Published in final edited form as:

Angiogenesis. 2015 January ; 18(1): 97–114. doi:10.1007/s10456-014-9449-y.

Genetic variation in retinal vascular patterning predicts variation in pial collateral extent and stroke severity

Pranay Prabhakar, BS¹, Hua Zhang, MD¹, De Chen, PhD², and James E. Faber, PhD¹

¹Department of Cell Biology and Physiology and the McAllister Heart Institute, University of North Carolina ²Optical Microscopy and Analysis Laboratory Leidos Biomedical Research, Inc, Frederick National Laboratory for Cancer Research, Frederick, Maryland

Abstract

The presence of a native collateral circulation in tissues lessens injury in occlusive vascular diseases. However, differences in genetic background cause wide variation in collateral number and diameter in mice, resulting in large variation in protection. Indirect estimates of collateral perfusion suggest wide variation also exists in humans. Unfortunately, methods used to obtain these estimates are invasive and not widely available. We sought to determine if differences in genetic background in mice result in variation in branch-patterning of the retinal arterial circulation, and if these differences predict strain-dependent differences in pial collateral extent and severity of ischemic stroke. Retinal patterning metrics, collateral extent, and infarct volume were obtained for 10 strains known to differ widely in collateral extent. Multivariate regression was conducted and model performance assessed using K-fold cross-validation. Twenty-one metrics varied with strain ($p < 0.01$). Ten metrics (eg, bifurcation angle, lacunarity, optimality) predicted collateral number and diameter across 7 regression models, with the best model closely predicting ($p < 0.0001$) number (± 1.2 -3.4 collaterals, K-fold $R^2 = 0.83$ -0.98), diameter (± 1.2 -1.9 μm , $R^2 = 0.73$ -0.88) and infarct volume ($\pm 5.1 \text{ mm}^3$, $R^2 = 0.85$ -0.87). These metrics obtained for the middle cerebral artery tree in a subset of the above strains also predicted ($p < 0.0001$) collateral number and diameter and diameter, although with less strength (K-fold $R^2 = 0.61$ -0.78) and 0.60-0.86, respectively). Thus, differences in arterial branch-patterning in the retina and the MCA trees are specified by genetic background and predict variation in collateral extent and stroke severity. If also true in human retina, and since genetic variation in cerebral collaterals extends to other tissues at least in mice, a similar “retinal predictor index” could serve as a non-or minimally invasive biomarker for collateral extent in brain and other tissues. This could aid prediction of severity of tissue injury in the event of an occlusive event or development of obstructive disease and in patient stratification for treatment options and clinical studies.

Corresponding author: James E. Faber, Department of Cell Biology and Physiology, 6309 MBRB, University of North Carolina, Chapel Hill, NC 27599-7545. Tel: 919-966-0327. Fax: 919-966-6927, jefaber@med.unc.edu.

Disclosures: None

Author Contributions: JEF conceived the original idea; JEF and PP designed the study; PP performed the experiments and statistical analysis; HZ performed cerebrovascular casting, MCA occlusion and determined collateral extent and infarct volume; DC derived fractal dimension and lacunarity; PP and JEF analyzed the data, interpreted the results, prepared the figures and wrote the manuscript; all authors approved the final version of manuscript.

Keywords

retinal circulation; collateral circulation; cerebral circulation; ischemic stroke; mouse genetics; vascular patterning

Occlusive vascular disease in brain, heart and peripheral limbs is caused by atherosclerosis, thrombosis and other disorders and imposes large social and economic burdens. Although available treatment options (eg, thrombolysis, thrombectomy, angioplasty/stent, bypass grafting) are successful in many patients, time window for treatment, co-morbidities, inaccessibility and diffuse obstructions exclude large numbers of patients. Irrespective of treatment availability or type, an abundant collateral circulation greatly reduces morbidity and mortality in these diseases.^{1,2} Collaterals are native (pre-existing) arteriole-to-arteriole anastomoses that cross-connect a small fraction of the outer branches of adjacent arterial trees and are present in most tissues. When the trunk of one of the trees becomes obstructed, collateral-dependent retrograde perfusion significantly decreases tissue injury. The amount of protection depends primarily on the extent (ie, number and diameter) of collaterals present, plus the perfusion pressure across the collateral network and vascular resistance above and below it.^{3,4}

The major determinants of severity of ischemic stroke are site of occlusion within the tree, time to endovascular revascularization when this is an option, and collateral blood flow. Unfortunately, collateral perfusion varies widely among individuals in brain, heart, lower extremities and other tissues. For example, in patients with occlusion of the middle cerebral artery (MCA), the most common cause of ischemic stroke, collateral-dependent perfusion of the MCA tree—which can be graded (scored) by neuroimaging—varies widely, with approximately twenty percent having poor pial (leptomeningeal) collateral scores (ie, poor collateral “status”).^{1,5-9} Notably, such individuals sustain larger infarct volumes, respond poorly to thrombolytic treatments, have increased risk for and severity of intracerebral hemorrhage, and suffer increased morbidity and mortality.^{1,5-9} Recent studies have confirmed a similar wide variation in collateral blood flow in other tissues among “healthy” humans, ie, without obstructive disease in the tissue under examination. In individuals without angiographically detectable coronary artery disease (CAD), collateral flow index (CFI) was distributed normally and varied by ~25-fold, with approximately twenty percent of individuals having low CFIs.¹⁰ Importantly, patients with CAD and poor CFIs had a sixty-four percent higher risk of mortality.² CFI also varied significantly in the lower extremities of individuals without peripheral artery disease (PAD).¹¹ Thus, assessing collateral status is increasingly regarded as an important means to identify optimal treatment and assess prognosis for recovery.^{1,2,5-9}

The mechanisms responsible for the wide variation in collateral status are unknown. However, recent studies in mice indicate that genetic background may be a major factor. Pial collateral extent was found to vary, remarkably, by 56-fold among 21 healthy adult strains with naturally occurring differences in genetic background, and was associated with a 30-fold variation in infarct volume after MCA occlusion.^{3,12,13} Moreover, a single polymorphic locus on chromosome 7, denoted “Determinant of collateral extent-1” (*Dce1*),

has since been identified that is critical in the pathway controlling collateralogenesis in the embryo and thus accounts for most of the above variation in the index strains.¹⁴ In that study, the at-risk allele of *Dce1* in the strain with poor collaterals was replaced with the allele from the strain with abundant collaterals. This restored the poor collateral phenotype to nearly that in the good strain: 83% correction of low pial collateral extent, and—after MCA occlusion—a 4.5-fold improvement in blood flow and 85% reduction of infarct volume; similar effects were achieved in skeletal muscle, resulting in 83% improvement in recovery of hindlimb perfusion after femoral artery ligation. Thus, ischemia, tissue injury and infarct volume were strongly reduced by exchanging a single discrete locus. Although the causative genetic element(s) at *Dce1* has not yet been identified, the candidate genes have been narrowed to a small number.¹⁴ Whether human *DCE1* and/or related loci are responsible for the wide variation in collateral status in humans is under investigation.¹⁵

A critical problem is that no non-invasive method exists for determining the extent of the collateral circulation in healthy humans or patients with obstructive disease. Since the diameters of most native collaterals are below the resolution of clinical imaging modalities, measurement of pial collateral score in acute stroke is used to indirectly estimate conductance of the collateral network. This method requires administration of a contrast agent, thus is invasive. It also relies on advanced neuroimaging not available at most treatment centers. Estimation of collateral-dependent perfusion in heart and lower extremities requires temporary intra-arterial balloon occlusion, which generally restricts its use to experimental studies.^{10,11} Thus, a non-invasive method or biomarker that predicts collateral extent would be an important development. Recent studies in mice have found that genetic differences in pial collateral extent are established late during gestation,^{16,17} and have noted a possible association with differences evident at the same time in the branch patterning of the MCA tree, with both differences being sustained in the adult.¹⁶ This apparent association of collateral extent with arterial tree geometry is also evident, at least qualitatively, in another tissue—skeletal muscle.¹⁸⁻²³ This suggests that naturally occurring genetic polymorphisms that cause differences in collateral extent may also cause differences in arterial tree patterning. This opens up the possibility that analysis of arterial tree arborization in a tissue amenable to non-invasive imaging could be used to predict the extent of the collateral circulation in the brain and other tissues, since genetic-dependent variation in pial collaterals extends to variation in other tissues in the same individual, at least in mice.^{4,14,18-21,23}

The retinal circulation stands alone as a candidate tissue. It is arranged in two-dimensions which aids geometric analysis and can be imaged directly and non-invasively in humans. Moreover, sophisticated methods have been developed for quantifying the geometry of its branch-patterning.²⁴⁻³⁰ In addition, it is well known that formation of the retinal and neocortical vasculatures share many anatomic similarities during development and maturation,²⁵ as well as structural and topographic changes that occur with aging, cardio/cerebrovascular diseases and hereditary angiopathies.²³⁻²⁹ Unfortunately, since the inner retinal circulation in human²⁵ and mouse (confirmed herein) lacks collaterals, it is not possible to simply quantify collaterals in retina to determine if their number and diameter predict collateral extent in brain and other tissues.

The purpose of this study was to determine if branch-patterning of retinal arterial trees varies with genetic background and correlates with differences in pial collateral extent, and in turn, infarct volume following MCA occlusion. We tested this hypothesis in a genetically diverse cohort of mice previously shown to have wide differences in collateral extent.^{3,4,16,18-20} A comprehensive set of previously reported retinal patterning metrics was examined, together with identification of several novel and more predictive metrics that define genetic-dependent retinal vascular complexity. Multivariate stepwise regression modeling was used to analyze the data in an unbiased manner. K-fold regression analysis was then used to reduce statistical bias and simulate external validation, thus improving the validity of the results. We also examined the same question for branch-patterning of the MCA tree, to determine whether the findings in retina extend to another tissue and thus offer insights into the underlying mechanism for the association.

Methods

An expanded Materials and Methods section is available in the Online Supplemental Material.

Pial collateral number and diameter, retinal vascular imaging, infarct volume, MCA imaging

The number and average diameter of pial collaterals (COL-N, COL-D) that cross-connect the MCA and anterior cerebral artery (ACA) trees of both hemispheres were obtained as detailed elsewhere³ from a population of ~3 month-old male mice (n=81, ~8 per strain) composed of 10 strains that differ widely in collateral extent (Figure 1).^{3,4,16,18-20} We have previously found no sexual dimorphism for genetic-dependent differences in pial collateral number and diameter in different strains of classic inbred, F1 or F2 crossed, or congenic mice.^{4,14,61} The deficient strains were: VEGFA^{lo/+} A/J, AKR/J, CLIC4^{-/-}, and BALB/cByJ; the abundant strains were: C57BLKS/J, DBA/2J, VEGFA^{hi/+}, C57BL/6J, and the CD1/CR strain (background strain for VEGFA^{lo/+}, VEGFA^{hi/+}, and CLIC4^{-/-}). The strains VEGFA^{lo/+}, CLIC4^{-/-}, VEGFA^{hi/+} and CD1 are closely related, although the gene targeted strains were generated from separate lines of CD1 which itself is maintained outbred.^{19,20} We selected them as part of the 10-strain population because we have shown previously that VEGF-A and CLIC4 are important in collaterogenesis, which occurs late in gestation and early postnatally, and are thus determinants of collateral number and diameter in adult and infarct volume after middle cerebral artery ligation and severity of hindlimb ischemia after femoral artery ligation; these proteins also regulate formation and patterning vessels of the general arterio-venous circulation.^{19,20} and references therein That is, these strains provided additional strains, besides the six classic inbred strains, with low, intermediate and high collateral number and diameter in their tissues. Although C57BL/6J (B6) and C57BLKS/J are also closely related genealogically, approximately 71% of the latter's genome derives from B6, 25% from DBA/2J and 4% from C57BL/10J, a 129 source and an unidentified source(s) (<http://jaxmice.jax.org/strain/000662.html>). Before the above procedures, one retina was collected from each mouse, flat-mounted, and stained (Alexafluor 568 GS-IB4). Other mice of the above strains received permanent occlusion of the right MCA trunk (MCAO), followed by determination of infarct volume 24h later using 2,3,5-

triphenyltetrazolium chloride staining.¹⁴ For the MCA study, the MCA artery tree was imaged as described previously³ in 5-6 mice of a subset of the above strains (AKR/J, BALB/cByJ, C57BLKS/J and C57Bl/6J). Different mice were used for the retina and MCA studies.

Vascular patterning metrics

We obtained 22 retinal patterning metrics (RPMs) to define complexity of the retinal artery trees. The metrics were determined in an inner zone region extending from the optic disc margin, and an outer zone region extending from there to the retinal periphery (Figures 2-3, Online Figure 1). The RPMs included several commonly used metrics,⁽²⁴⁻³⁰⁾ eg, vessel caliber, branch angle, central retinal artery and vein equivalent (CRAE and CRVE), artery-to-vein ratio (AVR), optimality, arterial tree area, fractal dimension and lacunarity (Figure 1, Online Table 1, Online Figure 2). We also measured additional RPMs that identify distribution (average, skewness, kurtosis) of length and tortuosity of branch segments. Measurements were limited to arterial trees because a preliminary analysis showed that the capillary bed, venous trees, and the distal-most arteriole branches beyond the 4th-order level lacked detectable strain-dependent differences in patterning and thus increased the noise in the data (Online Figures 3-5). A subset of the above metrics that were found to be most predictive in the retina study was obtained in the MCA study using the same methods for deriving the metrics as described below. All mice were coded to assure investigators remained blinded to mouse strain and collateral extent until all measurements had been obtained.

Image analysis

Three arterial trees were randomly chosen for each retina among the 5-7 trees present (Research Randomizer, www.randomizer.org). Ten RPMs were manually obtained from the inner-zone between the optic disc margin and inner zone margin, the latter defined as lying at 1/10th and 5/10th of the outer-zone margin (Figure 2). The remaining 12 RPMs (Figure 3) were obtained after binarization of the arterial trees (Online Figure 3), followed by semi-automatic application of Image J functions, a skeletonize plugin,³¹ and a MATLAB algorithm for measuring fractal dimension and lacunarity. A highly customizable macro recorder (<https://www.jitbit.com/macro-recorder/>) was used to create a variety of repetitive sequences of ImageJ commands and plugins followed by data-transfers to Microsoft Excel. The MATLAB code used to calculate fractal dimension and lacunarity is in Online Appendix 1. These action sequences were looped to allow more efficient acquisition of data from batches of a large number of images in a semi-automatic fashion. Preliminary results from 20 mice (5 each from 4 strains) indicated that analyzing 2 randomly chosen trees provided an optimal tradeoff between accuracy and time required for image binarization (Online Figure 6). Therefore, images were background-corrected using Photoshop CS5, and for the remaining 61 mice, 2 instead of 3 arterial trees from each retina were randomly chosen, binarized, and capillaries and branches beyond the 4th order vessel level were removed (Online Figure 3). RPMs across all measured arterial trees for a given retina were averaged.

Study design and statistical analysis

Statistical analyses were performed in JMP 9.0 (SAS, Cary, NC) and Microsoft Excel 2010 (Figure 4A). After obtaining COL-N, COL-D and the 22 RPMs for each mouse, an outlier analysis was conducted using Mahalanobis, T-squared and jackknife distances, resulting in identification of one common outlier (Figure 4B). ANOVA was then used to determine if RPMs vary with COL-N, COL-D and genetic background (Figure 5). Finally, to test the hypothesis that RPMs predict COL-N and COL-D, stepwise multivariate regression modeling was performed on the 80 remaining mice using 3 different stopping rules [minimum Akaike's Information Criterion (AIC), minimum Bayesian Information Criterion (BIC), and p-value cut-offs] in forward, backward, and mixed directions to obtain a set of 7 regression models for both COL-N and COL-D (RM1) (Online Figure 7). As an additional outlier analysis, mice with a significantly high Cook's D influence across RM1 of both COL-N and COL-D were identified and excluded (Figure 4C). The aforementioned modeling process was then repeated to obtain 2 additional sets of regression models (RM2 and RM3) (Online Figure 7). Leave-one-out K-fold cross-validation was performed³² for all 21 models for COL-N and COL-D to test predictive performance (Online Figure 7). In addition, direction and significance of independent correlation of predictive RPMs were compared across all models (Online Figure 7). The most predictive models for COL-N and COL-D were further examined to: 1) determine comparative predictive ability of individual RPMs as assessed by magnitude of scaled parameters and orthogonalized estimates (Figure 6) and 2) to obtain a formula—the Retinal Predictor Index—for collateral number and diameter (RPI_n and RPI_d). To aid presentation of the relationships that were identified, retinal and pial images of two mice with a wide difference in COL-N and COL-D were visually compared with values of the most predictive metrics (Figure 7). To test whether RPMs predict severity of stroke following MCAO, performance of RPI_n and RPI_d for the different mouse strains was evaluated for prediction of infarct volume (Figure 8).

Results

Patterning metrics of retinal arterial trees correlate strongly with genetic background-dependent differences in collateral number and diameter

To test the hypothesis that retinal artery tree geometry varies with genetic background, we performed a bivariate regression (ANOVA) of COL-N, COL-D, and RPMs across the 10 mouse strains (Figure 5A, Online Table 1, Online Figure 2). Differences in COL-N and COL-D were strongly associated with genetic background (adjusted R^2 of 0.86 and 0.87), confirming previous findings.^{3,4,16,18-20} Consistent with the above hypothesis, 10 of the 22 RPMs varied strongly with genetic background (adjusted $R^2 > 0.35$ and $p < 0.0001$) (Figure 5A), suggesting that, similar to the variation in COL-N and COL-D (Figure 1), differences in genetic background cause significant differences in retinal arterial tree patterning. This is confirmed by multivariate correlation matrices (Figure 5B,C), which show that many RPMs have a strong and significant correlation with COL-N and COL-D. These data support the hypothesis that RPMs predict collateral extent in the brain. However, the correlation matrices show that certain RPMs are also correlated with other RPMs. Thus to test the predictive hypothesis, a step-wise, multivariate regression modeling approach was required

to adjust for covariates, obtain a refined set of the most predictive RPMs, and eliminate any redundant metrics.

Genetic-dependent differences in patterning of retinal arterial trees predict differences in pial collateral number and diameter

To determine which RPMs and in what combination most strongly predict COL-N and COL-D, we performed stepwise multivariate regression modeling (Online Figure 7). The predictive performance of the models, evaluated using K-fold R^2 cross-validation, was strong across all models (0.73-0.83 for COL-N and 0.59-0.73 for COL-D). This supports the hypotheses that genetic background plays a significant role in specifying retinal patterning and that RPMs can be used to predict genetic differences in COL-N and COL-D. Removal of outliers did not change this conclusion: K-fold R^2 for COL-N and COL-D only improved by an average of ~ 0.05 and 0.02 for RM2 and ~ 0.09 and 0.1 for RM3 (Online Figure 7). In addition, all 7 models showed a similarly strong predictive performance and a nearly identical set of RPMs that were consistently predictive in the same direction, ie, 9 RPMs for COL-N, of which 7 were also predictive for COL-D. This verifies that the findings were not dependent on or biased by a particular modeling approach. Lacunarity and CRAE were the two RPMs that predicted COL-N but not COL-D. Lacunarity only became a significant predictor across all models after removal of outliers (RM2 and RM3), and CRAE was only predictive in 4 models. These results are consistent with our finding that lacunarity and CRAE only minimally contribute to predictive performance, in comparison to the other predictors (Figure 6). Only one of two RPMs for distribution of a metric—skewness and kurtosis of distribution of branch segment lengths—reached predictive significance across all models (ie, kurtosis) (Online Figure 7), a finding that is attributable to the strong covariance and redundancy of the two RPMs (Figure 5B).

A combination of nine retinal patterning metrics predicts differences in collateral extent

To compare the relative predictive strength of the RPMs, we obtained scaled estimates and orthogonalized estimates (ie, estimates that are set to a common relative scale and independent of other metrics after elimination of covariance) of the most strongly correlated RPMs arising from the most predictive models (identified in Online Figure 7) for COL-N and COL-D (Figure 6). The scaled estimates (Figures 6A,C) show relative amount of change in COL-N or COL-D (in raw values) that is expected when a specific RPM changes from its lowest to highest value in the data set. However, scaled estimates only provide limited information because of covariance between certain RPMs (Figure 5B) and because the metrics have unequal ranges and variances in the mouse cohort [eg, optimality and fractal dimension (FD) show less variation compared to vessel caliber (Online Figure 2)]. Pareto plots (Figures 6B,D) improve the assessment of relative predictive power because they also standardize and orthogonalize the RPMs so that they have equal variances and are uncorrelated. Accordingly, the average diameter of the larger branch vessel after a branch point (D2) is the strongest predictor of collateral extent, followed closely by average length of branch segments, and retinal area. Other predictive metrics in the model, eg, parent vessel caliber (D0), optimality ratio (a measure inversely related to equitability of distribution of blood flow from a trunk to daughter vessels at bifurcations), kurtosis of distribution (ie, ‘peakedness’ of distribution, or “self-similarity”) of branch segment lengths, branch angle at

bifurcations, retinal area, lacunarity and CRAE—although not strong contributors individually—together capture additional unique information that approximately doubles the net predictive ability beyond the three most predictive metrics. Thus, the combination of these nine features of retinal patterning strongly predicts genetic variation in collateral number and diameter. These RPMs were then combined with the most predictive models to obtain a formula—the Retinal Predictor Index—for collateral number and diameter (RPI_n and RPI_d) that were then found to be strong predictors of the actual collateral number (± 3.4 collaterals, K-fold $R^2 = 0.83$, $p < 0.0001$) and diameter ($\pm 1.9 \mu m$, 0.73 , $p < 0.0001$) in each mouse (Figures 6E,F).

Figure 7 combines retinal and pial images with RPM data from two mice that varied widely in collateral number (27 versus 0), to address the degree to which visual versus quantitative differences in retinal patterning correlate with differences in collateral number. Consistent with the models' predictive power, a mouse with no collaterals had a predicted COL-N of 0.5, lower values for vessel calibers (D0, D2), branch angles at branch points, retinal area and CRAE, but higher lacunarity, longer branch segments, and higher optimality. However, this mouse had a lower kurtosis of distribution of branch segment lengths (ie, lower proportion of branch segments with similar lengths)—a finding that is inconsistent with prediction and attributable to biological variance and experimental error. Also consistent with quantitative differences, the mouse with no collaterals had more acute branch angles, a smaller retinal area, lower vessel caliber overall, less vessel complexity, and fewer branch segments. On the other hand, visual differences in CRAE, optimality and kurtosis of distribution of branch segment lengths were less evident. Thus, the comparisons in Figure 7, showing that visual differences in retinal vascular patterning are subtle in mice with even wide differences in COL-N and COL-D, underscore the need for precise quantitative measurements and multivariate regression modeling in order to predict differences in collateral extent.

Retinal artery tree geometry predicts infarct volume

Previous studies have shown that differences in collateral extent are closely associated with (Figure 1)^{3, 19,20,23}—and causal for¹⁴—differences in severity of stroke following middle cerebral artery occlusion. Given that genetic variation in retinal geometry strongly predicts variation in collateral extent (Figure 6), we hypothesized that RPMs will also predict infarct volume (Figure 8), using the RPI_n and RPI_d derived from the above analysis. This test for association with infarct volume was necessarily conducted on a strain-wise basis, because infarct volumes for the ten strains (Figure 1) were a combination of historic data^{3,19,20} plus addition of more animals in VEGF^{hi/+}, VEGF^{lo/+} and BALB/c strains. Average RPIs for the ten mouse strains strongly predicted average COL-N and COL-D (K-fold R^2 of 0.98 and 0.88, Figure 8A). As suggested from our previous studies,^{3,19,20,23} average COL-N and COL-D among the strains strongly predicted infarct volume among the same strains (K-fold R^2 of 0.78 and 0.75, Figure 8B); these values are similar to those obtained with linear correlation of collateral number and diameter against infarct volume across fifteen mouse strains ($R^2 = 0.80$ and 0.71 , respectively).³ Importantly, average values for RPI_n and RPI_d among the strains thus strongly predicted average infarct volumes (K-fold R^2 of 0.85 and 0.87, Figure 8C).

The flow scheme at the top of Figure 8 derives from three observations: The data in panel A show that the average RPI for collateral number and diameter correlate closely with, ie, “predicts (A)”, the measured collateral number and diameter among the 10 strains. The data in panel B show that average collateral number and diameter also correlate well with, ie, “predicts (B)”, the measured infarct volume. These data also agree strongly with data obtained in a previous study of 15 classic inbred strains (6 of which were also studied in the present study) where infarct volume yielded correlation coefficients for collateral number and diameter ($r^2=0.80$, $p<0.0001$ and $r^2=0.71$, $p<0.0001$, respectively)³ very similar to those obtained in the present study (0.78, 0.75). Moreover, the present study confirms the conclusion reached previously³ that variation in pial collateral number and diameter are major determinants of variation in the severity of infarct volume. These data and conclusion agree with the observation that infarct volume in patients with acute ischemic stroke is strongly correlated with collateral status/score—an index of collateral-dependent flow whose major determinants are number and diameter of the pial collateral network serving the occluded territory.^{1,5-9} The data in panel C validate the inference that the RPI for collateral number and diameter therefore predict well ($r^2=0.85$ and 0.87) the actual measured infarct volume.

Relationship of fractal dimension and lacunarity with Euclidean metrics

Fractal dimension and lacunarity are global, non-Euclidean dimensionless metrics that have been used to define complexity of the retinal vasculature in association studies.^{24,25,27,34} However, how they relate to geometric measures of retinal arterial tree patterning has not been determined. Since we found that differences in fractal dimension and lacunarity were associated with differences in the other patterning metrics (Figure 5B,C; orange boxed-in metrics), our data set offered a unique opportunity to identify, in a complex vascular branching network, the relationship between these global metrics and the Euclidean metrics which are more intuitive and visualizable. No such analysis is extant in the literature. Online Figure 8A-I shows the association between RPMs and fractal dimension and lacunarity. As with our prior analysis, we performed forward and backward stepwise multivariate regression modeling of fractal dimension and lacunarity versus RPMs using a variety of criteria as detailed in Online Figure 7. Average fractal dimension and lacunarity of retinal artery trees (dependent variables) from the 80 mice that were used above were modeled with other RPMs (independent variables). Significance of RPMs for a given model and their strength of association with fractal dimension and lacunarity, as measured by K-fold R^2 , were identified as detailed in Figure 6 and Online Figure 7. Among RPMs that characterize retinal arterial tree complexity, high fractal dimension and low lacunarity were most strongly associated with differences in large vessel caliber (CRAE) and shorter and more tortuous branch segments, respectively.

Fractal dimension did not emerge as a significant predictor of COL-N and COL-D across most models (Online Figure 7), and lacunarity only moderately predicted COL-N (Figure 6B). The reasons for this become evident from the inspection of orthogonalized (uncorrelated, equal variance) metrics (Online Figure 8A-I), where it can be seen that these measures encompass contributions from multiple other patterning metrics that correlated either positively or negatively with collateral number and diameter (Online Figure 7).

Additional likely contributions to their low predictive power are the narrow range of fractal dimension (1.41-1.54) and high covariance of it and lacunarity with other RPMs (Figure 5B, orange boxed-in metrics), many of which defined more specific independent features of arterial tree complexity and thus emerged as stronger predictors of collateral number and diameter. These findings aid interpretation of previous studies and design of future studies using fractal dimension and lacunarity.

Middle cerebral artery tree analysis

Patterning of the MCA tree and arterial trees in skeletal muscle of C57Bl/6 mice differ qualitatively from BALB/c mice—strains with large differences in collateral number and diameter in these and other tissues.^{3,4,16,18,22,23} Given the above findings in the retina, we wondered if patterning in another tissue would evidence similar differences and correlate with/predict differences in pial collateral extent. We studied the MCA because it can be imaged in its entirety and in quasi-2-dimension without dissection of the brain, is the largest of the cerebral artery trees, and is cross-connected to the ACA and posterior cerebral artery (PCA) by the pial collaterals whose variation in extent is the subject of this study.

In agreement with previous qualitative observations,^{3,4,16,18,22,23} genetic-dependent differences in arborization of the MCA tree were also evident among a subset of the 10 mouse strains (BALB/c, C57BLKS, AKR and C57BL/6), when examined for the same predictive metrics that were identified in retina (Online Figures 9-11). Multivariate regression analysis showed that MCA tree geometry predicted pial collateral number and diameter, albeit with less strength than retina. However, many of the metrics correlated poorly or not at all, or with reversed slopes in bivariate regression against the same metrics in retina (Online Figure 9)—findings which also undoubtedly reflect, in part, the smaller number of strains and fewer animals per strain analyzed.

Discussion

The primary goal of this study was to test the hypothesis that genetic polymorphisms responsible for variation in collateral extent¹⁴ also cause strain-specific differences in branch-patterning of the retinal artery tree that correlate with and predict differences in collateral extent. No studies are extant in the literature that provide even an entrée to answering this question, ie, none has examined whether retinal vascular geometry varies with genetic background in mice or other laboratory species, wherein potential confounding effects of differences in environmental factors can be held constant. Our findings support this hypothesis. Retinal artery geometry varied with differences in genetic background and strongly predicted actual pial collateral number and diameter. Values for correlation and predictive strength derived from analysis of individual mice versus across strains were (an R^2 of 1.00 equals a perfect correlation/prediction): R^2 of 0.83 versus 0.98 for number, and 0.73 versus 0.88 for diameter, with predictive strength of ± 3.4 versus 1.2 for number, and ± 1.9 versus 1.2 μm for diameter; $p < 0.0001$ for all (Figures 6E,F, Figures 8A,B). Moreover, these predicted values for collateral number and diameter strongly predicted actual infarct volume after MCA occlusion: $R^2 = 0.85$ and 0.87 , $\pm 5.1 \text{ mm}^3$ for infarct volume, $p < 0.0001$ (Figure 8C). This is in agreement with the major role of differences in collateral blood flow

in determining differences in stroke severity.^{1,3,5-9,14,19,20} These results suggest that variation in arterial arborization in the retina may predict variation in collateral extent in humans and severity of ischemic injury if arterial obstruction occurs.

The two strongest metrics for predicting genetic differences in collateral number and diameter were D2 (diameter of the larger daughter vessel at bifurcations along the largest artery within the tree; positively correlated) and average length of branch segments (negatively correlated) (Figure 6). The latter, which was also true in the MCA tree study, is consistent with a larger number of branches in a tree providing a greater number of opportunities for collaterals to form between adjacent trees—a process termed collaterogenesis that occurs during development.^{16,17,34} The positive relationship with D2 may reflect artery trees that form, enlarge their diameters and extend outward faster during gestation to reach the crowns of adjacent trees, thus facilitating collaterogenesis.^{16,17} Other patterning metrics associated with greater collateral extent and thus lesser stroke severity were: larger retinal areas, wider branch angles, more equitable distribution of blood flow (lower optimality), and greater vessel complexity (higher lacunarity, shorter branch segments, greater variation in branch segment lengths). All of these metrics describe a larger, more complex tree with a greater number of distal-most arterioles that would thus provide more opportunities to form collaterals during collaterogenesis. Thus, one simple hypothesis to explain our results is that genetic background determines the timing of arterial tree formation, outgrowth and branch patterning during development; this in turn determines the number and proximity of distal branches in adjacent artery trees across which collaterals can then form (ie, collaterogenesis, which occurs in the embryo after arterial tree formation^{16,17}); thus patterning predicts collateral extent in the adult. Arguing against this “opportunity hypothesis” is that genetic-dependent differences in collateral diameter, which correlate closely with those for number,^{3,14,61} would not seem to need to follow this scenario. Also, genetic differences in arterial tree patterning in the adult primarily reflect subtle sculpting-like changes that occur during the first few weeks postnatal.^{16,17} Moreover, our previous findings have found that collaterogenesis is a unique process very different from arterial tree formation, and that the process itself, as well as the large difference in its vigor in the B6 and BALB/c strains, ie, the number and diameter of collaterals that form, does not appear to depend on whether distal branches of adjacent arterial trees are in proximity.¹⁶ An alternative hypothesis that thus arises is that one or more genes, which exist as naturally occurring variants, are shared by independent signaling pathways that control artery tree patterning and collaterogenesis (“shared gene hypothesis”). Additional studies will be required to distinguish between these hypotheses.

As suggested by previous qualitative observations,^{3,4,16,18} MCA tree structure also varied with genetic background. Greater collateral extent was associated most strongly with the same features associated in retina, ie, larger vessel calibers, wider branch angles at branch points, and smaller tree area. However, while multivariate regression analysis of the strongest predictive metrics identified in retina showed that MCA tree geometry also predicts pial collateral extent, statistical strength was approximately half that of retina. Also, some of the metrics derived from both the retina and MCA trees did not correlate on bivariate analysis. Several factors could contribute to these differences. Analysis of the MCA tree was done in a smaller number of strains (4) and mice per strain (5-6) due to

exigencies of resource allocation. The inability to image the MCA tree in a flattened orientation likely introduced a small amount of error. Differences in time of developmental of the brain and retina and their vascular trees may contribute (discussed below). In addition, retina lacks collaterals, while the presence of collateral connections between the MCA and adjacent trees during postnatal growth and maturation of the brain may contribute to normal activity-dependent changes in regional tissue blood flow. This could cause a difference in flow-dependent adaptive outward remodeling of the MCA tree as reflected in the larger arterial diameters (ie, D0 and D2), in strains with low collateral number and diameter. In other words, such “environmental” hemodynamic factors could change the value of a given metric, such that the metric will predict collateral extent because its value is affected, itself, by collateral extent. Irrespective of whether this speculation is true, our findings show that inherent genetic patterning differences in the retinal artery tree are stronger predictors, compared to the MCA tree, of genetic differences in collateral extent.

An intriguing question is why differences in branch-patterning in retinal artery trees so strongly predicts differences in collateral extent in the brain, when one tissue has collaterals (brain) while the other does not (retina). Before addressing this question, it should be noted that while most tissues have collaterals, several lack them, presumably because either they are supplied by a single artery and thus possess a single artery tree (eg, retina, kidney) or because their blood flow significantly exceeds the oxygen demand of their parenchymal cells thus eliminating the need for native collaterals to, as conjectured,⁴¹ optimize delivery of oxygen (discussed below) (eg, lung, kidney). Our finding that genetic-dependent differences in the patterning metrics in retina are shared in part in the arterial trees of another tissue, the MCA tree of the brain—and thus also closely correlate with collateral extent—allows us to propose a possible answer to this question. Formation of the retinal and neocortical vasculatures share many anatomic similarities during development and maturation.²⁵ In mouse, the MCA, ACA and posterior cerebral artery trees form from embryonic day (E) ~E9.5 to E16.5 (birth occurs at ~E19.5-21), while pial collaterals form from ~E15.5 to E18.5,^{16,17} and retinal arterial trees form from ~P5 to P12.^{35,36} In humans, there is a similar time-specific sequence, ie, the MCA tree is established by 9 weeks of gestation, pial collaterals by 14 weeks,^{37,38} and the retinal arterial trees begin formation at ~18 weeks.^{39,40} Accordingly, genetic variation in patterning of the MCA, collaterogenesis, and retinal trees are likely to be impacted similarly, and thus correlated, by developmental stage-specific differences in expression of vascular developmental genes possessing functional polymorphisms, eg, mouse *Dcel* and the genes that it potentially drives that control collaterogenesis.^{14,16-20} Of note, naturally occurring gene variants in some of these genes can be anticipated to have subtle effects on arterial branch-patterning in tissues, as shown herein, but large effects on the extent of the collateral circulation. This dichotomy is presumably due, on the one hand, to the evolution of redundant mechanisms that compensate for polymorphisms within genes in the pathway that drives development of the general arterial-venous circulation, as exemplified by the retinal and MCA trees, because a functional circulation is required early-on for embryogenesis to proceed. While on the other hand, in the case of the pathway that drives collaterogenesis (which presumably has some genes that overlap with the angiogenic pathway), little if any requirement and thus genetic selection pressure exists to have caused evolution of compensatory signaling mechanisms to

assure that an abundant collateral circulation forms.⁴¹ This is because possession of a poor collateral circulation does not have an effect on reproductive fitness, at least in healthy mice maintained in a vivarium.^{3,12}

Notwithstanding the above speculations, genetic differences in collateral extent in brain are mirrored in other tissues of the same mouse (eg, skeletal muscle and intestine).^{4,14,18-21,23} Thus, branch-patterning of the retinal arterial tree will likely also correlate with collateral extent in non-brain tissues. Whether genetic-dependent differences in arterial tree patterning in other tissues exist and link the same metrics seen in retina and the MCA tree to differences in collateral extent, will require additional study. However in support of this, arterial tree patterning in skeletal muscle is very different in C57BL/6 and BALB/cBy mice that have large differences in collateral extent (and that were studied herein).^{22,23}

Genetic background affects retinal tree structure in humans. In twin studies, estimates of heritability of CRAE, an indicator of retinal artery diameters, ranged from 56-70%,^{42,43} with lower values (20-21%) for sib-sib correlations in family-based studies.^{44,45} Consistent with this, we found that artery caliber showed the strongest genetic variation, up to 73%. In another twin study, heritability of arteriolar tortuosity was 82%.⁴⁶ Our finding that D2, D0, CRAE and tortuosity vary strongly with genetic background in mice support these findings, although tortuosity in retina did not emerge as a predictor of collateral extent in multivariate analysis but did so in the MCA. Several genetic loci have been associated with different retinal topologies. For example, a genome-wide SNP association study of more than 15,000 unrelated individuals identified 5 loci associated with a fraction, albeit small (1-3%), of the variation in retinal venular diameter.⁴⁷ One locus was associated with coronary artery disease and hypertension. Whether the other loci associate with additional “environmental” risk factors known to induce changes in venular diameter or other retinopathy signs, or instead link to intrinsic differences in retinal venular diameter, cannot be determined by such studies.

The present findings demonstrate that retinal patterning predicts stroke outcome in mice. We are unaware of previous studies in animals or humans that have examined whether severity of infarct volume in acute stroke or heart attack (ie, outcome rather than risk of occurrence) or tissue injury in chronic heart disease or PAD, which are all strongly impacted by known or presumed differences in native collateral extent,^{1-4,9-11} is associated with intrinsic differences in retinal patterning that are independent of retinopathy changes. The latter are known to occur with hypertension, diabetes and other cardiovascular risk factors and have also been linked to increased risk of small/large artery stroke, white matter disease, myocardial infarction, ischemic heart disease, PAD, and incidence of mortality.^{24-28,48-50} Doubal et al³³ reported that fractal dimension of the entire retinal circulation inversely correlated with age and incidence of lacunar stroke, after correcting for hypertension, diabetes, stroke severity and white matter hyperintensity. The authors suggested that a distinct vasculopathy that correlates with reduced retinal branching complexity (and/or vessel density) causes lacunar stroke. This was followed by speculation in an accompanying editorial that “loss of branching complexity of small vessels in the brain *leads to* the loss of collateral circulation..., which in turn increases the risk of lacunar infarcts.”⁵¹ Our finding that low retinal complexity correlates with/predicts low collateral extent supports this

speculation. However in our study, a difference in genetic background underlies (“leads to”) this association. Interestingly in regards to the above editorial comment, we have found that that “environmental factors”, eg, aging,⁵² endothelial dysfunction,²¹ and presence of other cardiovascular risk factors²³ cause loss of pial and skeletal muscle collaterals, reduced diameter of those that are still present, and increased infarct volume and hindlimb ischemia in mouse models of stroke and PAD. These findings have recently found support in patients with ischemic stroke.⁵³

Our study has several limitations. The correlations identified are dependent on the strains chosen. Mice were not randomly selected from a wild or wild-derived outbred population of mice, thus limiting the generalizability of our results to the ten strains studied. This was because the only practical way to test the hypothesis was in a cohort of inbred strains with known wide differences in collateral extent and infarct volume, and where multiple individuals with the same genetic background could be phenotyped to minimize measurement error and stochastic variation. In particular, the close relationship among the 4 strains on CD1 background may have led to an underestimate of the genetic contribution to each of the traits. A second limitation is that a better test of external validity would have been to take the most predictive metrics from the current study and test their performance on a new, independently obtained set of retinas belonging to the same strains. Because of fiscal exigencies, we instead used leave-one-out K-fold cross validation to reduce statistical bias and simulate such an external validation experiment.³² An even stronger validation for future studies would be to test the most predictive metrics on a subset of the Collaborative Cross, a genetic mapping population that captures more than 90% of the allelic variation in the mouse species.⁵⁴

An additional limitation is that we only studied healthy ~three month-old young adult mice (although note that they have comparable body weight and arterial pressure^{3,18-20,55}). However, in many patients where a collateral RPI would be of use, obesity, hypertension and other cardiovascular risk factors and disease (environmental factors) will often be present to potentially confound genetic determinants of retinal patterning and collateral extent. Aging, smoking, hypertension, basal metabolic rate and peripheral vascular disease have been associated with and presumed to cause changes in retinal artery patterning metrics.^{24-28,48-50} For example, aging and hypertension associate with narrowing of bifurcation angles and microvascular rarefaction.⁵⁶ Indeed, severity of these and other overt retinopathies have been used to predict extant end-organ damage and to monitor response to therapy in hypertension, diabetes, atherosclerosis and stroke.²⁸ Recent studies have shown that this genetic-environment interaction also extends to the collateral circulation. In mice, aging, hypertension, metabolic syndrome and endothelial dysfunction cause loss of collateral number and diameter in brain and skeletal muscle (collateral rarefaction) that is not seen in similarly-sized arterioles in the adjacent arterial trees.^{21,23,52} Supporting evidence has recently been reported in humans based on measures of collateral status in brain and heart.^{53,57} Thus, it will be important to investigate and adjust for such interactions in future animal and human studies aimed at extending the present findings. This should be possible since genetic background, at least in mice, has a stronger effect on collateral extent than environmental factors.^{3,4,12,14,16,18-23,52}

The translation of a retinal predictor index for use in patients with acute stroke will require rapid imaging and analysis. Fortunately, five out of seven and seven out of nine of the most informative predictive metrics for collateral number and diameter, respectively, were inner-zone metrics that do not require segmentation of the entire retinal artery trees. Moreover, these metrics have been obtained in many studies and large-population registries.²⁴⁻²⁸ However, whether these same metrics will be informative in humans requires confirmation. Recent development of fully automated software for quantification of the architecture of the retinal circulation and other two-dimensional vascular trees^{58,59} will greatly reduce the time required for the manual steps of image analysis used in the present study. Measurement of collateral status in brain requires neuroimaging^{1,9} and in heart and peripheral limb requires arterial catheterization.^{10,11} These requirements will present challenges in future studies seeking to extend the current approach to healthy humans. However, approximately 10% of patients presenting with angina have no detectable CAD or other cardiovascular disease on subsequent evaluation.¹⁰ Moreover, collateral status in the extremities of these “healthy” individuals, as well as those with CAD, can be readily obtained at the time of coronary evaluation.¹¹ Finally, if identification of the gene(s) responsible for variation in collateral extent in mice,¹⁴ can be confirmed in humans,¹⁵ this may permit combination of a rapid genetic test with a collateral retinal predictor index in prospective studies as well as in the analysis of retinal registries that integrate stroke, CAD or PAD with twin, family and population-based genetic data. Such studies could be aided if what has been found in mice^{3,4,14,16,18-21,23,52} is confirmed in human, ie, that collateral extent in one tissue is shared by other tissues of the same individual.

In conclusion, our findings in mice show that retinal arterial tree patterning varies with genetic background and predicts genetic-dependent differences in pial collateral number, diameter and infarct volume with high accuracy (80-90%). Our findings comparing retina and MCA trees suggest that the retina may be unique in the strength of its arterial geometry to predict variation in collaterals in brain and other tissues. If a similar retinal predictor index can be validated in humans, it may lead to development of a non-invasive, relatively inexpensive biomarker to aid existing neuroimaging and hemodynamic methods, as well as possible future genetic tests, in predicting variation in collateral extent. Such a multimodal screening would provide a means to predict the likelihood of severe tissue injury before obstructive disease develops. This knowledge could aid adoption of life-styles and treatments aimed at avoiding or reducing risk factors for obstructive disease. When obstruction does occur, combining a retinal predictor index with collateral scoring would improve the knowledge needed to guide treatment choice and tailor the time-window for recanalization therapy. With progression of time after onset of stroke, patients with poor collateral status are less likely to benefit from revascularization and more likely to develop intracranial hemorrhage after treatment.^{1,5-9} Thus, such tailoring seeks to identify patients with poor collaterals for exclusion of treatment, while providing an extended window to those with good collateral circulation.^{1,9} In addition, stratifying patients according to collateral extent would likely reduce the variability seen in past clinical studies. For example, recent findings suggest that variability in predicting functional outcome, based on NIHSS at baseline and early after stroke, can be significantly reduced by stratifying patients for collateral status.⁶⁰ Furthermore, in studies examining new treatments aimed at increasing

collateral flow, variability in efficacy would likely be reduced by stratifying patients for poor versus good collateral extent.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Stephen Lockett, director of the Optical Microscopy and Analysis Laboratory Leidos Biomedical Research, Inc, Frederick National Laboratory for Cancer Research for supporting Dr. Chen's contributions, Dr. Mary Hartnett and Nicarter Gordon for advice on retinal flat-mounting, Jonathan Alexander, Sadana Rangarao, Deepanshu Singh, and Dr. Siddharth Srivastava for assistance in image analysis, and Dr. Jennifer Lucitti for providing VEGFA^{hi/+}, VEGFA^{lo/+}, CD1 and CLIC4^{-/-} mice.

Sources of Funding: NIH grants HL090655, HL111070 and NS083633 (JEF), T35-DK007386 (PP). Dr. Chen's role in this project has been funded in part with federal funds from the National Cancer Institute, National Institutes of Health, under Contract No. HHSN261200800001E. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

References

1. Shuaib A, Butcher K, Mohammad AA, Saqqur M, Liebeskind DS. Collateral blood vessels in acute ischaemic stroke: a potential therapeutic target. *Lancet Neurol*. 2011; 10:909–921. [PubMed: 21939900]
2. Meier P, Hemingway H, Lansky AJ, Knapp G, Pitt B, Seiler C. The impact of the coronary collateral circulation on mortality: a meta-analysis. *Eur Heart J*. 2012; 33:614–621. [PubMed: 21969521]
3. Zhang H, Prabhakar P, Sealock RW, Faber JE. Wide genetic variation in the native pial collateral circulation is a major determinant of variation in severity of stroke. *J Cerebral Blood Flow Metab*. 2010; 30:923–934.
4. Chalothorn D, Faber JE. Strain-dependent variation in native collateral function in mouse hindlimb. *Physiol Genomics*. 2010; 42:469–479. [PubMed: 20551146]
5. Christoforidis GA, Karakasis C, Mohammad Y, Caragine LP, Yang M, Slivka AP. Predictors of hemorrhage following intra-arterial thrombolysis for acute ischemic stroke: the role of pial collateral formation. *AJNR Am J Neuroradiol*. 2009; 30:165–170. [PubMed: 18768718]
6. Miteff F, Levi CR, Bateman GA, Spratt N, McElduff P, Parsons MW. The independent predictive utility of computed tomography angiographic collateral status in acute ischaemic stroke. *Brain*. 2009; 132:2231–2238. [PubMed: 19509116]
7. Lima FO, Furie KL, Silva GS, Lev MH, Camargo EC, Singhal AB, Harris GJ, Halpern EF, Koroshetz WJ, Smith WS, Yoo AJ, Nogueira RG. The pattern of leptomeningeal collaterals on CT angiography is a strong predictor of long-term functional outcome in stroke patients with large vessel intracranial occlusion. *Stroke*. 2010; 41:2316–2322. [PubMed: 20829514]
8. Jung S, Gilgen M, Slotboom J, El-Koussy M, Zubler C, Kiefer C, Luedi R, Mono ML, Heldner MR, Weck A, Mordasini P, Schroth G, Mattle HP, Arnold M, Gralla J, Fischer U. Factors that determine penumbral tissue loss in acute ischaemic stroke. *Brain*. 2013; 136:3554–60. [PubMed: 24065722]
9. Nambiar V, Sohn SI, Almekhlafi MA, Chang HW, Mishra S, Qazi E, Eesa M, Demchuk AM, Goyal M, Hill MD, Menon BK. Collateral status and response to recanalization in patients with acute ischemic stroke. *AJNR Am J Neuroradiol*. 2014; 35:884–90. [PubMed: 24371030]
10. Meier P, Gloekler S, Zbinden R, Beckh S, de Marchi SF, Zbinden S, Wustmann K, Billinger M, Vogel R, Cook S, Wenaweser P, Togni M, Windecker S, Meier B, Seiler C. Beneficial effect of recruitable collaterals: a 10-year follow-up study in patients with stable coronary artery disease undergoing quantitative collateral measurements. *Circulation*. 2007; 116:975–83. [PubMed: 17679611]

11. Traupe T, Ortmann J, Stoller M, Baumgartner I, de Marchi SF, Seiler C. Direct quantitative assessment of the peripheral artery collateral circulation in patients undergoing angiography. *Circulation*. 2013; 128:737–744. [PubMed: 23817577]
12. Wang S, Zhang H, Wiltshire T, Sealock R, Faber JE. Genetic dissection of the Canq1 locus governing variation in extent of the collateral circulation. *PLoS One*. 2012; 7:31910.
13. Keum S, Marchuk DA. A locus mapping to mouse chromosome 7 determines infarct volume in a mouse model of ischemic stroke. *Circ Cardiovasc Genet*. 2009; 2:591–598. [PubMed: 20031639]
14. Sealock R, Zhang, Lucitti J, Moore S, Faber J. Congenic fine-mapping identifies a major causal locus for variation in the native collateral circulation and ischemic injury in brain and lower extremity. *Circ Res*. 2014; 114:660–71. [PubMed: 24300334]
15. Lee, Y.; Menon, B.; Huang, D.; Wilhelmsen, K.; Jovin, T.; Parsons, M.; Ribo, M.; Selim, M.; Sheth, K.; Al-Ali, F.; Marshall, R.; Shuaib, A.; Demchuk, A.; Powers, W.; Liebeskind, D.; Faber, J. GENetic Determinants of Collateral Status in Stroke - The GENEDCSS Study; AHA/ASA International Stroke Conference; 2014.
16. Chalothorn D, Faber JE. Formation and maturation of the murine native cerebral collateral circulation. *J Molec Cell Cardiol*. 2010; 49:251–259. [PubMed: 20346953]
17. Lucitti JL, Mackey J, Morrison JC, Haigh JJ, Adams RH, Faber JE. Formation of the collateral circulation is regulated by vascular endothelial growth factor-A and A Disintegrin and Metalloprotease Family Members 10 and 17. *Circ Res*. 2012; 111:1539–1550. [PubMed: 22965144]
18. Chalothorn D, Clayton JA, Zhang H, Pomp D, Faber JE. Collateral density, remodeling and VEGF-A expression differ widely between mouse strains. *Physiol Genomics*. 2007; 30:179–191. [PubMed: 17426116]
19. Clayton JA, Chalothorn D, Faber JE. Vascular endothelial growth factor-A specifies formation of native collaterals and regulates collateral growth in ischemia. *Circ Res*. 2008; 103:1027–1036. [PubMed: 18802023]
20. Chalothorn D, Zhang H, Smith JE, Edwards JC, Faber JE. Chloride intracellular channel-4 is a determinant of native collateral formation in skeletal muscle and brain. *Circ Res*. 2009; 105:89–98. [PubMed: 19478202]
21. Dai X, Faber JE. eNOS deficiency causes collateral vessel rarefaction and impairs activation of a cell cycle gene network during arteriogenesis. *Circ Res*. 2010; 106:1870–1881. [PubMed: 20431061]
22. MacGabhann F, Peirce SM. Collateral capillary arterialization following ligation in murine skeletal muscle. *Microcirc*. 2010; 17:333–47.
23. Moore SM, Waters MR, Faber JE. Hypertension and other cardiovascular risk factors lead to premature rarefaction of the native collateral circulation. *J Vasc Surg*. 2013; 57(5):79S.
24. Liew G, Wang JJ, Mitchell P, Wong TY. Retinal vascular imaging. *Circulation: Cardiovascular Imaging*. 2008; 1:156–161. [PubMed: 19808533]
25. Patton N, Aslam T, MacGillivray T, Pattie A, Deary IJ, Dhillon B. Retinal vascular image analysis as a potential screening tool for cerebrovascular disease: A rationale based on homology between cerebral and retinal microvasculatures. *J Anat*. 2005; 206:319–348. [PubMed: 15817102]
26. Witt N, Wong TY, Hughes AD, Chaturvedi N, Klein BE, Evans R, McNamara M, Thom SA, Klein R. Abnormalities of retinal microvascular structure and risk of mortality from ischemic heart disease and stroke. *Hypertension*. 2006; 47:975–81. [PubMed: 16585415]
27. Patton N, Aslam TM, MacGillivray T, Deary IJ, Dhillon B, Eikelboom RH, Yogesan K, Constable IJ. Retinal image analysis: Concepts, applications and potential. *Prog Retin Eye Res*. 2006; 25:99–127. [PubMed: 16154379]
28. Hughes AD, Wong TY, Witt N, Evans R, Thom SA, Klein BE, Chaturvedi N, Klein R. Determinants of retinal microvascular architecture in normal subjects. *Microcirculation*. 2009; 16:159–66. [PubMed: 19206002]
29. Witt NW, Chapman N, Thom SA, Stanton AV, Parker KH, Hughes AD. A novel measure to characterize optimality of diameter relationships at retinal vascular bifurcations. *Artery Res*. 2010; 4:75–80. [PubMed: 21072124]

30. Knudtson MD, Lee KE, Hubbard LD, Wong TY, Klein R, Klein BE. Revised formulas for summarizing retinal vessel diameters. *Curr Eye Res.* 2003; 27:143–149. [PubMed: 14562179]
31. Arganda-Carreras I, Fernandez-Gonzalez R, Munoz-Barrutia A, Ortiz-De-Solorzano C. 3D reconstruction of histological sections: Application to mammary gland tissue. *Microscopy Res and Tech.* 2010; 73:1019–1029.
32. Steyerberg, E. *Clinical Prediction Models.* Vol. Chapter 17. Springer Science+Business Media, LLC; 2009. Validation of prediction models; p. 299–311.
33. Doubal FN, MacGillivray TJ, Patton N, Dhillon B, Dennis MS, Wardlaw JM. Fractal analysis of retinal vessels suggests that a distinct vasculopathy causes lacunar stroke. *Neurology.* 2010; 74:1102–7. [PubMed: 20368631]
34. Cristofaro B, Shi Y, Faria M, Suchting S, Leroyer AS, Trindade A, Duarte A, Zovein AC, Iruela-Arispe ML, Nih LR, Kubis N, Henrion D, Loufrani L, Todiras M, Schleifenbaum J, Gollasch M, Zhuang ZW, Simons M, Eichmann A, le Noble F. Dll4-Notch signaling determines the formation of native arterial collateral networks and arterial function in mouse ischemia models. *Development.* 2013; 140:1720–1729. [PubMed: 23533173]
35. Saint-Geniez M, D'Amore PA. Development and pathology of the hyaloid, choroidal and retinal vasculature. *Int J Dev Biol.* 2004; 48:1045–1058. [PubMed: 15558494]
36. Fruttiger M. Development of the retinal vasculature. *Angiogenesis.* 2007; 10:77–88. [PubMed: 17322966]
37. Gielecki J, Zurada A, Kozłowska H, Nowak D, Loukas M. Morphometric and volumetric analysis of the middle cerebral artery in human fetuses. *Acta Neurobiol Exp (Wars).* 2009; 69:129–37. [PubMed: 19325646]
38. Okudera T, Ohta T, Huang YP, Yokota A. Developmental and radiological anatomy of the superficial cerebral convexity vessels in the human fetus. *J Neuroradiol.* 1988; 15:205–24. [PubMed: 3246599]
39. Provis JM. Development of the primate retinal vasculature. *Prog Ret Eye Res.* 2001; 20:799–821.
40. Fielder, AR.; Quinn, GE. Retinopathy of prematurity (chapter 51). In: Taylor, D.; Hoyt, CS., editors. *Pediatric Ophthalmology and Strabismus.* 3rd. Elsevier Saunders; Edinburgh: 2005. p. 506–530.
41. Faber, JE.; Dai, X.; Lucitti, J. Genetic and environmental mechanisms controlling formation and maintenance of the native collateral circulation. In: Deindl, IE.; Schaper, W., editors. *Arteriogenesis – Molecular Regulation, Pathophysiology and Therapeutics.* Vol. Ch 1. Shaker Verlag; Aachen, GE: 2011. p. 1–22.
42. Fahy SJ, Sun C, Zhu G, Healey PR, Spector TD, Martin NG, Mitchell P, Wong TY, Mackey DA, Hammond CJ, Andrew T. The relationship between retinal arteriolar and venular calibers is genetically mediated, and each is associated with risk of cardiovascular disease. *Invest Ophthalmol Vis Sci.* 2011; 52:975–81. [PubMed: 20926817]
43. Taarnhoj NC, Larsen M, Sander B, Kyvik KO, Kessel L, Hougaard JL, Sørensen TI. Heritability of retinal vessel diameters and blood pressure: A twin study. *Invest Ophthalmol Vis Sci.* 2006; 47:3539–3544.
44. Lee KE, Klein BE, Klein R, Knudtson MD. Familial aggregation of retinal vessel caliber in the beaver dam eye study. *Invest Ophthalmol Vis Sci.* 2004; 45:3929–33. [PubMed: 15505038]
45. Liu YP, Kuznetsova T, Jin Y, Thijs L, Asayama K, Gu YM, Bochud M, Verhamme P, Struijker-Boudier HA, Staessen JA. Heritability of the retinal microcirculation in Flemish families. *Am J Hypertens.* 2013; 26:392–9. [PubMed: 23382490]
46. Taarnhoj NC, Munch IC, Sander B, Kessel L, Hougaard JL, Kyvik K, Sørensen TI, Larsen M. Straight versus tortuous retinal arteries in relation to blood pressure and genetics. *Br J Ophthalmol.* 2008; 92:1055–60. [PubMed: 18653600]
47. Ikram MK, Sim X, Jensen RA, Cotch MF, Hewitt AW, Ikram MA, Wang JJ, Klein R, Klein BE, Breteler MM, Cheung N, Liew G, Mitchell P, Uitterlinden AG, Rivadeneira F, Hofman A, de Jong PT, van Duijn CM, Kao L, Cheng CY, Smith AV, Glazer NL, Lumley T, McKnight B, Psaty BM, Jonasson F, Eiriksdottir G, Aspelund T, Harris TB, Launer LJ, Taylor KD, Li X, Iyengar SK, Xi Q, Sivakumaran TA, Mackey DA, Macgregor S, Martin NG, Young TL, Bis JC, Wiggins KL, Heckbert SR, Hammond CJ, Andrew T, Fahy S, Attia J, Holliday EG, Scott RJ, Islam FM, Rotter

- Jl, McAuley AK, Boerwinkle E, Tai ES, Gudnason V, Siscovick DS, Vingerling JR, Wong TY. Global BPgen Consortium. Four novel Loci (19q13, 6q24, 12q24, and 5q14) influence the microcirculation in vivo. *PLoS Genet.* 2010; 6:e1001184. [PubMed: 21060863]
48. Wong TY, Klein R, Couper DJ, Cooper LS, Shahar E, Hubbard LD, Wofford MR, Sharrett AR. Retinal microvascular abnormalities and incident stroke: The atherosclerosis risk in communities study. *The Lancet.* 2001; 358(9288):1134–1140.
49. Doulal FN, Hokke PE, Wardlaw JM. Retinal microvascular abnormalities and stroke: a systematic review. *J Neurol Neurosurg Psychiatry.* 2009; 80:158–65. [PubMed: 18931002]
50. Lindley RI, Wang JJ, Wong M, Mitchell P, Liew G, Hand P, Wardlaw J, De Silva DA, Baker M, Rochtchina E, Chen C, Hankey GJ, Chang HM, Fung VS, Gomes L, Wong TY. Retinal microvasculature in acute lacunar stroke: A cross-sectional study. *The Lancet Neurology.* 2009; 8:628–634. [PubMed: 19481977]
51. Kwa VI, Lopez OL. Fractal analysis of retinal vessels: peeping at the tree of life? *Neurology.* 2010; 74:1088–9. [PubMed: 20368629]
52. Faber JE, Zhang H, Lassance-Soares RM, Prabhakar P, Najafi AH, Burnett MS, Epstein SE. Aging causes collateral rarefaction and increased severity of ischemic injury in multiple tissues. *Arterioscler Thromb Vasc Biol.* 2011; 31:1748–1756. [PubMed: 21617137]
53. Menon BK, Smith EE, Coutts SB, Welsh DG, Faber JE, Damani Z, Goyal M, Hill MD, Demchuk AM, Hee Cho KH, Chang HW, Hong JH, Sohn SI. Leptomeningeal collaterals are associated with modifiable metabolic risk factors. *Ann Neurol.* 2013; 74:241–8. [PubMed: 23536377]
54. Threadgill DW, Miller DR, Churchill GA, de Villena FP. The collaborative cross: a recombinant inbred mouse population for the systems genetic era. *ILAR J.* 2011; 52:24–31. [PubMed: 21411855]
55. Smolock EM, Ilyushkina IA, Ghazalpour A, Gerloff J, Murashev AN, Lusis AJ, Korshunov VA. Genetic locus on mouse chromosome 7 controls elevated heart rate. *Physiol Genomics.* 2012; 44:689–98. [PubMed: 22589454]
56. Stanton AV, Wasan B, Cerutti A, Ford S, Marsh R, Sever PP, Thom SA, Hughes AD. Vascular network changes in the retina with age and hypertension. *J Hypertens.* 1995; 13:1724–8. [PubMed: 8903640]
57. de Marchi SF, Gloekler S, Meier P, Traupe T, Steck H, Cook S, Vogel R, Seiler C. Determinants of preformed collateral vessels in the human heart without coronary artery disease. *Cardiology.* 2011; 118:198–206. [PubMed: 21701169]
58. Vickerman MB, Keith PA, McKay TL, Gedeon DJ, Watanabe M, Montano M, Karunamuni G, Kaiser PK, Sears JE, Ebrahim Q, Ribita D, Hylton AG, Parsons-Wingerter P. VESGEN 2D: automated, user-interactive software for quantification and mapping of angiogenic and lymphangiogenic trees and networks. *Anat Rec (Hoboken).* 2009; 292:320–32. [PubMed: 19248164]
59. Cheung CY, Hsu W, Lee ML, Wang JJ, Mitchell P, Lau QP, Hamzah H, Ho M, Wong TY. A new method to measure peripheral retinal vascular caliber over an extended area. *Microcirculation.* 2010; 17:495–503. [PubMed: 21040115]
60. Calleja AI, Cortijo E, García-Bermejo P, Gómez RD, Pérez-Fernández S, Del Monte JM, Muñoz MF, Fernández-Herranz R, Arenillas JF. Collateral circulation on perfusion-computed tomography-source images predicts the response to stroke intravenous thrombolysis. *Eur J Neurol.* 2013; 20:795–802. [PubMed: 23278976]
61. Wang S, Zhang H, Dai X, Sealock R, Faber JE. Genetic architecture underlying variation in extent and remodeling of the collateral circulation. *Circ Res.* 2010; 107:558–68. [PubMed: 20576932]

Non-standard abbreviations and acronyms

A	artery
ACA	anterior cerebral artery
AIC	Akaike's Information Criterion

AVR	artery-to-vein diameter ratio
B6	the C57BL/6 inbred mouse strain
BIC	Bayesian Information Criterion
CAD	Coronary artery disease
CFI	Collateral flow index
COL	collateral
COL-D	average diameter of all pial collaterals between the MCA and ACA trees
COL-N	the number of pial collaterals between the MCA and ACA trees
CRAE	central retinal artery equivalent
CRVE	central retinal vein equivalent
E1	embryonic day 1(2,3...etc)
FD	fractal dimension
IZ	inner zone (of the retina)
IQR	interquartile range
Lac	lacunarity
MCA	middle cerebral artery
MCAO	MCA occlusion
MV	marginal vein of the retina
OD	optic disk
OZ	outer zone (of the retina)
P1	postnatal day 1(2,3...etc)
PAD	peripheral artery disease
PBS	phosphate buffered saline
PFA	paraformaldehyde
RM	regression modeling (stepwise multivariate)
ROI	region of interest
RPI_n or d	retinal predictor index for collateral number or diameter
RPM	retinal patterning metric
V	vein

Figure 1. Collateral number and diameter vary widely with genetic background and are a major determinant of the accompanying wide variation in infarct volume (ANOVA, $p < 0.001$, n = number of 3-4 month-old male mice of each strain--given below columns). Representative images of neocortex of 2 strains of mice with high and low collateral number and diameter and infarct volume 24 hours after permanent middle cerebral artery ligation. Values for infarct volume for DBA/2 and AKR, CD-1, VEGFA^{hi/+}, VEGFA^{lo/+} and CLIC4^{-/-} were reported previously.^{3,19,20}

Figure 2. Measurement and definitions of retinal patterning metrics (RPM) numbers 1 to 10: retinal area, vessel diameter (D_0 , D_1 , D_2), optimality, branch angle, tortuosity index (inner zone), central retinal artery equivalent (CRAE), central retinal vein equivalent (CRVE), and artery-to-vein ratio (AVR)

Image of flat-mounted, stained (Alexafluor 568 GS-IB4) retina is imported into Photoshop CS4 (panel A). A circle centered on the optic disc and positioned over the outer zone-margin of the retina (OZ) is drawn so its circumference is tangential to the marginal veins (MV) along a majority of the flaps. The optic disc margin (OD) and inner-zone margins (IZ) are drawn at $1/10^{\text{th}}$ and $5/10^{\text{th}}$ of the OZ diameter. The borders of the retina are traced to measure the retinal area (RPM 10). All remaining manually obtained RPMs (numbers 1-4, 8, 9) are measured in the region lying between the OD and IZ from 4 randomly chosen arterial trees (yellow dashed boxes). To calculate inner-zone tortuosity index (RPM 4), a line (l) is traced on each tree along the vessel axis from the OD to IZ margin and a vector line (L) is drawn connecting the endpoints (B). A circle with a 25-pixel radius is manually placed encircling each branch point along the tree between OD and IZ. The center of B_c is approximately placed at the point of intersection (P) of the vessel axes of the Parent and Larger branch. Vessel diameters (Parent- D_0 , Smaller branch- D_1 , Larger branch- D_2) are measured perpendicular to the axis and used to calculate the optimality ratio; (RPM 9) branch angle is the angle of the rays bisecting the diameters (C). The above methods ensure consistent measurement of RPMs. All numbers obtained from the 4 trees are averaged to obtain a mean of each RPM for each mouse. CRAE and CRVE are measured using the revised Parr-Hubbard formulas^{29,30} and combined to obtain AVR. Using ImageJ, vessel calibers of retinal arterioles and venules were measured between 0.5 and 1 optic disc diameters from the OD and summarized into the CRAE and CRVE (D). Six of the largest arterioles and six of the largest venules are identified, and using an iterative procedure, the largest and smallest calibers are paired and entered into their respective formulas to obtain 3 vessel calibers (E). This procedure is repeated to obtain 2 and finally 1 central vessel equivalent, ie, the CRAE and CRVE and AVR (RPMs 5-7).^{30,31}

Figure 3. Measurement and definitions of semi-automatically obtained retinal patterning metrics (RPMs) numbers 11 to 22

Each artery tree was binarized using ImageJ (A) and skeletonized using the Analyze Skeleton plugin (B).³¹ Each tree is composed of branch segments defined as the length of tree lying between two branch points (B). Functions within ImageJ are used to obtain RPMs 13 and 14 from the binarized image, while the Skeletonize plugin is used to calculate scalar length (ℓ), vector length (L), and tortuosity of each branch segment (B). Distributions (average, skew, and kurtosis) of these values are calculated using Microsoft Excel to obtain RPMs 17-22 for each arterial tree (C, D). A MATLAB program is used to calculate fractal dimension and lacunarity using the sliding box-count method (E). All numbers obtained from the 2-3 randomly chosen trees (Online Figure 1) are averaged to obtain a mean of each RPM for each mouse.

Figure 4. Summary of study design and statistical and outlier analysis

Collateral number and diameter (COL-N and COL-D) and 22 RPMs were obtained from 81 mice ((panel A) and Figures 1-3, Online Table 1, Online Figure 1). A preliminary multivariate outlier analysis of all observations found one mouse with significantly high Mahalanobis, jackknife, and T^2 distances ($n=1$) (A, B). Bivariate regression (A) of COL-N, COL-D, and RPMs was performed on the remaining 80 mice to determine degree and significance of covariance and correlation with genetic strain (Figure 5). Forward, backward, and mixed-direction stepwise multivariate regression modeling of RPMs versus COL-N and COL-D was performed using three different criteria—minimum AIC and BIC, and p-value cutoffs—to obtain 7 different models (RM1) ($n=80$) (Online Figure 7). A K-fold R^2 value for each model was obtained using leave-one-out cross-validation to assess the average predictive performance of the modeling process for each of the 7 models repeated n-times (correlation between actual and predicted COL-N or COL-D for a given model developed on $n-1$ observations) (Online Figure 7). Observations with significantly high Cook's D influence averaged across RM1 of COL-N and -D, COL-N, or COL-D models were identified (C) and excluded to repeat the aforementioned modeling process (RM2 and RM3) ((A) and Online Figure 7). The most predictive models for COL-N and COL-D (ie, combination of RPMs) among RM1, RM2, and RM3 were used to obtain a retinal predictive index $-RPI_N$ and RPI_D , respectively (Figure 6). Performance of the average RPI_N and RPI_D for each strain in predicting historic average infarct volume for each strain was tested (Figure 8). In the outlier box plots (B and C), the vertical line within the box represents the mean; top and bottom points of diamond represent the 95% confidence interval of the mean; the ends of the box represent the 25th and 75th quantiles (ie, 1st and 3rd quartile (Q)) and span the interquartile range (IQR) of the data; the box-lines that extend from the top and bottom end of the box (whiskers) represent the 1st Q - $1.5 \cdot (IQR)$ and 3rd Q + $1.5 \cdot (IQR)$, respectively. The bracket outside identifies the most dense 50% of the observations.

Figure 5. Retinal patterning metrics (RPMs) vary with genetic background and correlate with collateral number (COL-N), diameter (COL-D) and other RPMs

Bivariate regression (one-way ANOVA adjusted R^2 and p-value) of COL-N, COL-D and RPMs versus mouse strain (panel A) show that COL-N, COL-D and 10 out of 22 RPMs strongly vary with genetic background (black columns, relative strength of correlation; red dashed line, adjusted R^2 of > 0.35 , $p < 0.0001$). These data suggest that, similar to its contribution to the variation in COL-N and COL-D, genetic background also plays a significant role in specifying variation in features of retinal vascular patterning, such as vessel caliber, branch angle, etc. Multivariate correlation matrices (B and C) of COL-N, COL-D and RPMs show significant covariance; matrices show direction and strength ($+/-$ adjusted R^2 (B)), and significance (p-value, (C)) of covariance. Highlighted regions of matrices (B,C; yellow boxes) show that a number of RPMs (1-3,5,6,10,11,13-15,17,20) also vary strongly with COL-N and COL-D, suggesting that they may also predict collateral extent. Fractal dimension and lacunarity (RPMs 11 and 12), global descriptors of pattern complexity, both correlate with other RPMs and are inversely related to each other (B,C; orange boxes).

Figure 6. Among the most predictive RPMs, average diameter of larger branch vessel at a arterial tree bifurcation along the length of the retinal arteries emanating from the optic disc (D2) contributes the most, statistically, to predicting collateral number and diameter (COL-N and COL-D)

To compare the relative predictive power of the RPMs, parameter estimates from the 2 most predictive models for RPI_n and RPI_d (Online Figure 7) were obtained and plotted as scaled estimates (ie centered by mean and normalized to have identical range) (A,C); in addition, the parameter estimates were standardized to have equal variances, orthogonalized to be uncorrelated, and plotted—in descending order of scaled estimates—as a pareto plot (B,D). The scaled estimates show the relative extent of change in COL-N or COL-D as a specific RPM is varied from the lowest to highest value in the population of mice. The pareto plot accounts for covariance and extent of variability of an RPM in the mouse population to estimate and arrange the RPMs in descending order of relative “explanatory power.” Plots of predicted COL-N and COL-D versus RPI_n and RPI_d , along with K-fold R^2 reveals the spread of data and the strength of correlation (E,F). **** $P<0.0001$.

Figure 7. Comparison of retinal and pial images of a VEGF^{hi/+} and BALB/c mouse with wide differences in number of MCA-to-ACA collaterals (COL-N)—27 vs. 0 collaterals, respectively “Branch points”, magnified images of selected branch points (5 small boxes in retina). “Trees”, regions of retinal arterial trees (3 large boxes in retina). Values of predictive retinal metrics for the two mice are shown for comparison. Consistent with the retinal prediction modeling (Figure 6), the mouse with a high COL-N (27) has a higher RPI_n (25.6), larger branch vessel caliber (D2), branch angles at branch points, retinal area, and CRAE, but lower lacunarity, shorter branch segments, and lower optimality (ie, more equitable distribution of vessel calibers of daughter vessels at bifurcations). However, contrary to expectations, this mouse had a larger kurtosis of distribution of branch segment lengths (ie, higher proportion of branch segments with similar lengths) and larger parent vessel caliber (D0) attributable to both biological variance and experimental error.

Figure 8. Average retinal predictor index for collateral number and diameter (RPI_n and RPI_d) predict average infarct volume following middle cerebral artery occlusion (MCAO) in 10 mouse strains

Average strain RPI_n and RPI_d predict pial collateral number (COL-N) and (COL-D), respectively (A), which in turn predict average MCAO infarct volume for strains (B, Figure 1). Accordingly, RPI_n and RPI_d predict average infarct volume after MCAO (C). This indicates that retinal patterning, which predicts COL-N and COL-D, is a strong non-invasive biomarker of infarct volume in ischemic stroke in mouse. K-fold R^2 values obtained by leave-one-out cross-validation and p-value (p-value***, ****<0.001, 0.0001) show strength and significance of correlation between actual and predicted parameters. ***, ****P<0.001, 0.0001.