfully used to predict cardiovascular risk factors and outcomes. Whereas this potentially powerful approach indicated that vascular regions of the retina appeared important, it cannot provide a molecular

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Arterioscler Thromb Vasc Biol is available at www.ahajournals.org/journal/atvb
pathoetiologcal basis for the link between retinal vasculature and cardiovascular disease.

Population-based studies have demonstrated a significant genetic component to variation in retinal blood vessel width. Evidence suggests that retinal tortuosity, a potentially important vascular parameter, is also associated with a range of cardiovascular risk factors. Heritability estimates for retinal arterial tortuosity range from 50% to 82% and 21% for retinal venular tortuosity, indicating a substantial genetic contribution to the variation in these parameters. Recent genome-wide association studies (GWAS) found a number of loci for the previously published GWAS results. There is ample evidence indicating that increased resting heart rate is linked to various cardiovascular events and associated with the increased risk of plaque rupture in coronary atherosclerosis patients. Therefore, we also checked whether there is any association between TortV-associated variants with heart rate in independent samples from United Kingdom Biobank.

SUBJECTS AND METHODS

Data analysis, methods, and all other supporting materials are available in the online-only Data Supplement document. The data that support the findings of this study are available to the researchers from the corresponding author upon request.

Study Participants

Participants in the discovery phase of this study were obtained from 2 independent cohorts, the GoDARTS (Genetics of Diabetes Audit and Research in Tayside) and the ORCADES (Orkney Complex Disease Study). Three independent cohorts of individuals of European ancestry were used at the replication stage, including the Lothian Birth Cohort 1936 (LBC1936), Croatia-Korčula, and Croatia-Split study. Detailed descriptions of each cohort are presented in the online-only Data Supplement note.

Retinal Vascular Parameter Measurement

Standard digital retinal photographs used for diabetes mellitus retinal screening were obtained from the clinical record of
patients with type 2 diabetes mellitus in GoDARTS. A total of 1744 images (661 images from the GoDARTS data set 1 and 1083 images from GoDARTS data set 2) were selected for analysis after quality control (QC). Similarly, 1595 individual’s retinal images from ORCADES were used for this study after QC. The Vascular Assessment and Measurement Platform for Images of Retina 3.1, semi-automatic software, was used to measure retinal vascular traits in fundus images (Figure I in the online-only Data Supplement) from both GoDARTS and ORCADES. Standard protocols were followed to measure the retinal vessel parameters, including CRAE, CRE, AVR, ODradius, TortA, TortAm, TortV, and TortVmax (online-only Data Supplement). Tortuosity mean values were normalized by natural log transformation for association analysis (Figure II in the online-only Data Supplement). Smaller values indicate straighter vessels. After quality assessment and processing, a total of 644, 387, and 382 individuals’ retinal fundus images from the LBC1936, Croatia–Korčula, and Croatia-Split cohorts, respectively, were selected for the analysis. In these cohorts, retinal tortuosity traits were quantified using SIVA v3.124 (Singapore I Vessels Assessment), semi-automated software, and normalized by natural log transformation for association analysis (online-only Data Supplement).

Genotyping, QC, and Imputation
GoDARTS participants were genotyped using the Affymetrix 6.0 (n=927) and Illumina Human Omni Express (n=809) platforms. ORCADES samples were genotyped with either the Illumina HumanHap300 bead chip (n=890) or the Illumina Omni1M (n=304) or Illumina Omni Express bead chip (n=1073). Genotype data quality was assessed and imputed on the basis of 1000 Genome Genotypes reference panel. Imputed genotypes for 658, 1078, 1358 individuals assessed from the GoDARTS data set 1, GoDARTS data set 2, and ORCADES cohorts, respectively, were used for the three independent GWAS analysis.

LBC1936 samples were genotyped using the Illumina Human 610Quad BeadChip. A total of 1398 participants from the 2 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). More details on QC, imputation, and processing can be found in the online-only Data Supplement.

Statistical Analyses
We performed association analyses with each data set from GoDARTS separately for each of the 8 retinal traits using SNPTEST v2.525 linear regression assuming an additive genetic model, adjusting for 3 ancestry principal components, age at eye examination, and gender. Association analysis in ORCADES was performed using linear mixed modeling to account for relatedness and assuming an additive genetic model, adjusting for 3 ancestry principal components, age and gender, using the mmscore function in ProbABEL. Then, we performed the meta-analysis using a fixed-effects model in GWAMA27 with the QC filtered GWAS summary results (imputation quality score >0.4 and minor allele frequency >0.03) from the GoDARTS and ORCADES. Manhattan plots, Quantile-Quantile plots, and forest plots were generated using in-built R scripts, and metafor—R package. Regional plots were generated using the Locus Zoom tool.26

Conditional analyses were performed in SNPTEST v2.5 using the genome-wide significant loci in the COL4A2 region, conditioned on the lead single-nucleotide polymorphism (SNP) (rs56399312). In addition, this newly discovered locus was conditioned on previously reported genome-wide significant SNPs (rs4773144,29 rs11617955,30 and rs951520331) associated with coronary artery disease (CAD). We performed an association test with an additive model adjusted for age, gender, and the first 3 principal components ancestry in the diabetes mellitus cohort (GoDARTS) using 759 samples without any cardiovascular events before the retinal screening date. We investigated the in-silico functional effects of the sentinel variants for each retinal vascular traits using various bioinformatics databases.23–37 Details can be found in the online-only Data Supplement.

The top 3 SNPs (P<1.07x10^{-7}) near ACTN4, TMEM132D, and COL4A2 from the discovery stage for the tortuosity traits were taken forward for examination in 3 replication cohorts of European ancestry. In the LBC1936 cohort, association analyses were performed for arterial and venular tortuosity traits using linear regression model adjusting for age, sex, and 3 ancestry principal components, using mach2qtl. Similarly, in the Croatia–Split, and Korčula cohorts, association analyses were performed for each trait separately using linear mixed models implemented in the hglm R package, accounting for kinships derived using the skin function of the GenABEL package.28

Summary association statistics for lead SNPs associated with TortA and TortV from the 2 discovery and 3 replication cohorts were combined and effect estimates from each cohort were presented in the forest plots using metafor—R package. Because of the variability in the β values and SE between the discovery and replication studies arising from different measurement algorithms, we standardized the effect estimates (using Cohen d) from each of the individual study cohorts. Cohen d is a scale-free interpretation which provides a standardized effect size measurement between the studies.29 In this study, Cohen d was calculated by the effect estimate divided by the SD.

In-Silico Look-Ups of the Novel Variants for Clinical Outcomes
To investigate the association of the lead SNPs for TortA, and TortV with cardiovascular outcomes, we performed in-silico look-ups using summary association results from the Coronary Artery Disease Genome-wide Replication and meta-analysis plus CAD consortium,29 Global Lipid Genetics Consortium analysis41, and International consortium for blood pressure GWAS analysis.41 A recent study reported the association of ACTN4 locus with heart rate.40 To examine whether the lead SNPs associated with TortV in ACTN4 were also associated with heart rate, we checked the linkage disequilibrium (r>0.8) between our SNPs and the index SNP (rs11083475) for heart rate in that study. Furthermore, we investigated the association of these SNPs with pulse rate in UK Biobank data.33 Additionally, we explored the relationship between the TortV-associated variants with atrial fibrillation (AF) using the summary statistics data from a recent large-scale meta-analysis of GWAS of AF.44 To identify the potential causal/target gene
for TortV-associated variants, colocalization analysis was performed eCAVIAR. Details of these studies and online in-silico functional annotation resources have been described in the online-only Data Supplement on methods.

RESULTS

Meta-Analysis of Discovery GWAS

The characteristics of the discovery study cohorts and overall study design can be found in Table I in the online-only Data Supplement and Figure 1. We combined the summary GWAS results from the GoDARTS and ORCADES cohorts for each trait using a fixed-effect meta-analysis, which overall showed no evidence of excessive amount of false-positive associations (genomic inflation factor of 0.99). Table II in the online-only Data Supplement presents the results from the meta-analysis and independent cohort GWAS analysis. Manhattan plots, Quantile-Quantile plots, and regional plots are shown in Figures 2 and 3 and Figures III through V in the online-only Data Supplement, respectively.

This analysis revealed one genome-wide significant (P<5×10⁻⁸) SNP, rs56399312, associated with TortA at 13q34, in COL4A2 with moderate heterogeneity (I²=0.50; β=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 (β=0.151, SE=0.028, P=8.59×10⁻⁶). Conditional analysis on the lead SNP indicated that these are not independent signals (Table III in the online-only Data Supplement). Two genome-wide significant SNPs were associated with TortV at 19q13 in ACTN4 (lead SNP rs1808382; β=−0.123, SE=0.022, P=1.55×10⁻⁶; no heterogeneity, I²=0.00), and at 12q24.33 near TMEM132D (lead SNP rs73157566; β=−0.294, SE=0.054, P=4.07×10⁻⁶; low heterogeneity, I²=0.10); these associations at both these loci have not been reported previously with any retinal vascular parameters.

Moreover, we replicated 3 loci out of 8 previously reported loci for CRVE but did not replicate any of the previously reported SNPs associated with CRAE. Finally, we replicated a previously reported genome-wide significant locus for OD radius at 10q21.3 near PBLD (lead SNP rs18054835; β=−3.840, SE=0.575, P=4.06×10⁻¹₁) and confirmed a number of other loci for this trait (Table IV in the online-only Data Supplement).

Replication of Novel Associations With Vessel Tortuosity in Independent Cohorts

As candidates to carry forward for replication, we selected 3 lead SNPs from both loci (total 6 SNPs) (ACTN4/CAPN12 and TMEM132D) for TortV and COL4A2 for TortA that reached significance P≤1.07×10⁻⁰⁷ and had...
similar effect size and direction across the discovery cohorts. The characteristics of the replication cohorts are presented in Table V in the online-only Data Supplement.

Two TortA-associated SNPs, rs7991229, and rs9515212 in COL4A2 reached nominal significance ($P<0.05$) in the LBC1936, Croatian cohorts. $P$ values were significant after multiple testing in stage 2 and combined analyses (based on 2 independent tests). The lead SNP (rs56399312) from discovery stage did not reach significant $P$ value in the LBC1936 but had similar effect size and direction. Two TortV-associated SNPs, rs1808382 and rs3786835 in ACTN4/CAPN12 reached suggestive significance ($P<1 \times 10^{-4}$) in the combined analysis of replication cohorts whereas rs73157566 near TMEM132D did not replicate. Table contains the summary statistics from replication cohorts and meta-analysis of these cohorts.

### Meta-Analysis of Discovery and Replication Cohorts

In the overall meta-analysis, SNPs at 13q34, COL4A2 (TortA) and 19q13, ACTN4 (TortV) display genome-wide

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**Figure 2.** Manhattan plots for meta-analysis of genome-wide association results from 2 independent discovery cohorts. **A,** The results for the arteriolar tortuosity (TortA) and **B** represents the results for the venular tortuosity trait (TortV). The blue and red horizontal lines indicate the suggestive and genome-wide significance threshold ($P<5 \times 10^{-8}$), respectively.

**Figure 3.** Regional association and recombination plots of variants that reached $P$ value $<5 \times 10^{-7}$ in the meta-analysis of the 2 discovery study cohorts (GoDARTS and ORCADES). **A,** Lead SNP for TortA; **B,** lead SNP for TortV. Each plot was created using LocusZoom for the lead single-nucleotide polymorphism (SNP) in genomic region 400 kb in either side of the significant signal. Blue spikes represent the estimated recombination rates. Color scale (high to low $r^2$) circles depicts the pairwise correlation ($r^2$) between lead SNP and other SNPs in the loci. The lead SNP in that region is indicated by purple color solid diamond, and gene annotations in this region are shown in the bottom. GoDARTS indicates Genetics of Diabetes Audit and Research in Tayside; and ORCADES, Orkney Complex Disease Study.
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<td>−0.033</td>
<td>0.009</td>
<td>0.0006</td>
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<td></td>
<td>Stage 2</td>
<td>−0.130*</td>
<td>0.027*</td>
<td>1.10×10⁻⁶†</td>
<td>0.84 (0)</td>
<td></td>
</tr>
<tr>
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<td></td>
<td></td>
<td>Combined</td>
<td>−0.109*</td>
<td>0.015*</td>
<td>3.31×10⁻¹⁰†</td>
<td>0.53 (0)</td>
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<tr>
<td>rs73157566</td>
<td>12</td>
<td>129533847</td>
<td>TMEM132D</td>
<td>A (0.04)</td>
<td>GODARTS</td>
<td>−0.289</td>
<td>0.061</td>
<td>3.28×10⁻⁵</td>
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<td>Stage 1</td>
<td>−0.097*</td>
<td>0.018*</td>
<td>4.07×10⁻⁶†</td>
<td>0.28 (0.1)</td>
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<td></td>
<td>LBC1936</td>
<td>−0.061</td>
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<td></td>
<td>All croatia</td>
<td>−0.003</td>
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<td>0.9045</td>
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<td>Stage 2</td>
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<td>0.027*</td>
<td>0.30795</td>
<td>0.35 (0)</td>
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<td></td>
<td>Combined</td>
<td>−0.075*</td>
<td>0.015*</td>
<td>2.61×10⁻⁰⁸†</td>
<td>0.08 (0.6)</td>
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Natural log-transformed—TortA and TortV. BP indicates base position; Croatia, Croatia island of Korcula+Croatia-Split; EA, effect allele; GoDARTS, Genetics of Diabetes Audit and Research in Tayside; LBC1936, Lothian Birth Cohort 1936; ORCADES, Orkney Complex Disease Study; TortA, retinal arteriolar tortuosity; and TortV, retinal venular tortuosity.

*Standardized β (s.d.) estimate (Cohen d) and SE, β values. β, change in natural log-transformed retinal tortuosity traits for each copy of the effect allele; SE, base position is based on build 37 of the reference genome.

†Significant P value <0.05 in Stage 1, Stage 2, and combined.
significant associations. Although TortA-associated SNPs, rs9515212 and rs7991229, were not genome-wide significant in the discovery meta-analysis, they reached genome-wide significance in the overall meta-analysis, and no heterogeneity ($I^2=0.00$) was observed across different cohorts; $P_{\text{overall}}=4.66\times10^{-12}$ and $P_{\text{overall}}=4.71\times10^{-12}$, respectively. The lead SNP in COL4A2 for TortA (rs56399312) at the discovery stage did not reach genome-wide significance in the overall meta-analysis ($P_{\text{overall}}=1.95\times10^{-07}$), suggesting it is not likely to be the causal association at this locus that warrants further study. For TortV the lead SNPs, rs1808382 ($P_{\text{overall}}=2.39\times10^{-13}$) and rs3786835 ($P_{\text{overall}}=3.31\times10^{-13}$) near ACTN4/CAPN12, increased genome-wide significance and show no heterogeneity of effect sizes across studies ($I^2=0.00$; Table). These SNPs are in tight linkage disequilibrium and, therefore, do not represent independent signals. The effect estimates from each of the individual study cohorts were standardized using Cohen’s $d$. Forest plots for lead SNPs in the combined analysis are shown in Figure 4.

**Functional Annotation: TortA-Associated Variants**

COL4A2 encodes collagen alpha-2(IV) (collagen type IV $\alpha_2$), one of the 6 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. Recent GWAS report that common variants around COL4A2 and COL4A1 (a paralogue immediately proximal to COL4A2, with which it shares a promoter and is co-expressed), are associated with coronary artery calcification, arterial stiffness, and CAD. Gene expression data from GeneAtlas, a human protein-coding transcriptome study validated the high expression of COL4A2 in retinal microvessel endothelial cells (Figure VI in the online-only Data Supplement) whereas COL4A1 is weakly expressed in retina indicating a specific role of COL4A2 in the retinal vasculature. TortA-associated variants near COL4A2 significantly alter transcription factor binding motifs and have putative effects on transcription as annotated by ENCODE (Table VI in the online-only Data Supplement). Additionally, expression data from the GTEx database confirmed that these significant SNPs are associated with the expression of COL4A2 in heart left ventricle and artery aorta, shown in Table VII and Figure VII in the online-only Data Supplement, and these SNPs are in linkage disequilibrium (rs9515212 and rs7991229; $I^2=0.99$, $D^2=1$).

**Association of TortA-Associated Variants With Cardiovascular Risk Factors**

Lead SNPs associated with TortA remained significant after conditioning on the previously reported cardiovascular risk variants in COL4A2 (rs11617955, rs4773144, and rs9515203; Figure VIII and Table VIII in the online-only Data Supplement). Conversely, the lead SNPs for TortA were not associated with CAD and myocardial infarction risk in the Coronary Artery Disease Genome-wide Replication and meta-analysis plus C4D consortium meta-analysis (Table IX in the online-only Data Supplement). Finally, the CAD-associated variants specifically in COL4A1 from Coronary Artery Disease Genome-wide Replication and meta-analysis plus C4D consortium meta-analysis (Table X in the online-only Data Supplement). Retinal arterial tortuosity traits have been previously associated with blood pressure, which may, therefore, link these variants with CAD; however, we found no evidence for an association between these lead variants and blood pressure in the International consortium for blood pressure GWAS analysis (Table XI in the online-only Data Supplement).
**Functional Annotation: TortV-Associated Variants**

*ACTN4* encodes α-actinin 4 (alpha-actinin-4), a crosslinking protein belonging to the spectrin superfamily and mutations in this gene cause focal segmental glomerulosclerosis in humans. *ACTN2*, a homolog of *ACTN4*, interacts with *ACTN4* and missense mutations in *ACTN2* are linked to a range of cardiac diseases.\(^5,6\) Annotation by ENCODE\(^23\) indicates that the 2 genome-wide significant variants (rs1808382 and rs3786835) associated with TortV near *ACTN4* may have direct regulatory effects as they are located within a DNase I hypersensitivity site and in genomic regions enriched for promoter/enhancer histone marks in heart tissues (Table VI in the online-only Data Supplement). *ACTN4* and *CAPN12* (calcium-activated neural proteases 12) overlap by 339 bases at their 3′ ends and multitissue 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 (Table VII and Figure IX in the online-only Data Supplement). Additionally, this analysis indicates that the T allele at rs1808382 is correlated with lower *ACTN4* (artery aorta; \(P = 2.1 \times 10^{-03}\)) and this correlation is even stronger with *CAPN12* (artery aorta; \(P = 2.0 \times 10^{-29}\)). Whereas gene expression data using Genevestigator validated the high expression of *ACTN4* in arterial tissue, the highest expression of *CAPN12* appears to be in the hematopoietic system.

**Colocalization Analysis of SNPs in ACTN4/CAPN12**

We further conducted eQTL/trait colocalization analyses with eCAVIAR\(^45\) and estimated the colocalization posterior probability for the lead variants that are associated with TortV and eQTLs. We found that the *CAPN12* has substantially higher colocalization posterior probability in heart left ventricle and artery tibial tissues than the *ACTN4* which indicates moderate colocalization between *CAPN12*eQTL and TortV (Table XIII in the online-only Data Supplement). The heart left ventricle is considered as the most relevant tissue (colocalization posterior probability; 0.38) for the TortV-associated lead variant (rs1808382)-*CAPN12* eQTL whereas for the other 3 tissues (heart atrial appendage; 0.22, artery aorta; 0.27, and artery tibial; 0.37) are considered as the relevant tissues for the second top variant (rs3786835) associated with TortV-*CAPN12* eQTL (Table XIII in the online-only Data Supplement).

**Association of TortV-Associated Variants With Cardiovascular Risk Factors**

Lead SNPs in *ACTN4* were significantly associated with CAD in the Coronary Artery Disease Genome-wide Replication and meta-analysis plus C4D consortium meta-analysis\(^26\) (Table IX in the online-only Data Supplement) and were associated with CAD risk factors include HDL (high-density lipoprotein) cholesterol and triglycerides in the Global Lipid Genetics Consortium analysis\(^10\) but not associated with LDL (low-density lipoprotein) cholesterol in the Global Lipid Genetics Consortium analysis or blood pressure in the International Consortium for blood pressure GWAS analysis\(^41\) (Table XI in the online-only Data Supplement). Furthermore, we have confirmed the association between TortV and top variants in *ACTN4*/*CAPN12* in a sensitivity analysis that only included GoDARTS samples without any cardiovascular events before the retinal screening date (Table XII in the online-only Data Supplement). Moreover, a recent meta-analysis of 35 GWAS studies reported the association of SNP (rs11083475) in the *ACTN4* locus with increased resting heart rate,\(^42\) which may increase cardiovascular disease risk. This signal appears the same as that for TortV with strong linkage disequilibrium being observed between the lead SNPs for TortV and the index SNP for heart rate. Furthermore, we found that these SNPs were associated with heart rate in UK Biobank\(^43\) (Table XIV and Figure X in the online-only Data Supplement). Interestingly, TortV-associated SNPs also associated with AF in the recent AF GWAS analysis\(^44\) (Table XI in the online-only Data Supplement).

**DISCUSSION**

In this first GWAS meta-analysis for quantitative retinal vascular tortuosity traits, we identify a novel locus for retinal arteriolar tortuosity (*COL4A2*) and for retinal venular tortuosity (*ACTN4/CAPN12*), which were firmly established by replication in 3 independent cohorts. Power calculations indicated that a sample size of 4507 (stage 1 and stage 2), and 3094 (stage 1) and the effect size of 0.10 using Bonferroni correction (\(<5\times10^{-8}\)) was adequate to provide 80% statistical power to detect the associations. Notably, we also identified in the discovery studies a genome-wide significant signal at a previously reported locus in near *ATOH7/PBLD* for the optic disc radius and replicated previously identified variants for *CRVE* which validate our retinal traits measurement methods. However, we did not replicate the previously reported SNPs for *CRAE*, but the direction of the effect was consistent. This may due to differences in the phenotyping, smaller effect sizes for certain SNPs compared to the previous studies and need for more power to replicate this association (Table XV in the online-only Data Supplement).\(^15,16\)

There are few limitations in this study. The meta-analysis of the lead SNPs for TortA and TortV in the replication cohorts (stage 2) lacks genome-wide significant \(P\) values which may due to the limited sample sizes. Other possible limitation of this study is that the retinal vascular...
traits were measured using different software in the discovery (eg, Vascular Assessment and Measurement Platform for Images of Retina) and replication cohorts (eg, SIVA). In spite of this limitation, our findings show consistent, homogeneous effects on tortuosity across 5 fairly diverse cohorts of European ancestry comprising individuals with and without diabetes. This highlights the robust nature of these genetic effects on retinal vascular topology. Also, these aspects strongly support the robustness our study design and findings.

Previous studies have reported association between COL4A2 and CAD but the TortA-associated variants in COL4A2 in the present study are not associated with cardiovascular disease and similarly COL4A2 variants that are associated with CAD do not appear to be associated with arteriolar tortuosity suggesting that variants in this gene complex may be involved differentially in the pathophysiology of microvascular and macrovascular diseases. However, more work has to be done to determine the distinct role of genetic variants in COL4A2/COL4A1 in different clinical conditions. In contrast, we found that retinal venular tortuosity-associated variants near ACTN4/CAPN12 were associated with CAD, heart rate, and AF. Furthermore, the lead variant influences the expression of the ACTN4/CAPN12 genes in the heart and blood vessel tissues. But our colocalization eQTL analysis found CAPN12 as the most likely causal gene at the chromosome 19 locus. Our sensitivity analyses including samples without CAD before the date of acquisition of the measured retinal image indicates that the relationship between genetic predictors of retinal venular tortuosity and cardiovascular diseases is not due to reverse causation and demonstrate the robustness of our findings.

Notably, the TortV-associated variants have shared genetic architecture with other cardiovascular-related traits including HDL cholesterol and AF. A recent study reported the relationship between retinal venular tortuosity and lower HDL cholesterol in the Asian-based cross-sectional cohorts.12 Our in-silico look-ups indicate that the genetic determinants for TortV near ACTN4 also associated with HDLc in the Global Lipid Genetics Consortium analysis GWAS but not associated with the LDLc which is consistent with the Asian-based study.12 A study from the ORCADES and Croatia-Korcula cohorts reported a weak association between retinal arteriolar tortuosity and systolic blood pressure and no significant association between retinal venular tortuosity trait and blood pressure.10 A recent larger epidemiological study in European-based prospective cohort reported an association with systolic blood pressure for TortA, and a weaker association for TortV.15 In this regard, neither the TortA nor TortV-associated variants were associated with systolic and diastolic blood pressure in the International Consortium for blood pressure GWAS analysis; therefore, it seems unlikely that observed associations with CAD or related traits are mediated through blood pressure.

In summary, this first GWAS for retinal arteriolar and venular tortuosity reveals associated SNPs of strong effects influencing the expression of COL4A2 and ACTN4/CAPN12, respectively. Our results demonstrate that the TortA-associated variants in COL4A2 are independent of CAD, myocardial infarction, and blood pressure, 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, HDL cholesterol, AF, and heart rate but not associated with blood pressure. Our findings appear to indicate CAPN12 as the causal gene for TortV through colocalization analysis. However, detailed investigation and functional validation of this new finding are essential to elucidate the causal role of this locus and the relative contribution of ACTN4 and CAPN12 in the observed cardiovascular pathophysiology. These findings highlight the potential genetic impacts of retinal vasculature to provide new insights into cardiovascular disease.

ARTICLE INFORMATION
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