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Age-Related Reductions in Retinal Vessel Density Partly Reflect Vessel Calibre: A Two-Centre Fundus Imaging Study

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Abstract—Purpose: To examine relationships among age, retinal vessel calibre, and vessel density, and determine whether arterial and venous calibre statistically account for part of the association between age and corresponding vessel density. Materials and Methods: This retrospective cross-sectional study included 384 eligible right-eye colour fundus image records from two clinical centres. Retinal vascular measurements were obtained using AutoMorph. Composite scores were derived for arterial and venous calibre and vessel density using measurements from the full analysed field and standard retinal zones. A parallel partial-mediation model evaluated arterial and venous pathways simultaneously, adjusting for sex, centre, and age-by-centre interaction. Indirect effects were assessed using 2,000 centre-stratified bootstrap resamples. Results: Mean age was 40.54 ± 16.13 years; 159 records (41.4%) were male. Older age was associated with smaller arterial ($\beta = -0.531$) and venous calibre ($\beta = -0.413$; both $P < 0.001$). Larger calibre was associated with greater corresponding vessel density (arterial $\beta = 0.267$; venous $\beta = 0.356$; both $P < 0.001$). Age remained directly associated with lower arterial ($\beta = -0.211$) and venous density ($\beta = -0.241$; both $P < 0.001$). Indirect effects through arterial and venous calibre were -0.142 (95% CI, -0.183 to -0.100) and -0.147 (95% CI, -0.191 to -0.105), accounting statistically for 40.2% and 38.0% of the respective total associations. Conclusions: Retinal vessel calibre accounted statistically for approximately two-fifths of the association between older age and lower arterial and venous vessel density, while substantial direct age associations remained. These findings demonstrate that age-related reductions in area-based retinal vessel density cannot be interpreted as vascular rarefaction alone, because vessel width contributes directly to the segmented vascular area. The indirect effects therefore represent statistical decomposition rather than evidence of causal mediation. Joint assessment of vessel calibre, density, and vascular complexity may provide a more informative characterisation of retinal vascular ageing.

Keywords—ageing, fundus photography; retinal vessel calibre; vessel density

I. INTRODUCTION

Ageing is accompanied by gradual changes in the structure and function of the microcirculation. These changes are difficult to study directly in most organs, but the retinal vasculature can be photographed non-invasively and quantified in considerable detail. Retinal photographs therefore offer a

practical window into vascular ageing and, more broadly, biological ageing. This concept is supported by studies showing that age-related information extracted from retinal photographs is associated with subsequent risks of major morbidity and mortality [1].

Several features of the retinal vascular network change with increasing age [2]. Retinal arterioles and venules generally become narrower, as reflected by reductions in the central retinal artery equivalent and central retinal vein equivalent [2,3]. These calibre changes have also been associated with measures of early vascular ageing, including arterial stiffness and carotid vascular structure [4]. Although the strength of the association varies across populations and measurement systems, the overall evidence suggests that narrowing of both retinal arterioles and venules is a reproducible feature of ageing [2–4].

Age-related change is not limited to vessel calibre. Studies using segmented fundus photographs have reported reductions in retinal vessel density or vessel area with increasing age [3,5,6,7]. Retinal vascular complexity, commonly quantified using fractal dimension, also tends to decline with age [3,8–10]. In healthy eyes, average vessel diameter, vessel area rate, and fractal dimension have been shown to decrease in parallel across age groups, suggesting that ageing affects several related aspects of the retinal vascular network [3]. Vessel density and fractal dimension are strongly correlated, although they represent different properties of the vascular image [7].

The importance of distinguishing these related vascular traits extends beyond descriptive retinal ageing. Retinal vascular tortuosity has been associated with blood pressure and cardiovascular risk factors [11], while quantitative retinal vascular measurements may vary depending on fundus camera systems and image resolution [12]. Furthermore, the interpretation of relationships among these vascular traits requires methodological caution, particularly when cross-sectional mediation analyses are used, as indirect effects may be subject to temporal bias and should not be interpreted as evidence of causality [13]. Retinal calibre has also been linked to systemic cardiovascular risk factors and preclinical large-artery function, and meta-analytic evidence supports associations between retinal vascular phenotypes and cardiovascular disease [14–16].

What remains less clear is how these age-related vascular changes relate to one another. Vessel calibre and area-based vessel density are often examined as separate outcomes, yet they are not completely independent. Area-based density is calculated from the proportion of segmented image pixels occupied by vessels. It is therefore influenced by both the extent of the visible vascular network and the width of the vessels contributing to that network. A narrower vessel occupies fewer pixels even when the vessel remains present. Consequently, part of the observed association between age and lower vessel density may reflect age-related narrowing of the retinal vessels rather than the disappearance of vascular branches alone.

Automated retinal image-analysis systems now make it possible to examine these relationships using multiple vascular measurements from the same fundus photograph. AutoMorph combines image-quality assessment, vessel and artery–vein segmentation, optic-disc analysis, and quantitative measurement of retinal vascular morphology [6]. Its outputs include regional and whole-image measurements of vessel width, central retinal artery and vein equivalents, vessel density, fractal dimension, and tortuosity. Combining related measurements into domain-specific composite scores may provide more stable representations of arterial calibre, venous

calibre, and their corresponding vessel densities than relying on a single regional measurement.

Previous studies have established that older age is associated with narrower retinal vessels and lower retinal vessel density [2,3,5,7]. However, it remains uncertain whether arterial and venous calibre statistically account for part of the corresponding age–density associations. Clarifying this relationship is important because a reduction in area-based vessel density may represent a combination of vessel narrowing and broader vascular rarefaction.

The present study therefore examined the relationships among age, retinal vessel calibre, and vessel density using AutoMorph-derived measurements from two clinic-based cohorts. Arterial and venous pathways were evaluated simultaneously using an observed-composite parallel partial-mediation path model. We hypothesised that older age would be associated with smaller arterial and venous calibre and lower corresponding vessel density, and that calibre would statistically account for part, but not all, of the age–density association. Importantly, the analysis was intended to decompose contemporaneous associations rather than establish a causal sequence. By quantifying the shared and residual components of the age–density relationship, we aimed to clarify how area-based vessel density should be interpreted in retinal ageing research.

II. METHODS

A. Study Design and Participants

This cross-sectional study used de-identified colour fundus photographs from two clinic-based cohorts. The IIUM cohort consisted of images obtained at the IIUM Optometry Clinic using a Nidek AFC-230/210 non-mydratic fundus camera. The tertiary hospital cohort consisted of images acquired at the tertiary hospital Ophthalmology Clinic using a Topcon TRC-NW7SF Mark II camera during routine health screening between 1 January and 31 December 2025. The study was conducted in accordance with the Declaration of Helsinki and received ethical approval under IREC-2025-53 and IREC 2024-261. The requirement for informed consent for this secondary analysis was waived because the datasets were de-identified.

Participant metadata were linked to AutoMorph outputs using image filenames. AutoMorph quality filtering defined the eligible image set. Records without a matching metadata entry were excluded. All retained images had been clinically reviewed and were free of identifiable retinal disease. Only right-eye fundus photographs were included in the analytical sample; no left-eye images were analysed.

B. Retinal Vascular Measures and Composite Scores

AutoMorph provided arterial and venous measurements from the full analysed field and from zones B and C. Four primary analysis-derived composites were constructed: arterial calibre, venous calibre, arterial vessel density, and venous vessel density.

The arterial calibre composite included arterial average width from the full field, zone B, and zone C, together with Knudtson central retinal artery equivalent values from zones B and C. The venous calibre composite was constructed in the

same way using venous average width and Knudtson central retinal vein equivalent. Arterial and venous density composites each included the corresponding full-field, zone B, and zone C density measurements.

Secondary composites were created for arterial and venous fractal dimension, arterial and venous distance tortuosity, cup-to-disc ratio, and arteriovenous ratio. Hubbard calibre estimates were excluded from the primary composites because they were nearly redundant with the corresponding Knudtson estimates. Arteriovenous ratio was not entered into the primary mediation model because it is algebraically derived from arterial and venous calibre.

C. Data Preparation

Implausible arteriovenous-ratio values of ≤ 0.20 or > 1.50 were treated as missing. Missing indicator values were replaced by the median of the corresponding variable within each centre. Distance-tortuosity measures were log-transformed before standardisation.

Raw absolute calibre values differed substantially between centres, reflecting camera and image-scale differences. Each absolute calibre indicator was therefore standardised within centre before the composite score was calculated. Scale-invariant indicators were standardised across the pooled sample. Each composite was formed by averaging its standardised indicators and then standardising the resulting score.

Age was standardised across the pooled sample; one standard deviation corresponded to approximately 16.13 years. Sex was coded as male = 1 and female = 0. Centre was coded as IIUM = 0 and tertiary hospital = 1. Sex and centre were mean-centred, and an age-by-centre interaction term was calculated.

D. Statistical Analysis

The primary analysis used an observed-composite parallel partial-mediation path model. Two pathways were estimated simultaneously: age $\rightarrow$ arterial calibre $\rightarrow$ arterial vessel density and age $\rightarrow$ venous calibre $\rightarrow$ venous vessel density. Direct paths from age to arterial and venous density were retained, allowing assessment of partial mediation. Sex, centre, and age by centre were included as covariates. Residual correlations were allowed between arterial and venous calibre and between arterial and venous density.

Standardised coefficients were reported with robust sandwich standard errors. Model fit was assessed using the chi-square test, comparative fit index (CFI), Tucker–Lewis index (TLI), goodness-of-fit index, adjusted goodness-of-fit index, normed fit index, and root-mean-square error of approximation (RMSEA). The same model was fitted separately within each cohort, with adjustment for sex. As no fellow-eye observations were included, there was no inter-eye correlation from bilateral sampling requiring a clustering adjustment; robust sandwich standard errors were not used to address such inter-eye dependence.

Indirect effects were evaluated in the pooled sample using 2,000 nonparametric bootstrap resamples stratified by centre. Percentile-based 95% confidence intervals were calculated for the indirect effects and proportions mediated. Total age effects on arterial and venous density were estimated using ordinary least-squares regression with HC3 robust standard errors.

Because calibre and density were measured contemporaneously from the same fundus image, mediation terminology is used in the statistical sense: the indirect path represents decomposition of an association and is not interpreted as evidence of temporal or causal mediation.

Secondary outcomes were analysed in separate adjusted regression models. Benjamini–Hochberg false-discovery-rate correction was applied to the secondary age and age-by-centre tests. Sensitivity analyses repeated the mediation model using average-width-only and Knudtson-only calibre composites. All tests were two-sided, with $P < 0.05$ considered statistically significant.

Analyses were performed in Python using pandas, NumPy, semopy, statsmodels, and SciPy.

III. RESULTS

A. Study Sample

The analysis included 384 right-eye retinal image records: 205 from IIUM and 179 from the tertiary hospital. Mean age was 40.54 ± 16.13 years, and 159 records (41.4%) were from male participants. The tertiary hospital cohort was older and had higher raw arterial and venous width values than the IIUM cohort (Table I). The indicators within each primary composite showed strong within-domain coherence, with Cronbach's α ranging from 0.880 to 0.958.

B. Model Fits and Primary Pathways

The pooled model showed close fit: $\chi^2(12) = 4.98$, $P = 0.959$, CFI = 1.000, TLI = 1.000, and RMSEA = 0.000. The cohort-specific models also met conventional fit criteria (Table II).

Older age was associated with smaller arterial calibre ($\beta = -0.531$, $P < 0.001$) and smaller venous calibre ($\beta = -0.413$, $P < 0.001$). Larger arterial calibre was associated with greater arterial density ($\beta = 0.267$, $P < 0.001$), while larger venous calibre was associated with greater venous density ($\beta = 0.356$, $P < 0.001$). Age remained directly associated with lower arterial density ($\beta = -0.211$, $P < 0.001$) and lower venous density ($\beta = -0.241$, $P < 0.001$), indicating partial mediation. The direction of all primary paths was consistent in both cohorts (Table III).

The total adjusted age effect was -0.349 for arterial density and -0.387 for venous density, both $P < 0.001$. The pooled models explained 56.6% of the variance in arterial density and 36.3% of the variance in venous density.

C. Model Fits and Primary Pathways

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TABLE I. CHARACTERISTICS OF THE ANALYTICAL SAMPLE AND PRIMARY COMPOSITE INDICATORS

A. Cohort characteristics

Characteristic	Combined	IIUM	Tertiary hospital
Retinal image records, n	384	205	179
Age, years, mean $\pm$ SD	40.54 $\pm$ 16.13	34.94 $\pm$ 14.41	46.94 $\pm$ 15.64
Age range, years	7–75	10–73	7–75
Male, n (%)	159 (41.4)	92 (44.9)	67 (37.4)
Raw arterial average width, mean	139.22	121.84	159.14
Raw venous average width, mean	155.58	130.76	184.00

B. Within-domain coherence of the primary composite indicators

Composite	Included indicators, n	Cronbach's α	Mean inter-item correlation
Arterial calibre	5	0.921	0.699
Venous calibre	5	0.880	0.594
Arterial vessel density	3	0.958	0.883
Venous vessel density	3	0.918	0.789

NOTE: Raw average-width values are presented only to demonstrate the center-associated measurement offset. Absolute calibre indicators were standardised within center before composite construction. The α values describe coherence among the AutoMorph-derived indicators included within each analysis-generated composite; they do not represent agreement between imaging systems or repeated measurements.

The total adjusted age effect was -0.349 for arterial density and -0.387 for venous density, both $P < 0.001$. The pooled models explained 56.6% of the variance in arterial density and 36.3% of the variance in venous density.

D. Model Fits and Primary Pathways

The pooled model showed close fit: $\chi^2(12) = 4.98$, $P = 0.959$, CFI = 1.000, TLI = 1.000, and RMSEA = 0.000. The cohort-specific models also met conventional fit criteria (Table II).

Older age was associated with smaller arterial calibre ($\beta = -0.531$, $P < 0.001$) and smaller venous calibre ($\beta = -0.413$, $P < 0.001$). Larger arterial calibre was associated with greater arterial density ($\beta = 0.267$, $P < 0.001$), while larger venous calibre was associated with greater venous density ($\beta = 0.356$, $P < 0.001$). Age remained directly associated with lower arterial density ($\beta = -0.211$, $P < 0.001$) and lower venous density ($\beta = -0.241$, $P < 0.001$), indicating partial mediation. The direction of all primary paths was consistent in both cohorts (Table III).

The total adjusted age effect was -0.349 for arterial density and -0.387 for venous density, both $P < 0.001$. The pooled models explained 56.6% of the variance in arterial density and 36.3% of the variance in venous density.

E. Indirect Effects and Centre Differences

All 2,000 bootstrap models converged. The arterial indirect effect was -0.142 (95% confidence interval [CI], -0.183 to -0.100 ; $P < 0.001$), corresponding to 40.2% of the total age–arterial density association. The venous indirect effect was -0.147 (95% CI, -0.191 to -0.105 ; $P < 0.001$), corresponding to 38.0% of the total age–venous density association (Table IV).

The estimated proportions mediated were 28.6% and 29.2% for the arterial and venous pathways in IIUM, compared with 58.1% and 45.5% in the tertiary hospital. These cohort-specific values were descriptive because bootstrap confidence intervals were generated only for the pooled model.

The arterial calibre-to-density association was stronger in tertiary hospital than in IIUM (interaction $\beta = 0.187$, 95% CI, 0.037 – 0.337 ; $P = 0.015$). The corresponding venous interaction was not significant ($P = 0.377$).

TABLE III. FIT INDICES FOR THE PARALLEL PARTIAL-MEDIATION MODELS

Fit index	Combined	IIUM	Tertiary hospital
Sample size	384	205	179
χ^2 (degrees of freedom)	4.98 (12)	0.59 (5)	8.93 (5)
Chi-square P value	0.959	0.989	0.112
Comparative fit index	1.000	1.000	0.989
Tucker–Lewis index	1.000	1.000	0.964
Goodness-of-fit index	0.996	0.999	0.977
Adjusted goodness-of-fit index	0.990	0.996	0.921
Normed fit index	0.996	0.999	0.977
Root-mean-square error of approximation	0.000	0.000	0.066

NOTE: Comparative fit index and Tucker–Lewis index estimates slightly exceeding 1.00 were bounded at 1.00 for presentation.

TABLE III. STANDARDISED ESTIMATES FOR THE PRIMARY RETINAL VASCULAR PATHWAYS

Pathway	Combined β (robust SE)	P	IIUM β (robust SE)	P	Tertiary hospital β (robust SE)	P
Age $\rightarrow$ arterial calibre	-0.531 (0.049)	<0.001	-0.515 (0.072)	<0.001	-0.468 (0.066)	<0.001
Arterial calibre $\rightarrow$ arterial density	0.267 (0.029)	<0.001	0.277 (0.034)	<0.001	0.465 (0.043)	<0.001
Age $\rightarrow$ arterial density, direct	-0.211 (0.037)	<0.001	-0.356 (0.052)	<0.001	-0.157 (0.047)	0.014
Age $\rightarrow$ venous calibre	-0.413 (0.053)	<0.001	-0.397 (0.072)	<0.001	-0.372 (0.078)	<0.001
Venous calibre $\rightarrow$ venous density	0.356 (0.035)	<0.001	0.311 (0.042)	<0.001	0.492 (0.046)	<0.001
Age $\rightarrow$ venous density, direct	-0.241 (0.040)	<0.001	-0.300 (0.063)	<0.001	-0.219 (0.047)	<0.001

NOTE: All estimates are standardised coefficients. The pooled model was adjusted for sex, center, and age by center. The cohort-specific models were adjusted for sex.

TABLE IV. BOOTSTRAP DECOMPOSITION OF THE POOLED AGE-VESSEL DENSITY ASSOCIATIONS

Pathway	Direct effect	Indirect effect	Bootstrap 95% CI for indirect effect	Total effect	Proportion mediated, %	Bootstrap 95% CI, %	P for indirect effect
Arterial	-0.211	-0.142	-0.183 to -0.100	-0.352	40.2	28.6–54.4	<0.001
Venous	-0.241	-0.147	-0.191 to -0.105	-0.388	38.0	27.7–49.5	<0.001

NOTE: Indirect effect = age-to-calibre coefficient $\times$ calibre-to-density coefficient. Confidence intervals were obtained from 2,000 nonparametric bootstrap samples stratified by center. Mediation is interpreted statistically because the study was cross-sectional.

F. Secondary and Sensitivity Analyses

Older age was associated with lower arterial fractal dimension ($\beta = -0.271$), lower venous fractal dimension ($\beta = -0.252$), and lower arteriovenous ratio ($\beta = -0.220$); all false-discovery-rate-adjusted P values were <0.001. No overall age association was found for arterial tortuosity, venous tortuosity, or cup-to-disc ratio. The age-by-centre interaction was significant only for venous tortuosity (adjusted P = 0.043).

The primary findings remained directionally unchanged when calibre was reconstructed using average-width-only or Knudtson-only measurements. Indirect effects ranged from -0.082 to -0.178 for the arterial pathway and from -0.088 to -0.163 for the venous pathway.

IV. DISCUSSION

A. Principal Findings and Study Contribution

The principal finding is that retinal vessel calibre accounted statistically for a substantial, but incomplete, component of the inverse association between age and area-based retinal vessel density. In the pooled analysis, the indirect component through arterial calibre represented 40.2% of the total age–arterial density association, while the corresponding venous component represented 38.0%. At the same time, age retained significant direct associations with both arterial and venous density after calibre was included. The data therefore do not support a simple interpretation in which lower density with increasing age is explained only by narrower vessels, nor do they support interpreting the entire density reduction as loss of

vascular branches. Instead, the results indicate that age-related variation in an area-based density metric contains at least two distinguishable components: one that covaries with vessel width and another that remains after width-related variation is considered.

This distinction is the main contribution of the study. Previous fundus-photography studies have generally described age associations with calibre, vessel area or density, and fractal dimension as separate vascular phenotypes [2–5, 7–10]. The present analysis brings arterial calibre and arterial density, and venous calibre and venous density, into the same statistical model. Doing so changes the interpretation of vessel density. A lower density value is not a unitary phenotype; it can arise because the visible vessels occupy less area as they become narrower, because fewer or shorter vessel segments are represented, because branching complexity is reduced, or because some combination of these processes occurs. The partial rather than complete statistical mediation observed here is consistent with that multi-component interpretation.

The approximately 40% proportions should nevertheless not be read as fixed biological fractions of an ageing mechanism. They are model-dependent quantities derived from the particular definitions of calibre and density used here. This caution is reinforced by the sensitivity analyses: the direction of the indirect effects was stable when calibre was reconstructed using average-width-only or Knudtson-only measures, but the magnitude of the indirect effects varied. Thus, the qualitative inference that calibre contributes materially to the age–density association was robust, whereas the exact numerical proportion depended on how calibre was

operationalised. For biomarker development, this difference between directional robustness and magnitude robustness is important.

B. Age-Related Calibre Change in the Context of Vascular Ageing

The inverse associations between age and both arterial and venous calibre are consistent with a broad retinal vascular literature. Streese et al. reported progressive reductions in central retinal arteriolar equivalent and central retinal venular equivalent across adulthood [2], while Feng et al. found lower average vessel diameter in older age groups using automated macular vascular analysis [3]. Gómez-Sánchez et al. linked smaller arteriolar calibre and a lower arteriovenous index with age and early vascular ageing phenotypes [4]. Earlier synthesis by Sun et al. similarly identified older age as a consistent correlate of narrower retinal arterioles and venules. It emphasised that calibre also reflects blood pressure, metabolic factors, inflammation, smoking, and endothelial function [14]; systematic and meta-analytic evidence further links retinal microvascular calibre with systemic vascular health. Narrower retinal arteriolar calibre has been associated with faster pulse-wave velocity and greater carotid intima-media thickness [15]. At the same time, retinal vascular calibre has also been associated with cardiovascular outcomes including coronary artery disease, stroke, heart failure, and mortality [16]. A recent comprehensive review further highlighted associations between retinal vascular calibre and a broad range of systemic conditions, including metabolic, cardiovascular, and cerebrovascular diseases, supporting its potential relevance as a non-invasive marker of systemic vascular health [17].

The present results therefore fit a model in which retinal calibre is both age-sensitive and systemically informative. Still, they cannot identify the biological process responsible for the observed narrowing. Vascular ageing involves endothelial dysfunction, altered vasomotor regulation, arterial stiffness, remodelling, and cumulative exposure to cardiovascular risk factors. Each of these processes may influence the retinal circulation, and several were not measured in the present datasets. The stronger age association for arterial than venous calibre is compatible with the established sensitivity of arteriolar calibre to blood pressure and resistance-vessel physiology, but this study did not measure blood pressure or haemodynamic function. It therefore cannot attribute the arterial finding to those mechanisms. Furthermore, quantitative retinal imaging research has demonstrated the value of distinguishing vessel area density, skeleton density, and diameter-related indices when characterising vascular morphology [18]. Although these measures were developed using optical coherence tomography angiography, the underlying distinction is relevant to fundus photography, where vessel calibre can influence area-based density independently of changes in the extent of the visible vascular network.

Longitudinal evidence is particularly relevant because it separates within-person change from cross-sectional cohort differences. In a healthy working population, Büttner et al. observed small but significant reductions in both CRAE and CRVE over a three-year interval, alongside cross-sectional associations with cardiovascular risk factors [19]. Such findings support the plausibility of progressive calibre change over time, while also illustrating why a cross-sectional age

coefficient should not be equated with an individual ageing trajectory. The current data show an age gradient across participants; longitudinal imaging is needed to determine how much of that gradient reflects true within-eye vascular change.

C. Why Area-Based Vessel Density Is Mechanically Linked to Vessel Width

The interpretation of the indirect pathway depends critically on how vessel density is defined. AutoMorph calculates vessel density from the proportion of the analysed image area occupied by segmented vessel pixels [6]. For a simplified vascular mask containing vessel segments with lengths L and widths w , the occupied vascular area can be approximated by the sum of $L \times w$ across segments, with adjustments for branch overlaps and local geometry. Dividing this occupied area by the retinal region of interest produces an area fraction. Consequently, even if the centreline length, number of branches, and topology of the network remain unchanged, a reduction in average width will reduce the number of vessel pixels and therefore lower area-based density. This is a geometric property of the metric, not necessarily evidence of disappearance of vessels.

This point explains why the statistical mediation is scientifically informative but should not be interpreted as a mechanistic statement that narrowing causes rarefaction. Calibre and density were extracted from the same segmented vascular representation. Their association therefore combines at least two sources of covariance: biological covariance, because ageing may alter both vessel width and vascular architecture, and mathematical or measurement covariance. After all, width directly contributes to the area occupied by the segmentation. Shared segmentation errors may add a third source. For example, reduced contrast or lower resolution can make a vessel appear both thinner and less extensive, strengthening the apparent calibre-to-density relationship even without an equivalent biological change.

A useful methodological comparison comes from quantitative OCT angiography, where vessel area density and skeletonised vessel length density are explicitly distinguished. Skeletonisation reduces each vessel to a one-pixel-wide centreline, so a length-based density is much less dependent on vessel diameter than an area-based measure; vessel area divided by skeleton length can also be used as a diameter-related index [18]. The imaging modality in the present study is colour fundus photography rather than OCT angiography, so these metrics are not directly interchangeable. Nevertheless, the underlying image-analysis principle is general. Future fundus studies could supplement area-based density with skeletonised vessel length, branch count, endpoint count, or other width-insensitive network measures to determine how much of the age association persists when vessel width is mathematically removed from the density definition.

D. Residual Age Effects and Retinal Vascular Complexity

The significant direct age effects after adjustment for calibre suggest that width-related occupancy did not explain the entire age–density association. Approximately three-fifths of the pooled total association remained outside the estimated indirect component. In a cross-sectional model this residual should not be treated as a single biological pathway, but it is consistent with the possibility that other structural features of the vascular

network vary with age. Potential contributors include total centreline length, the visibility of fine vessels, branch and endpoint counts, branching geometry, and the spatial distribution of vessels within the analysed field.

The secondary fractal-dimension findings provide complementary evidence. Older age was associated with lower arterial and venous fractal dimension after false-discovery-rate correction. Fractal dimension characterises aspects of branching complexity rather than vessel width alone, and population studies have repeatedly reported lower retinal vascular fractal dimension with increasing age [8–10]. Lemmens et al. further showed that the age–fractal relationship can be nonlinear across a wide lifespan [9]. Because vessel density and fractal dimension are correlated but not interchangeable [7], the combination of lower density and lower fractal dimension in the present study is compatible with age-related changes in network organisation beyond simple calibre narrowing.

Even here, the term rarefaction should be used cautiously. Fundus photographs visualise the retinal vascular tree only above the limits imposed by optics, contrast, image resolution, and the segmentation algorithm. A reduction in the number of detected fine vessels could represent biological loss, reduced visibility of vessels that remain anatomically present, or both. The present analysis cannot separate anatomic rarefaction from detectability. This is another reason why combining complementary vascular measures is preferable to assigning a single biological meaning to one image-derived density value.

The absence of a clear overall age association with distance tortuosity also deserves context. Previous results are inconsistent: Cheung et al. reported lower arteriolar and venular tortuosity with older age [11], whereas Feng et al. did not observe a significant age relationship for macular vessel tortuosity [3]. Tortuosity is especially sensitive to vessel selection, region of interest, formula, image resolution, and segmentation smoothness. The significant age-by-centre interaction for venous tortuosity in the current dataset therefore appears more consistent with a technically and population-sensitive phenotype than with a universally monotonic marker of ageing.

E. Arterial–Venous Differences and Between-Centre Heterogeneity

Separating arterial and venous networks was important because the two vascular compartments did not behave identically. Age showed a stronger association with arterial than venous calibre, whereas the calibre-to-density coefficient was numerically stronger for the venous pathway in the pooled model. These patterns are plausible because arterioles and venules are exposed to different haemodynamic and metabolic influences, and the systemic correlates of CRAE and CRVE are known to differ [14]. They also caution against collapsing all vessels into a single average-width or total-density measure. Indeed, the total average-width sensitivity model produced a much smaller indirect component than the artery- and vein-specific models, suggesting that combining vessel types can obscure opposing or unequal relationships.

The between-centre findings require equally careful interpretation. Raw average arterial width was 121.84 in the IIUM cohort and 159.14 in the tertiary-hospital cohort, while raw venous width was 130.76 and 184.00, respectively. These

large offsets are unlikely to represent biological differences alone and are consistent with differences in camera magnification, image geometry, pixel scale, resolution, and processing. Cunnac et al. demonstrated experimentally that retinal vascular measurements can differ materially across fundus-camera systems and image resolutions even when the underlying eye is unchanged [12]. Within-centre standardisation in the present analysis was therefore appropriate for constructing relative calibre composites, but standardisation can remove a location and scale offset without making two imaging systems biologically equivalent.

The stronger arterial calibre-to-density association in the tertiary-hospital cohort may have several explanations. First, the cameras and clinical centres were completely confounded, so a centre interaction is simultaneously a camera-system interaction. Second, the tertiary-hospital cohort was older on average, and a linear age-by-centre term may not capture nonlinear age-related differences in the calibre–density relationship. Third, the clinical settings may differ in unmeasured cardiovascular risk, refractive status, media clarity, and other participant characteristics. Fourth, image-specific factors such as field geometry, illumination, compression, and segmentation performance may alter the covariance between width and area occupancy. Because the present design cannot distinguish these possibilities, the interaction should be treated as evidence of heterogeneity that needs calibration rather than as a biological centre effect.

For multicentre retinal biomarker research, this has practical consequences. Harmonisation should ideally begin before statistical modelling, using standardised acquisition protocols, explicit scale calibration, common image-quality thresholds, and, where possible, same-eye cross-device datasets. Statistical standardisation remains useful, but it cannot substitute for direct assessment of inter-camera agreement. A future multicentre validation in which the same eyes are imaged on both camera systems would allow the technical component of the centre difference to be separated from cohort composition.

F. Clinical and Research Implications

The immediate implication is interpretive rather than diagnostic. An area-based retinal vessel density value should not be described automatically as the amount of vasculature present or as direct evidence of vessel loss. In an area-occupancy metric, vessel width is part of the signal. A lower density can therefore arise from narrower vessels even when the underlying centreline network is unchanged. Conversely, two eyes with similar area density could achieve that value through different combinations of vessel width and network length. This ambiguity is clinically important if retinal density is proposed as a marker of microvascular rarefaction, cardiovascular ageing, or systemic disease burden.

A more informative reporting framework would pair at least three classes of measurements: calibre, area-based density, and network complexity or length. Arterial and venous measures should be reported separately where the segmentation permits. If the research question concerns the area occupied by perfused or visible vessels, area density is a relevant outcome, but its width sensitivity should be acknowledged. If the question concerns rarefaction or the presence of vascular paths independent of width, a skeletonised length metric is conceptually preferable. Fractal dimension, branch statistics, or

graph-based measures can add information about network organisation. The objective is not to identify one universally superior metric, but to align the metric with the biological construct being claimed.

This multi-metric approach may also improve retinal oculomics. Retinal vascular calibre has established associations with cardiovascular risk and disease [14–17], while deep-learning retinal age markers demonstrate that fundus photographs contain distributed information about biological ageing and future health outcomes [1,20]. Interpretable vascular phenotypes could complement such high-dimensional models by identifying which visible vascular components contribute to risk. However, the present results show that even apparently intuitive metrics such as vessel density may contain built-in geometric dependencies. Interpretability therefore requires attention not only to statistical associations but also to the mathematical definition of each imaging feature.

The current findings are not sufficient to establish clinical thresholds or to recommend vessel density as a stand-alone screening test. The cohorts were clinic-based, important cardiovascular and ocular covariates were unavailable, and the measurements were standardised rather than expressed on a common calibrated physical scale. The main clinical message is therefore one of measurement interpretation: retinal vascular phenotypes should be viewed as a profile rather than isolated numbers, especially when they are derived from the same segmentation.

G. Strengths and Limitations

Several design features strengthen the analysis. Arterial and venous pathways were estimated simultaneously rather than in separate unrelated models. Multiple measurements from the full analysed field and standard retinal zones were combined into domain-specific composites, reducing reliance on any single regional value. The primary composites showed strong within-domain coherence. Robust sandwich standard errors and centre-stratified bootstrap resampling were used; all bootstrap models converged, and the principal direction of the indirect effects persisted when alternative calibre definitions were used. The inclusion of two centres also exposed an important problem, inter-camera and inter-cohort heterogeneity, which would have been invisible in a single-camera dataset.

The limitations, however, define the boundaries of interpretation. First, this was a cross-sectional analysis. Chronological age necessarily precedes the retinal image, but calibre and density were measured at the same time and from the same segmentation. The temporal ordering required for causal mediation between calibre and density therefore was not observed. Cross-sectional indirect effects can be biased when they are interpreted as longitudinal processes, and the direction or magnitude of mediation may differ when variables are measured over time [13]. The terms indirect effect and proportion mediated in this manuscript should consequently be read as statistical decomposition, not causal attribution.

Second, calibre and area-based density are mathematically coupled. This is not merely an unmeasured confounder that can be adjusted away; it is partly built into the feature definitions. The common source image and segmentation may further induce correlated measurement error. This limitation is central to the paper rather than incidental, because it explains why the indirect pathway is expected to be non-zero even if the

biological network length were unchanged. The sensitivity of the indirect magnitude to the calibre composite reinforces this point.

Third, the centre was fully linked to the camera system and clinical setting. Within-centre standardisation controlled the large raw scale offset in absolute calibre but could not remove differences in resolution, field geometry, image processing, or cohort composition. Fourth, potentially important covariates, including blood pressure, diabetes status, smoking, refractive error, axial length, and lens opacity, were unavailable or not incorporated. These variables may influence calibre, vessel visibility, or both, and residual confounding is therefore likely. Axial length and refractive error are particularly relevant to image scale and retinal magnification when absolute geometric measures are compared.

Fifth, the sample spanned 7 to 75 years. The estimated age coefficient therefore represents a cross-sectional age gradient across a broad life-course range rather than senescent ageing alone. Developmental vascular changes in younger participants may contribute to the fitted linear relationship, and the modest number of older participants limits inference specifically about late-life vascular ageing. Age was modelled linearly in the primary analysis, although prior work suggests that retinal vascular complexity can change nonlinearly across the lifespan [8,9]. Future analyses with larger samples should use spline or other flexible age functions and report age-stratified results.

Finally, automated quantitative imaging is constrained by image quality and segmentation performance even after quality filtering. The study included clinically reviewed images without identifiable retinal disease, which improves internal interpretability but limits generalisability to eyes with retinal pathology, media opacity, or more challenging image quality. The strong Cronbach alpha values for the composites demonstrate coherence among included indicators; they should not be interpreted as validation of biological construct equivalence, inter-camera agreement, or repeatability over time.

H. Future Directions

The next step should be longitudinal, within-eye imaging. Repeated fundus photographs obtained with a stable acquisition protocol would allow investigators to ask whether change in calibre precedes or accompanies change in area-based density and whether baseline calibre predicts subsequent density decline. A stronger mediation design would temporally separate the proposed mediator and outcome, for example, modelling baseline-to-follow-up calibre change in relation to later change in density, while adjusting for baseline levels and cardiovascular risk factors. Such a design would not remove the geometric dependence of area density on width, but it would distinguish cross-sectional covariance from true within-eye change.

Future studies should also measure width-dependent and width-independent vascular features in parallel. In addition to conventional calibre and area density, candidate outcomes include skeletonised vessel length density, total centreline length, branch and endpoint counts, fractal dimension, and graph-based topology. Comparing age associations across these metrics would help determine whether the residual age effect observed here corresponds to genuine reduction in network extent or complexity. Simultaneous estimation of a vessel-diameter index and centreline-density metric could further

separate changes in width from changes in network length, following the general quantitative framework used in angiographic image analysis [18].

Multimodal imaging could then connect visible structural geometry with perfusion and tissue-level consequences. Colour fundus photography provides scalable vascular morphology, OCT angiography provides depth-resolved flow-related vascular maps, and structural OCT can quantify retinal and choroidal tissue changes. Combining these modalities with systemic measurements such as blood pressure, arterial stiffness, glycaemic status, lipid profile, smoking exposure, and renal function would allow retinal vascular ageing to be studied as part of a broader cardiovascular phenotype. Recent retinal-age models illustrate the value of longitudinal and high-dimensional fundus information [20], but mechanistic interpretation will benefit from pairing those models with transparent vascular features.

Finally, external validation should address device and population transportability explicitly. Same-eye cross-camera calibration, harmonised acquisition, prospective quality control, and independent cohorts spanning different ethnicities and cardiovascular-risk profiles are needed before numerical density or calibre thresholds can be generalised. The present two-centre heterogeneity should therefore be viewed not only as a limitation but also as a guide to the design of the next generation of retinal vascular biomarker studies.

V. CONCLUSION

In conclusion, older age was associated with smaller arterial and venous calibre and lower corresponding area-based vessel density. Calibre accounted statistically for a substantial but incomplete part of both age–density associations. In contrast, significant residual age effects and lower fractal dimensions indicated that the observed age gradient extended beyond vessel width alone. The indirect component should not be interpreted as proof that narrowing causes vascular rarefaction, because area-based density is mathematically dependent on vessel width and both measurements were obtained from the same segmentation. These findings support joint interpretation of calibre, area density, and width-independent measures of vascular complexity or length, with separate consideration of arterial and venous networks. Longitudinal, multimodal, and cross-device calibrated studies are needed to determine which components represent true within-eye vascular ageing and which arise from image geometry or measurement system effects.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

DATA ACCESS STATEMENT

The datasets generated during the current study are available from the corresponding author upon reasonable request and subject to ethics committee approval.

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DECLARATION OF AI-ASSISTED TOOLS USAGE

We used an AI-assisted language tool (ChatGPT 5.6) to refine grammar, wording, and overall clarity of the manuscript. All authors reviewed and edited the AI-assisted text and take full responsibility for the accuracy, integrity, and validity of the content, including all reported results and interpretations.

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