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RESEARCH ARTICLE

Retinal microvascular complexity as a putative biomarker of biological age: a pilot study

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Abstract Physiological changes associated with aging increase the risk for the development of age-related diseases. This increase is non-specific to the type of age-related disease, although each disease develops through a unique pathophysiologic mechanism. People who age at a faster rate develop age-related diseases earlier in their life. They have an older "biological age" compared to their "chronological age". Early detection of individuals with accelerated aging would allow timely intervention to postpone the onset of age-related diseases. This would increase their life expectancy and their length of good quality life. The goal of this study was to investigate whether retinal microvascular complexity could be used as a biomarker of biological age. Retinal images of 68 participants ages ranging from 19 to 82 years were collected in an observational cross-sectional study. Twenty of the old participants had age-related diseases such as hypertension, type 2 diabetes, and/or Alzheimer's dementia. The rest of the participants were healthy. Retinal images were captured by a hand-held, non-mydriatic fundus camera and quantification of the microvascular complexity was performed by using Sholl's, box-counting fractal, and lacunarity analysis. In the healthy subjects, increasing chronological age was associated with lower retinal microvascular complexity measured by Sholl's analysis. Decreased box-counting fractal dimension was present in old patients, and this decrease was 2.1 times faster in participants who had age-related diseases ($p=0.047$). Retinal microvascular complexity could be a promising new biomarker of biological

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age. The data from this study is the first of this kind collected in Montenegro. It is freely available for use.

Keywords Retinal microvascular complexity · Biological age · Chronological age · Fractal dimension · Sholl's analysis · Age-related diseases

Introduction

Life expectancy is increasing globally, regardless of the socioeconomic development of the region (Roser et al. 2023). The incidence of age-related diseases is also expected to increase, therefore health care systems will need to adapt accordingly.

Common molecular processes play important role in both aging and age-related diseases (Franceschi et al. 2018). The process of aging creates a permissive environment for development of age-related diseases and usually precedes their onset. Although the aging process itself makes the individual more prone to develop one or more age-related diseases, each one of the age-related diseases may be the result of a unique pathophysiologic mechanism. People who age at a faster rate develop these diseases earlier in life and have a shorter life expectancy. These people have an older "biological age" compared to their "chronological age" (Franceschi et al. 2018).

There is a need for the development of biomarkers of biological age, which would detect people with an increased risk for developing age-related diseases. Early detection of these individuals, in the preclinical phase with no overt signs of age-related diseases would enable us to intervene more effectively and postpone the onset of age-related diseases in general, instead treating each individual disease in the phase of its evident manifestation. This would not only increase their life expectancy, but would also increase their length of good quality life. For now, those interventions are mostly limited to adopting a healthy lifestyle such as decreased caloric intake, intermittent fasting, increased proportion of fish, fruits, and vegetables in the diet, and increased physical activity (American Diabetes Association Professional Practice Committee 2021; Johnson et al. 2022; Longo et al. 2015). Identification of medications that could decrease the rate of aging would make these interventions even more effective, and some preliminary studies examining this theory show promising results (Johnson et al. 2022; Longo et al. 2015; McIntyre et al. 2021; Fahy et al. 2019; Mohammed et al. 2021).

The American Federation for Aging Research defined five essential characteristics of a good biomarker of biological age. Such biomarker has to measure a parameter that changes in correlation with the chronological age. It has to be able to predict the rate of aging better than the chronological age and to detect people with accelerated aging. The observed changes should not represent specific effects of a disease. This method of measurement has to be non-invasive, easy to use and affordable, not requiring excessive specialized training. Finally, the method must be applicable across the species (Austad et al. 2023).

Some of the most prominent and the most studied biomarkers of aging are Horvath's and Hanum's clock, which are methods based on quantification of DNA methylation patterns in blood or other tissues (Horvath and Raj 2018). This analysis has already been proposed as a tool for determination of the age of the tissue donor in forensic medicine (Vidaki et al. 2013), but its frequent population-wide application for determination of biological age is still not feasible. This method can also be used in chimpanzees, but not in any other species (Horvath and Raj 2018).

There is increasing evidence that retinal microvascular complexity is decreased with age (Azemin et al. 2012; Orlov et al. 2019), and that it also decreases non-specifically in many age-related diseases such as hypertension (HTN), type 2 diabetes (DM2) (Azemin et al. 2012), and Alzheimer's dementia (AD) (Cheung et al. 2012, 2014; Cabrera DeBuc et al 2020). Decrease in microvascular complexity is more pronounced in individuals with AD compared to age-matched healthy counterparts (Cabrera DeBuc et al 2020), suggesting that microvascular complexity could potentially be used to identify individuals with accelerated aging. The goal of the present study was to investigate whether retinal microvascular complexity could be used as a biomarker of biological age. To test this, we used retinal images of 68 participants with ages ranging from 19 to 82 years, in which 20 of the old participants had HTN, DM2, and/or AD. Retinal images were captured by a hand-held, non-mydriatic fundus camera and microvascular complexity was measured using Sholl's, box-counting fractal, and lacunarity analysis. This type of camera is inexpensive, easy to use, and it does not require expensive

training. It visualizes small arteries and veins, as well as the first three generations of arterioles and venules which belong to retinal microcirculation. Other methods such as optical coherence tomography angiography and fluorescent angiography could be used for very detailed visualization of vascular and even neural tissue in retina, but they require the use of non-portable, expensive sophisticated equipment, extensive specialist training, so their use would not fit the requirements for a good biomarker of aging.

Materials and methods

Patient recruitment and study design

This is a pilot observational cross-sectional study that examines differences in retinal microvascular complexity among three groups of patients: young healthy (YH), old with no chronic disease (O_NCD), and old with one or more chronic diseases (O_CD). These chronic diseases included HTN, DM2, and/or AD. The sample size for this study was empirically derived based on previously published studies also examining retinal microvascular complexity with similar sample size. Our group published a study in 2019, which compared two groups of retinal images-72 from young participants and 10 from old participants, and confirmed a significant difference in microvascular complexity measured by box-counting fractal dimension (Db) (Popovic et al. 2021). In addition, a study by Cabrera DeBuc et al. examined retinal images from 69 old patients, of which 32 had cognitive impairment, and demonstrated that although the two groups were not different in age, there was a significant difference in Db of retinal microvasculature between the groups (Cabrera DeBuc et al. 2018). The numbers of patients screened and enrolled in the present study are shown in Fig. 1.

Finally, sixty-eight participants were enrolled in this study and divided into 3 groups: YH (n = 40), O_NCD (n = 8), and O_CD (n = 20). The comorbidity profiles in O_CD group of patients are shown in the Fig. S1.

For the 40 young participants (YH group) recruitment and participation in the study was approved by the Ethical Committee of the Faculty of Medicine of the University of Montenegro

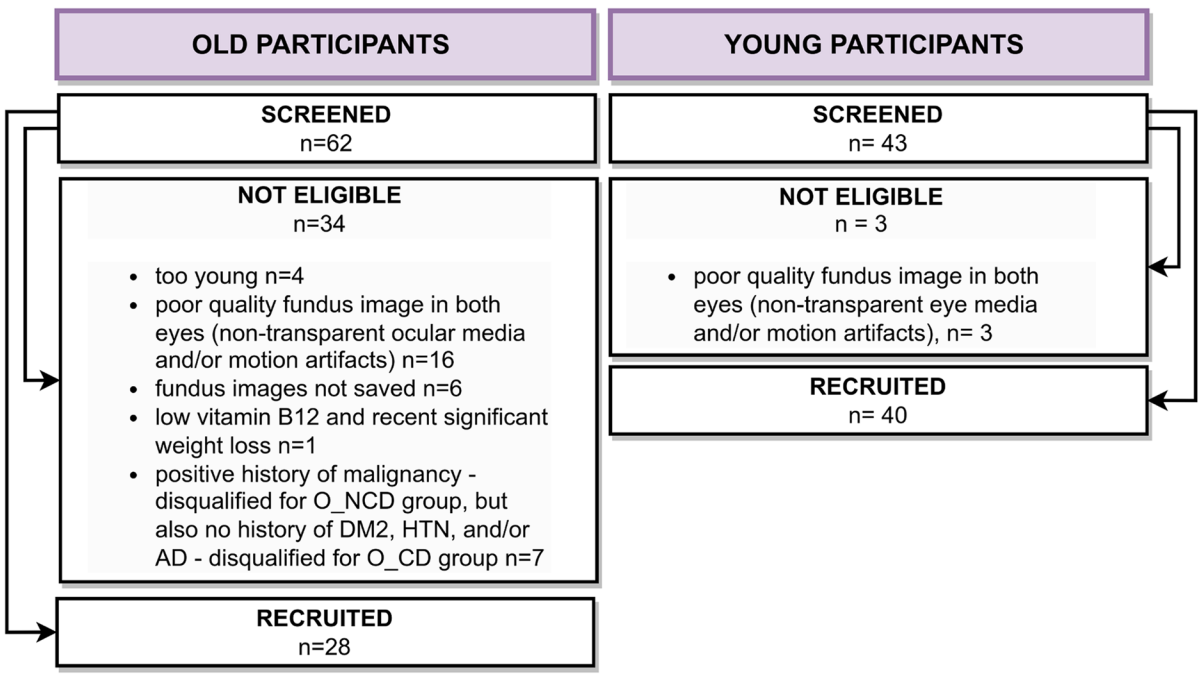

Fig.1 The numbers of patients screened and enrolled Abbreviations: YH- young healthy, O_NCD- old with no chronic disease, O_CD- old with chronic disease, AD- Alzheimer's dementia, HTN- hypertension, DM2- diabetes mellitus type 2

(Protocol No. 2487/4), as previously described (Popovic et al. 2021). To be recruited, YH participants had to be between 18 and 25 years old and without history of chronic diseases requiring medical management. They were recruited at the Faculty of Medicine of the University of Montenegro during the period between February 18th and March 11th, 2020.

For the 28 old participants (O_CD and O_NCD groups) recruitment and participation in the study was approved by the Ethical Committee of the Faculty of Medicine of the University of Montenegro and by the Ethical Committee of the Clinical Center of Montenegro (Protocol No. 3824/4 and No. 03/01–11417/1). Participants who were included in the O_CD and O_NCD groups had to be at least 56 years old, and could not have history of psychiatric disease, substance or alcohol abuse, or current acute disease. O_CD-specific inclusion criteria were: controlled HTN and/or DM2 (blood pressure < 140/90 mmHg, HbA1C < 10%), and/or established diagnosis of AD. O_NCD-specific criteria for inclusion was negative previous history of any chronic disease. These participants were recruited at the Clinical Center of Montenegro and at the Faculty of Medicine of the University of Montenegro from September 26th, 2019 to August 19th, 2021.

Presence of opacities of the transparent media in both eyes affecting the visualization of the microvascular network, and myopia ≥ 5 diopters, represented exclusion criteria for all 3 groups of participants (YH, O_CD and O_NCD).

All procedures were conducted in accordance with the Declaration of Helsinki. Only authors in charge of recruitment of participants had access to information related to their identity. Each participant was assigned a study number during the recruitment, and any data collection was related to the study number, so anonymity of all study participants has been preserved. After signing the informed consent, height and body weight were measured, and demographic and basic health information were collected from the participants by using standardized questionnaires. Body mass index (BMI) was calculated and participants were considered obese if BMI ≥ 30 (Centers for Disease Control and Prevention 2022).

Montreal cognitive assessment (MoCA) test

The MoCA test was used as a cognitive screening instrument in the old participants (normal test scores ≥ 26; mild cognitive impairment 18–26; dementia ≤ 18) (Naserddine 2023).

Retinal imaging and image processing

All images were captured by a family medicine physician (NP) by using the hand-held non-mydriatic fundus camera MiiS HORUS scope DES 200 with U-RETINAL control unit. The images were captured at 45° field of view with 2560 × 1920 pixel resolution. The automatic focus and capture settings were centered between the optic disc (OD) and macula and IR/White light LED was set to 3/7.

Image quality control was done automatically during the image acquisition by using the built-in control unit software, so the images containing blur or shadow were not used. All participants with at least one good quality image from at least one eye were included in the further analysis. If images from both eyes were available, the image with higher box-counting fractal dimension (Db) was used because Db can be negatively affected by the lower quality of the image (Lyu et al. 2022). The presence or absence of retinal pathology was assessed independently by two ophthalmologists (AAZ and MR).

The color fundus images were manually segmented by using Vampire software to generate binarized 8-bit black-and-white images in the PNG format which contained only topological information of the retinal microvascular network from each image (Vampire group 2023). Subsequently, a circular region of interest (ROI) centered on the OD was cropped from each image using custom-made software, and vascular network was skeletonized by using Skeletonize 2D/3D plug-in in ImageJ 1.53a (ImageJ group 2023). The radius of ROI was 350 pixels, which was the maximum size that still allowed the ROI to retain a circular shape and fall within the limits of the nasal area of the retinal image (Fig. 2).

Evaluation of microvascular complexity

Microvascular complexity was quantified by using Sholl's sum, box-counting fractal dimension (Db), and lacunarity dimension (Λ). All 3 parameters can

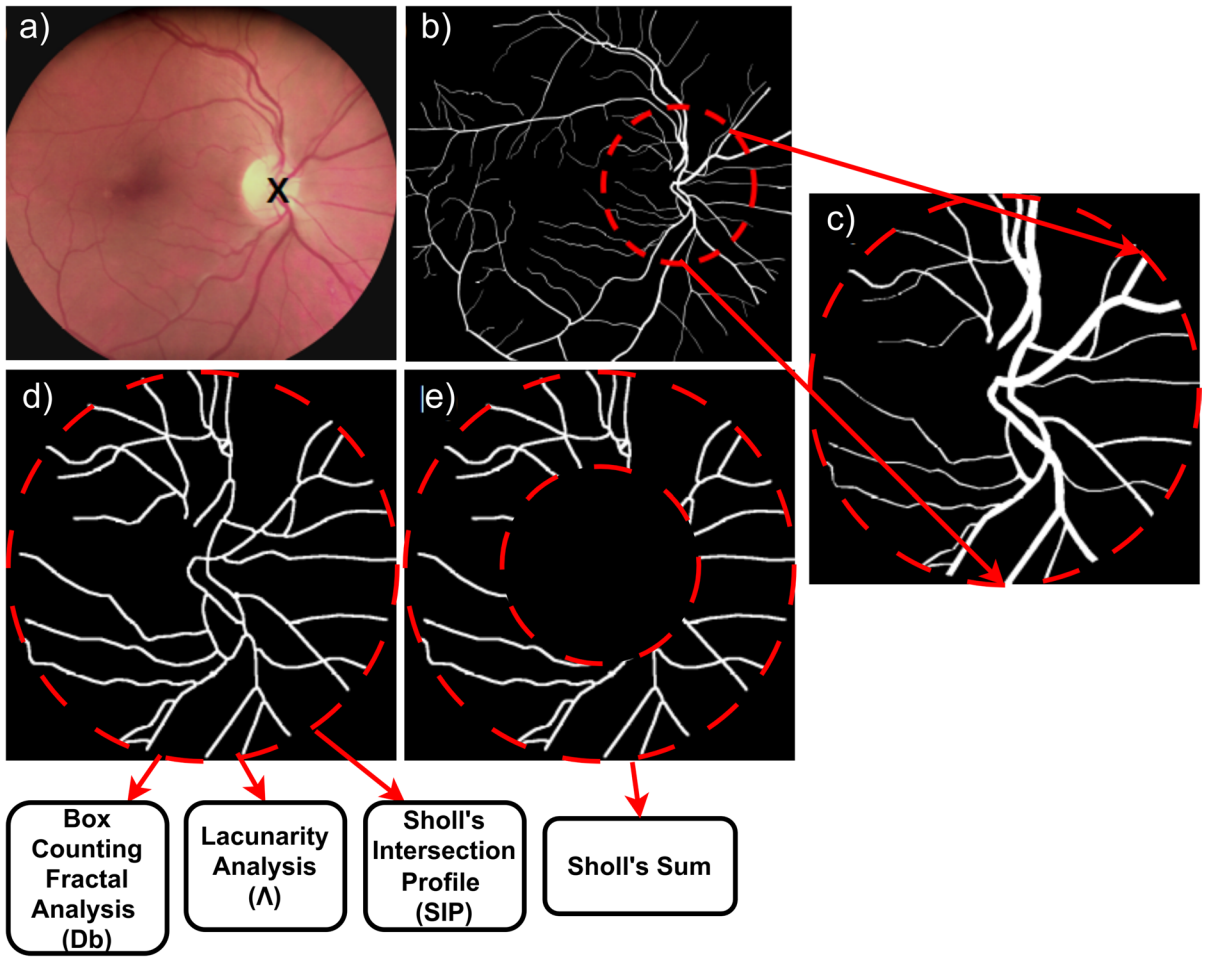

Fig. 2 Retinal image processing steps **a** Color fundus image- X denotes the OD center; **b** Manually segmented retinal microvascular network and selection of a circular ROI- centered on OD, diameter 350 pixels; **c** Cropped ROI; **d** Skeletonized microvascular network in the ROI was used for evaluation of microvascular complexity by Db, Λ (lacunarity dimension), and Sholl's analysis- Sholl's intersection profile (SIP) and Sholl's sum. **e** Calculation of the Sholl's sum was performed in the doughnut-shaped area encompassing the outer ½ of the ROI

be used to quantify complexity of patterns found in nature, but they capture different aspects of these patterns. Sholl's sum measures the extent of line branching in a certain region of an image.

Fractal is a geometric shape that exhibits self-similarity at different scales. If we zoom in on a detail of a fractal shape, we will find a smaller copy of the same shape. Higher fractal dimension means that the examined region of an image is filled with a more intricate pattern. A fern would be a typical example of a shape with a high fractal dimension, while a maple leaf would be an example of a shape with lower fractal dimension. Lacunarity quantifies the arrangement of empty spaces in a given area (gaps in the image) and homogeneity of these empty spaces. The higher the lacunarity dimension, the more empty spaces are present in a given area with more variations in spacing between the components. For example, memory foam, which is a synthetic material, typically has a low lacunarity. It is engineered to have small pores uniformly spaced throughout the material. Contrary to this, a sea sponge has higher lacunarity as it contains larger pores that vary in size and distribution throughout the material.

Sholl's analysis was performed using ImageJ 1.53q and the Sholl's analysis plug-in under the

Neuroanatomy plug-in window. This approach is most commonly used for measurements of dendritic complexity of the neurons (Bird and Cuntz 2019), but in the present study it was used to measure the complexity of the retinal microvascular network by counting the number of skeletonized vascular branches intersecting with the concentric circles drawn at increasing distances from the OD center in each ROI. The number of intersections plotted as a function of the circle radius is termed Sholl's intersection profile (SIP, see Fig. S2). Sholl's sum is the sum of all intersections in a certain area of the image. In the present study, we used SIP to determine the most critical area of the image that should be used to calculate the Sholl's sum (Fig. S2 and Fig. 3a).

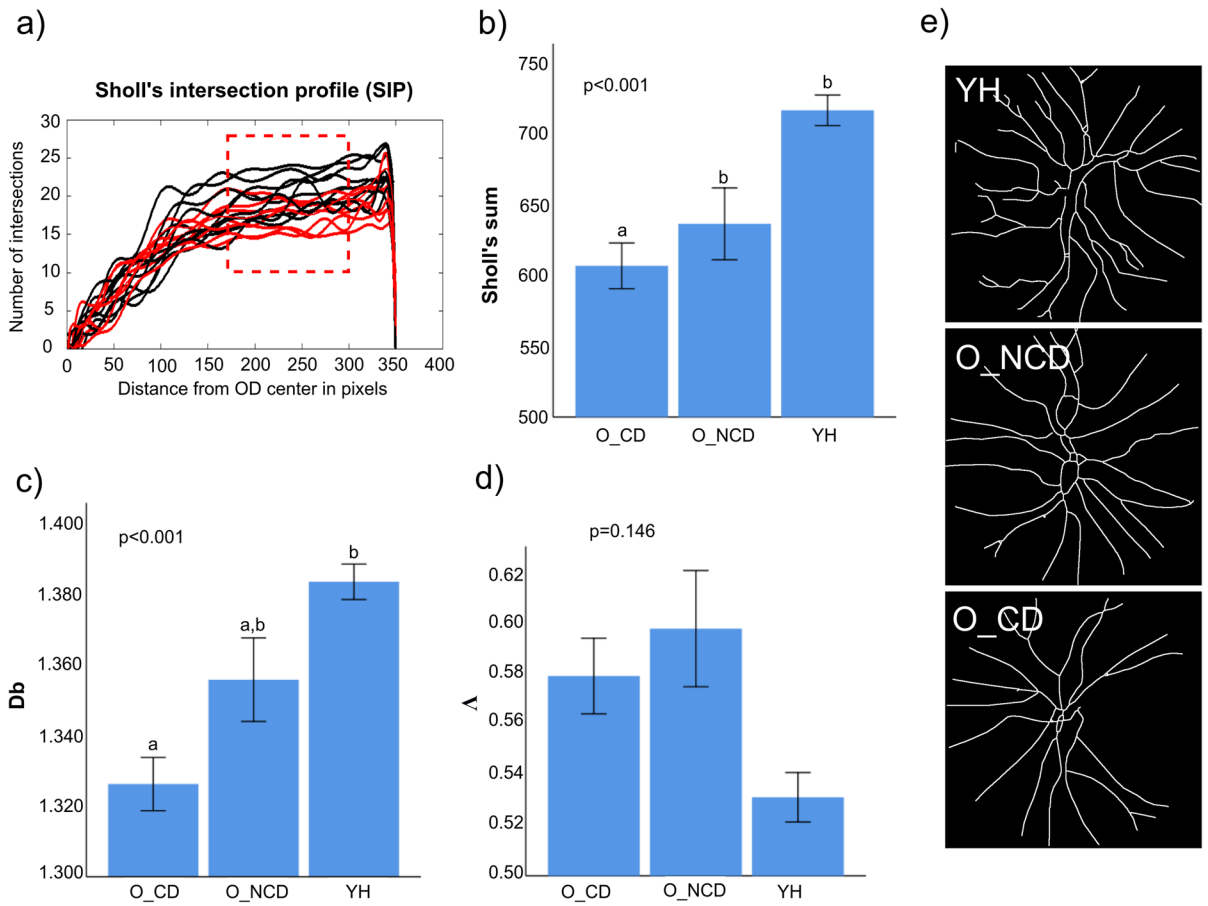

Fig. 3 One-way ANCOVA comparison of parameters of microvascular complexity among the groups of participants **a** Representative images of SIP. Full red (lighter) lines = O_NCD + O_CD group, black lines = YH group. Dashed rectangle points out the area in which the SIPs are different in old compared to the young participants; **b** Results for Sholl's sum at a distance of 175 to 350 pixels from the center of the OD (estimated marginal mean ± SD: O_CD vs. N_CD vs. YH = 606.41 ± 16.09 vs. 636.01 ± 25.28 vs. 715.89 ± 10.71). Covariates included in this model are gender and education; **c** Results for Db in the entire ROI (estimated marginal mean ± SD: O_CD vs. N_CD vs. YH = 1.326 ± 0.007 vs. 1.355 ± 0.012 vs. 1.383 ± 0.005). Covariates included in this model are gender and education; **d** Results for Λ in the entire ROI (estimated marginal mean ± SD: O_CD vs. N_CD vs. YH = 0.580 ± 0.015 vs. 0.599 ± 0.023 vs. 0.531 ± 0.010). Covariates included in this model are gender, education, smoking ever, and BMI. The bar graphs show estimated marginal mean ± error for each group. Different letters on the graph denote groups that are significantly different. **e** Representative images of skeletonized microvascular network displaying different complexity in the ROIs for each group of participants; Abbreviations: YH- young healthy (n = 40), O_NCD- old with no chronic disease (n = 8), O_CD- old with chronic disease (n = 20), OD- opic disc, SIP- Sholl's intersection profile, Db- box-counting fractal dimension, Λ- lacunarity dimension. (Color figure online)

Since SIP represents a number calculated with the assumption that the branches are evenly distributed at a certain distance from the center ROI (Bird and Cuntz 2019), which is frequently not the case in reality (Fig. 3e), we also used Db and Λ to measure spatial complexity of the microvascular network. The skeletonized ROIs were analyzed by using FracLac plug-in of the ImageJ 1.53q, and Db, as well as lacunarity dimension (Λ) were determined as previously described (Popovic et al. 2018).

Statistical analysis

SPSS 29.0.0.0 and GraphPad Prism 9.3.1. were used for statistical analysis. Chi-Square test was used to analyze nominal data (presence or absence of a certain condition or a disease). After assessing normality of distribution by using D'Agostino & Pearson and Shapiro–Wilk tests, numerical data that were not normally distributed were analyzed by using non-parametric Kruskal–Wallis one-way ANOVA with the post hoc multiple comparison Dunn's test. The MoCA test results were analyzed by using one-way ANOVA with Tukey's post hoc test. The effect of age and presence of age-related diseases (health) on Db, Λ, Sholl's sum were determined by ANCOVA with Bonferroni post hoc test. To determine the contribution of age and age-related diseases to the change of each parameter of microvasccular complexity, we used hierarchical multiple linear regression. To test the equality of the regression slopes for Db and Λ in healthy vs. unhealthy aging, we ran a continuous by categorical variable interaction test in linear regression. P-values < 0.05 were considered statistically significant.

Data availability

The images and associated data for YH group of participants (TREND database) are available at https://doi.org/10.5281/zenodo.4521044. The images and associated data for O_NCD and O_CD group of participants (TREND2 database) are freely available at https://doi.org/10.5281/zenodo.7678656.

Results

Participant characteristics

First, we checked if there were differences in demographic and basic health factors among the 3 groups (YH, O_NCD, O_CD), other than age. For each of the three groups demographic and basic health factors are shown in the Table 1.

The results showed that the O_NCD group had on average the most years of education. This could partly be explained the fact that the YH group consisted of college students whose education was still in progress at the time of the study. Furthermore, both O_NCD and O_CD group had higher BMI with respect to YH group, but prevalence of obesity was not different among the groups. O_NCD group had a significantly higher proportion of participants who consumed alcohol, both ever and currently, with respect to the O_CD group. Regarding the history of smoking, YH group showed a significantly lower proportion of smokers with respect to both the O_NCD and O_CD groups, but no significant differences were noted in the number of current smokers among the groups. Smoking was significantly less represented in the YH group compared to both groups of older participants, most likely because the legal smoking and drinking age in Montenegro is 18 years, which is very close to the average age of the YH group of participants. The groups were homogeneous in regard to gender. The comorbidity profile in the O_CD group is shown in Table 1 and in Fig. S1.

To address the inhomogeneity of the groups of participants with regard to certain health factors we performed ANCOVA analysis. We explored if health factors such as gender, education, BMI, obesity, history of smoking and alcohol use affect: 1. the relationship between each parameter of microvacular complexity with the chronological age, and 2. the relationship between each parameter of microvacular complexity with the health status (i.e. presence or absence of age-related diseases) (Table 2). Gender and education (used here as an indicator of socioeconomic status) are non-modifiable health factors. The health factors such as BMI, obesity, smoking ever, smoking current, alcohol ever and alcohol current are modifiable health factors. The ANCOVA showed that Db and Sholl's sum represent robust parameters of microvascular complexity as their association with chronological

Table 1 Demographic and basic health characteristics of study participants

	YH n = 40	O_NCD n = 8	O_CD n = 20	YH vs. O_NCD vs. O_CD *p*-value
Age (mean ± SD)	19.90 ± 1.03^{a}	63.63 ± 7.87^{b}	68.50 ± 8.33^{b}	$< 0.001^{*}$
MoCA score (mean ± SD)	not performed	26.25 ± 2.05	21.80 ± 6.47	not performed
Gender (Male %)	43	50	35	0.704
Education (mean ± SD)	12.00 ± 0^{a}	13.63 ± 1.85^{b}	10.50 ± 3.25^{a}	$< 0.001^{*}$
BMI (mean ± SD)	22.80 ± 3.13^{a}	25.68 ± 2.61^{b}	26.26 ± 4.55^{b}	0.019^{*}
Obese (%)	5	0	17	0.262
Alcohol use**				
Ever (%)	28^{a}	63^{ab}	15^{ac}	0.045^{*}
Current (%)	28^{a}	63^{ab}	10^{ac}	0.017^{*}
Smoking				
Ever (%)	13^{a}	50^{b}	40^{b}	0.015^{*}
Current (%)	13	25	15	0.659
Chronic diseases				
AD (%)	0	0	35	Not performed
HTN (%)	0	0	90	Not performed
DM2(%)	0	0	40	Not performed

*Statistically significant results. Statistical tests were not performed when the mean and SD of 2 out of 3 groups were 0. *P*-values were calculated using the Chi-Square test for nominal data, non-parametric Kruskal–Wallis one-way ANOVA for numerical data

**Up to 3 drinks, each equivalent of 30 ml of hard liquor 3 times per week

[abc]Results denoted with different letters are significantly different

YH young healthy, *O_NCD* old with no chronic disease, *O_CD* old with chronic disease, *MoCA* Montreal Cognitive Assessment, *BMI* body mass index, *AD* Alzheimer's dementia, *HTN* hypertension, *DM2* diabetes mellitus type 2

Table 2 Association of parameters of microvascular complexity (Db, Λ, Sholl 's sum) with chronological age and with health status

Covariate included in model	AGE (young vs. old)			HEALTH (with vs. without age-related disease)		
	Db *p*-value	Λ *p*-value	Sholl 's sum *p*-value	Db *p*-value	Λ *p*-value	Sholl 's sum *p*-value
None	< 0.001	0.060	< 0.001	< 0.001	0.412	< 0.001
Gender	< 0.001	0.058	< 0.001	< 0.001	0.382	< 0.001
Education	< 0.001	0.034*	< 0.001	< 0.001	0.140	< 0.001
Gender + Education	< 0.001	0.034	< 0.001	< 0.001	0.137	< 0.001
BMI	< 0.001	0.016*	< 0.001	< 0.001	0.195	< 0.001
Obesity	< 0.001	0.028*	< 0.001	< 0.001	0.247	< 0.001
Smoking current	< 0.001	0.030*	< 0.001	< 0.001	0.384	< 0.001
Smoking ever	< 0.001	0.014*	< 0.001	< 0.001	0.266	< 0.001
Alcohol current	< 0.001	0.063	< 0.001	< 0.001	0.429	< 0.001
Alcohol ever	< 0.001	0.062	< 0.001	< 0.001	0.385	< 0.001
Gender + Education + BMI	< 0.001	0.016*	< 0.001	< 0.001	0.132	< 0.001
Gender + Education + Smoking ever	< 0.001	0.007*	< 0.001	< 0.001	0.076	< 0.001

*p-values that became significant after confounding variable was included in the statistical model. *P*-values were calculated using the one-way ANCOVA

Db box-counting fractal dimension, *Λ* lacunarity dimension, *BMI* body mass index

age and with the presence of age-related diseases was significant even after adjusting for non-modifiable health factors. However, the association of Λ and chronological age, and association of Λ and the health status were not significant. The association of Λ and chronological age became significant when the confounding variables such as education, BMI, obesity, smoking ever, and smoking current, were included in the statistical model.

MoCA test results in old participants

The MoCA test was not performed in YH group. As expected, MoCA scores were lower in the O_CD group compared to the O_NCD group, because some of the participants in O_CD group had AD. To determine if the presence of HTN and/or DM2 affected cognitive performance, we performed one-way ANOVA analysis and compared MoCA scores in AD vs. DM2 + HTN vs. O_NCD. The results showed that AD group had the lowest average score (AD vs. DM2 + HTN vs. O_NCD, mean ± SD = 16.00 ± 7.16 vs. 24.92 ± 3.23 vs. 26.25 ± 2.05, $p < 0.001$). Pairwise comparison showed that AD group was different from the other two groups, while DM2 + HTN was not different from O_NCD group. Age was not different among these 3 groups of participants (data not shown).

Retinal pathology

Out of the 28 old participants, 14 had normal-looking retina, 8 had hypertensive retinopathy grade 1–2, 1 had moderate non-proliferative diabetic retinopathy (DR), 1 had age-related wet macular degeneration, 1 had atherosclerotic changes, and 3 had drusen. Retinas of all young participants were normal.

Microvascular complexity, chronological age, and biological age

To determine the effects of chronological and biological age on the retinal microvascular complexity, we compared SIP, Sholl's sum, Db, and Λ, for each of the three groups of participants by ANCOVA analysis with adjustments for appropriate confounding variables (Fig. 3a-e).

Topological characterization of the retinal microvascular network by visual inspection of SIP graphs demonstrated that the most prominent difference between YH and O_NCD + O_CD group is in the doughnut-shaped area of the periphery of the ROI, at a distance of 175 to 350 pixels from the center of the OD (Fig. 3a). Sholl's sum results confirmed that the number of intersecting vessels at the periphery of the ROI is higher in young participants when compared to either group of old participants (Fig. 3b). At this distance all vascular branches attain arteriolar/venular status (Cabrera DeBuc et al. 2018; Cheung et al. 2011).

The results showed that old participants with chronic diseases (O_CD) have lower retinal microvascular complexity measured by Db compared to young healthy group (YH) (Fig. 3c). There were no significant differences in Λ among the groups (Fig. 3d). To test if the observed associations were different in men and in women, we performed the same analysis separately on males and females (Table S1). The results showed that Sholl's sum and Db were different in O_CD group compared to YH group in both genders, while Λ was not different in any of the comparisons.

To further explore the effects of chronological age and health status on the 3 parameters of microvascular complexity, we applied hierarchical multiple linear regression analysis. First, we verified which of health factors are in linear correlation with the age of participants, because only those variables could be included in this type of analysis (Table 3). Table 3 shows that neither age nor health are in linear correlation with Λ,

Table 3 Association between the parameters of microvasuclar complexity with each health parameter (linear regression performed on all 68 participants together)

	Sholl's sum	Db	Λ
Age	< 0.001*	< 0.001*	0.068
Gender	0.406	0.805	0.513
Education	0.180	0.038*	0.184
BMI	0.049*	0.030*	0.822
Obesity	0.376	0.343	0.430
Smoking current	0.157	0.385	0.011*
Smoking ever	0.190	0.155	0.247
Alcohol current	0.202	0.057	0.844
Alcohol ever	0.491	0.184	0.838
Health	< 0.001*	< 0.001*	0.412

*p-values that are statistically significant

Db- box-counting fractal dimension, Λ- lacunarity dimension, BMI- body mass index

so hierarchical multiple linear regression was not performed for this parameter of microvascular complexity. Next, we started with a simple model based only on age as a prediction variable for Db- Model 1, and made it more complex by adding the presence of age-related diseases as an additional variable in Model 2, education in Model 3, and finally BMI in Model 4 (Table 4). The analysis showed that all 4 models are able to explain changes in Db ($p < 0.001$ for each model), but the second model was the best (based on high R square combined with the lowest standard error of the estimate). The R square change in this hierarchical analysis shows that 36.2% of variation in Db is explained by the chronological age of the participants, while additional 4.6% could be additionally explained by the presence of age-related diseases. Both prediction variables, age, and presence of age-related diseases were significantly negatively correlated with Db (Table 4, the last two columns). The hierarchical linear regression for Sholl's sum showed that the only variable that significantly explains variability of Sholl's sum is the age of participants, and that adding variables such as health and BMI did not contribute significantly to the model.

Next, we used linear regression analysis to explore whether the rate of change in retinal microvascular

Table 4 Hierarchical multiple linear regression analysis on Sholl's and Db results from all participants

Db						
Model #:-predictors	R square	R square change	Standard. error of the estimate	Sig. of the model (*p*-value)	Standard. regression coefficients for the model predictors	Sig. of the regression coefficient (*p*-value)
Model 1:	0.362	0.362	0.0319	< 0.001*		
- Age					− 0.001	< 0.001*
Model 2:	0.408	0.046	0.0309	< 0.001*		
- Age					− 0.001	0.031*
- Health					− 0.030	0.031*
Model 3:	0.409	0.001	0.0312	< 0.001*		
- Age					− 0.001	0.034*
- Health					− 0.028	0.064
- Education					0.001	0.792
Model 4:	0.409	0.000	0.0314	< 0.001*		
- Age					− 0.001	0.045*
- Health					− 0.028	0.067
- Education					0.001	0.797
- BMI					− 2.112E-5	0.985
Sholl's sum						
Model #:-predictors	R square	R square change	Standard. error of the estimate	Sig. of the model (*p*-value)	Standard. regression coefficients for the model predictors	Sig. of the regression coefficient (*p*-value)
Model 1:	0.365	0.365	67.189	< 0.001*		
- Age					− 2.137	< 0.001*
Model 2:	0.375	0.010	67.165	< 0.001*		
- Age					− 1.695	< 0.001*
- Health					− 30.043	0.311
Model 3:	0.376	0.000	25.259	< 0.001*		
- Age					− 1.724	0.004*
- Health					− 30.237	0.312
-BMI					0.454	0.852

Db box-counting fractal dimension, *BMI* body mass index

complexity in healthy aging is different compared to the rate of change in unhealthy aging. Since the majority of the people start their life as young healthy individuals with no age-related diseases, healthy aging trends were modeled by the linear regression line for the Db values for O_NCD + YH group, while for unhealthy aging O_CD + YH group was used. The analysis showed that Db decreases with age. The slope of the line is negative, but it is close to 0. This means that the observed decrease is subtle, especially in healthy subjects. However, in this model the decrease in Db occurs at a 2.1 times faster rate in participants with age-related diseases compared to those with healthy aging (Fig. 4a). Sholl's sum decreases in both, in the model for healthy and for unhealthy aging, and the rate of this decrease is not significantly different in the two models (Fig. 4b). These results show that both parameters together could be used as a biomarkers for chronological and biological age. This analysis did not account for any potential confounding variables because most of the health factors measured in this study were not in linear correlation with the parameter of microvascular complexity (Table 3), and because hierarchical multiple linear regression identified only age and presence of age-related diseases (i.e. health) as significant predictors of Db, and the only significant predictor for Sholl's sum was the chronological age of participants. These findings are in agreement with the findings of the ANCOVA analysis (Fig. 3), and this reinforces the conclusion that Sholl's sum and Db are robust parameters of microvascular complexity not affected by the other health factors in the study.

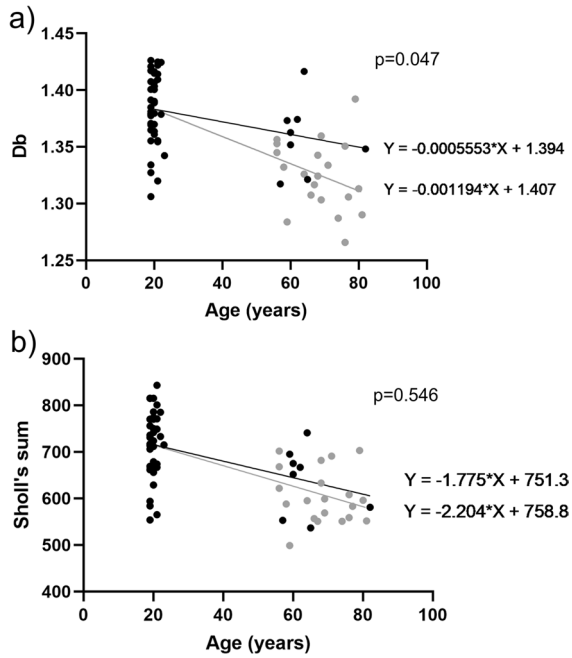

Fig. 4 Microvascular complexity measured by Sholl's sum and by Db in healthy vs. unhealthy aging Healthy aging model-YH + O_NCD (shown in black), unhealthy aging model- YH + O_CD group (shown in gray). **a** Db decreases in both, the model for healthy and for unhealthy aging, but this decrease is 2.1 times faster in people with age-related diseases compared to those who age healthy; **b** Sholl's sum decreases in both, in the model for healthy and for unhealthy aging, but the rate of this decrease is not significantly different between the two models. *P*-values were calculated by using continuous by categorical variable interaction test in linear regression. Abbreviations: YH- young healthy (n = 40), O_NCD- old with no chronic disease (n = 8), O_CD- old with chronic disease (n = 20). (Color figure online)

Discussion

This study showed that microvascular complexity decreases with age. More precisely, advanced chronological age is associated with decreased number of arteriolar branches in the peripapillary area, regardless of the health status of the individual as demonstrated by the SIP and Sholl's sum analysis (Fig. 3a, and b). However, in people with age-related diseases, decreased complexity is additionally manifested by the loss of self-similarity of vascular geometrical patterns over different scales (Fig. 4c).

The observed decrease in complexity of the microvascular network might be a consequence of progressive damage of elastin and increased stiffness of the extracellular matrix (ECM) in large conduit vessels, a process associated with vascular aging in mammals (Avolio et al. 1998; Levine 1997; Komutrattananont et al 2020). Levine noted that species with a faster resting heart rate have a shorter lifespan and hypothesized that higher metabolic rate might be the cause for this observation (Levine 1997). Others however, argued that this might be caused by mechanical fatigue and damage to elastin associated with repeated heart cycles (Avolio et al. 1998; Komutrattananont et al 2020). Elastin is produced and cross-linked only during the fetal period and early infancy, and it has very long estimated average half-life of about 74 years (Shapiro et al. 1991).

Increased vascular stiffness is also the result of an increased collagen/elastin ratio, calcification of the remaining elastin, phenotypic switching of smooth muscle cells (SMC) from the contractile to proliferative phenotype, endothelial dysfunction (Duca et al. 2016; Mochizuki et al. 2002), decreased ability of SMC to sense mechanical changes in the environment, and decreased SMC contractility (Ojha et al. 2022). Decrease in aorta elasticity results in the loss of the Windkessel effect and increased pulse pressure. As a reaction to increased pulse pressure, the microvascular network is remodeled though rarefaction (Mitchell 2018).

The presence of more than one age-related diseases in the same person commonly occurs in real life. To mimic real life, the present study analyzed the effect of one or more age-related diseases on retinal microvascular complexity. One explanation for how HTN might negatively affect microvascular complexity was given by a study on transient high oxygen exposure in newborn rats (Huyard et al. 2014). This study demonstrated that changes in the ECM of the aorta might be the initial event preceding the development of HTN. More importantly, people with a genetic predisposition for primary HTN exhibit rarefaction of dermal capillaries even before HTN develops (Noon et al 1997; Antonios 2006). These studies suggest that microvascular rarefaction represents an early event in the development of HTN. HTN itself might accelerate this process through a positive feedback loop. This is supported by the findings that microvascular rarefaction is more pronounced in people with untreated and poorly controlled HTN (Hughes et al. 2006; Sng et al 2010).

Studies report opposing findings on Db trends in DM2. The presence or absence of diabetic retinopathy (DR), as well as different stages of DR in different studies, might be the reason for these conflicting observations. Decreased vessel density close to the OD has been detected by using optical coherence tomography angiography even before overt clinical signs of DR are present (Vujosevic et al. 2018). Early stages of DR are associated with arteriolar pruning and increased size of the foveolar avascular zone. Since neovascularization represents the hallmark sign of advanced staged DR, there may be increased complexity in some parts of the retina at advanced stages of DR (Parsons-Wingerter et al. 2010). In the present study, only one patient with DM2 had moderate stage DR, while the other 7 did not have signs of DR. Consequently, the presence of DM2 together with the increasing age of participants could negatively affect microvascular complexity.

Increased macrovascular stiffness and consequent microvascular damage are recognized risk factors for the development of cognitive decline and dementia. Increased arterial stiffness is associated with increased amyloid deposition in brains of cognitively preserved adults (Hughes et al. 2014), indicating that increased arterial stiffness might be one of the early events associated with pathogenesis of AD. Increased aortic stiffness is associated with increased transmission of pulsatility to the cerebral circulation, cerebral microvascular disease, and lower brain volume (Mitchell 2018).

DM2, HTN, and AD are characterized by many overlapping pathophysiologic processes that directly affect structure and function of neurovascular tissue in brain and retina (Exalto et al. 2014; Little et al. 2022). DM2 is characterized by microvascular and macrovascular complications, and both are known to affect brain function. People with DM2 have almost twofold increased risk for developing AD when compared to the general healthy population (Zhang et al. 2017). This risk persisted after adjusting for increased vascular risk factors in people with DM2 (Zhang et al. 2017). In addition, elderly patients with DM2 have a 42% higher risk for the development of AD if they also have severe diabetic retinal disease compared to those without it (Exalto et al. 2014). This risk persisted after adjusting for diabetes-related and macrovascular disease-related factors. Collectively, these studies suggested that there is a causative relationship between microvascular disease and dementia (Exalto et al. 2014).

According to the hypothetical model that describes temporal evolution of major biomarkers of AD (Jack et al. 2013), there is a lag time usually measured in years, between appearance of AD biomarkers and detection of cognitive impairment. This lag time varies depending on genetic risk, lifestyle, and other comorbidities, as well as the cognitive reserve of the individual. Exalto's group hypothesized that retinal microvascular disease and changes in the brain associated with AD start to develop approximately at the same time, years before overt clinical signs of dementia occur (Exalto et al. 2014). In the present study, the majority of the patients with DM2

also had AD, which is in agreement with the observation that AD occurs more frequently in patients with DM2 compared to the patients without it (Jack et al. 2013). However, none of these patients had severe retinal microvascular disease. One of the factors that predisposed these patients for early development of overt cognitive impairment might be lower cognitive reserve, because O_CD group of patients was less educated relative to O_NCD group.

The present study did not test the applicability of the retinal microvascular complexity as a marker of biological age in animal models, but a study by Nazari et al. showed that vascular complexity decreases with aging in mice, and that this change is more pronounced in the mouse model of AD (Nazari et al. 2022).

The study has several limitations. The sample size is small, so the power of statistical tests is limited. The results show only temporal association between advanced age, age-related diseases, and decreased microvascular complexity. The causal relationships and precise temporal dynamics of their change are yet to be determined by longitudinal follow up of a larger number of participants. A study aimed at participants of all age groups including the ages between 26 and 55 (that were not included in the present study) would be important since some studies have shown that decrease in fractal dimension as a function of chronological age might be better explained by a quadratic rather than linear function (Che Azemin et al. 2013). Finally there is a possibility that the study could have a potential bias due to residual confounding. For example, no adjustment was made for genetic factors and environmental factors such as air pollution, and if exposure, outcome, or other confounders were poorly measured this could have impacted the results.

Conclusion

We demonstrated that retinal microvascular network visualized by a hand-held fundus camera can be used for quantification of microvascular complexity. This method captures changes in retinal microvascular complexity associated with chronological age, and is also sensitive enough to detect accelerated aging due to age-related diseases. Thus, retinal microvascular complexity represents a promising potential biomarker of biological age. Longitudinal studies on larger data sets with computer-aided analysis for faster segmentation of blood vessels (Mookiah et al. 2021), evaluation of the correlation of microvascular complexity with other age-related diseases such as cancer, as well as with longevity biomarkers (Roth et al. 2002) such as levels of dehydroepiandrosterone sulphate, melatonin, insulin, plasma triglyceride levels, glucose tolerance, and oxidative damage are needed for further validation. Lastly, the images and associated data from this study is one of the first of this kind collected in Montenegro, and they are freely available for researchers to use.

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Declarations

Competing interests The authors declare no relationships and financial support that may have posed as a conflict of interest.

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