

Ageing effect on flicker-induced diameter changes in retinal microvessels of healthy individuals

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Abstract

Purpose: To compare flicker-induced retinal vessel diameter changes in varying age groups with low cardiovascular risk.

Methods: Retinal vascular reactivity to flicker-light was assessed by means of dynamic retinal vessel analysis in 57 participants aged 19-30yrs, 75 participants aged 31-50yrs, and 62 participants aged 51-70yrs participants. Other assessments included carotid intima-media thickness (c-IMT), augmentation index (AIx), blood pressure profiles, blood lipid metabolism markers, and Framingham risk scores (FRS).

Results: Retinal arterial dilation amplitude (DA) and post-flicker percent constriction (MC%) were significantly decreased in the oldest group compared to the middle-aged ($p = 0.028$; $p = 0.021$) and youngest group ($p = 0.003$; $p = 0.026$). The arterial constriction slope ($Slope_{AC}$) was also decreased in the oldest group compared to the youngest group ($p = 0.027$). On the venous side, MC% was decreased in the middle-aged and older groups in comparison to the youngest group ($p = 0.015$; $p = 0.010$, respectively). Additionally, men exhibited increased arterial DA ($p = 0.007$), and percent dilation (MD%, $p < 0.001$) in comparison to women, but only in the youngest age group. Both AIx and c-IMT scores increased with age (both $p < 0.001$), however, no correlations were found between the observed differences in the measured retinal vascular function and systemic parameters.

Conclusion: In individuals with low cardiovascular risk, there are age-related differences in flicker-induced retinal vessel diameter changes throughout the entire functional response curve for arteries and veins. Gender differences mainly affect the arterial dilatory phase and are only present in young individuals.

Key words: ageing, retina, vascular function, cardiovascular risk, dynamic retinal vessel analysis

Introduction

It is well known that the incidence and prevalence of cardiovascular disease (CVD) increases exponentially with age (McDermott 2007; Rosamond et al. 2008; Nichols et al. 2013). At present, the current identification of at-risk individuals for primary prevention efforts relies on classical risk factors for CVD such as lipid profiles, smoking, and hypertension (Grundy et al. 1999; Committee 2012; Goff et al. 2014). Although some of these variables increase with age, the predictive accuracy of traditional risk estimates that include the aforementioned variables, such as Framingham risk scores (FRS), the Prospective Cardiovascular Münster (PROCAM) score, and the European Society of Cardiology Systematic Coronary Risk Evaluation (SCORE) either over- or under-estimate actual risk in a large number of individuals (Vasan 2006; Koenig 2007; Cohn 2013). Therefore, other measures such as genetic, inflammatory, and coagulation markers, as well as, various tests for subclinical disease have been sought (Helfand et al. 2009; Wang 2011; Ge & Wang 2012). The quantification of vascular and endothelial dysfunction (Deanfield et al. 2007) is a recently emerging early marker for CVD and is usually achieved by employing techniques such as ultrasound flow mediated dilation (FMD), pulse wave analysis (PWA), plethysmography, and iontophoresis (Ray et al. 2014). These tests can, however, be complex and time consuming, and are performed only in highly specialized services. Among the various methods developed to measure microvascular function, dynamic retinal vessel analysis (DVA) features as a non-invasive method that allows for continuous recordings of retinal arterial and venous diameter changes in response to flicker-light stimulation. The main advantage of the DVA assessment is that it provides integrated and dynamic data analysis that is specific to each individual. In addition, its output has proven to be modified not only by overt disease but also in the presence of more subtle risk factors for CVD (Pemp et al. 2009; Reimann et al. 2009; Kotliar et al. 2011) including ethnicity (Patel et al. 2011) and impaired glucose tolerance (Patel et al. 2012). Therefore, it is possible to use the assessment of retinal microvascular function as an early marker for vascular and endothelial dysfunction.

Besides pathologies, however, normal ageing as assessed by DVA can also influence retinal microvascular dynamics. Indeed, published data allude to an age-related decrease in retinal arterial response profiles (Kotliar et al. 2008), as well as, to a general decline in overall vessel dilation amplitudes during flicker-light stimulation (Nagel et al. 2004; Kneser et al. 2009; Gugleta et al. 2013). Nevertheless, a more complex analysis of the dynamics of both vasodilation and vasoconstriction responses, as well as, of the capacity to re-establish a pre-flicker diameter after the cessation of stress is needed for a better understanding of healthy individual vascular dynamics. Therefore, the present study, using a more detailed approach for the evaluation of retinal vascular function, seeks to characterize the entire retinal microvascular response to flicker provocation in individuals with low cardiovascular risk belonging to various age groups.

Methods

Study participants

Community-dwelling volunteers (aged above 18 years) were recruited through local advertisements at the Vascular Research Laboratory and Health Clinics at Aston University (Birmingham, UK). Ethical approval for the study was received from the relevant local and institutional ethics committees. Written informed consent was received from all participants prior to study enrolment and all study procedures were designed and conducted in accordance with the tenets of the Declaration of Helsinki.

Study exclusion criteria were defined as a history or current diagnosis of cardiovascular or cerebrovascular disease including coronary artery disease, heart failure, arrhythmia, stroke, transient ischemic attacks, peripheral vascular disease, as well as, smoking, hypertension, diabetes, and or severe dyslipidaemia (defined as plasma triglyceride levels above 6 mmol/L or cholesterol levels above 7 mmol/L). The use of vasoactive medications such as dietary supplements containing vitamins or antioxidants and bronchodilators also served as exclusion criteria. In addition, participants with elevated intraocular pressures (IOP > 21

mmHg), retinal disease, intraocular surgery, neuro-ophthalmic disease, cataract or other media opacities that may affect the ocular vascular system or prevent retinal vascular examination were also excluded from the study.

General investigations

All study-related measurements were performed between 8 and 11 AM following a 12-hour overnight fast, which included refraining from alcohol or caffeine.

Standard anthropometric measures of height and weight were recorded to determine body mass index ($BMI = \text{weight}/\text{height}^2$). Systolic blood pressure (SBP), diastolic blood pressure (DBP), and heart rate (HR) were measured using an automatic BP monitor (UA-767; A&D Instruments Ltd, UK) to determine mean arterial pressure ($MAP = 2/3 \text{ DBP} + 1/3 \text{ SBP}$). IOP readings were obtained using non-contact tonometry (Pulsair; Keeler Ltd, UK) to determine ocular perfusion pressure ($OPP = 2/3 \text{ MAP} - \text{IOP}$).

In addition, blood and plasma samples drawn from the antecubital fossa vein were assessed immediately for fasting glucose (GLUC), triglycerides (TG), total cholesterol (CHOL), and high-density lipoprotein cholesterol (HDL-c) using the Reflotron Desktop Analyzer (Roche Diagnostics, UK). Low-density lipoprotein cholesterol (LDL-c) values were calculated as per the Friedewald equation (Friedewald et al. 1972).

Framingham risk score (FRS)

The FRS is a widely used gender-specific algorithm originally developed to estimate CVD risk (Wilson et al. 1987). In the present study FRS was calculated using the current version of the FRS published by an expert panel of the National Heart, Lung and Blood Institute (NHLBI) (2002) and is based on risk factors such as age, gender, CHOL, HDL-c, SBP, treatment for hypertension, smoking status, and diabetes. Risk factors such as age, treatment for hypertension, smoking status and diabetes were identified from self-report questionnaires and CHOL, HDL-c, and SBP values were as those determined on the day of

study assessment. The scoring algorithm is based on gender-specific points assigned for each risk factor variable that can be determined using FRS tables i.e. point scores by age group; age group and total CHOL; age group and smoking status; HDL-c level; SBP and treatment status. Ten-year risk percentage is then calculated by total points (1 point, 6%; 2 points, 8%; 3 points, 10%; 4 points, 12%; 5 points, 16%; 6 points, 20%; 7 points, 25%; 10 points or more, > 30%). Absolute CVD risk percentage over 10 years was classified as low risk (< 10%), intermediate risk (10-20%), and high risk (> 20%) (Ford et al. 2004).

Carotid intima-media thickness (c-IMT)

Intima-media thickness measurements of the left and right common carotid arteries were obtained for all participants as described previously (Seshadri et al. 2015), and in accordance with an already published protocol (Salonen et al. 1991).

Pulse-wave analysis (PWA)

Arterial stiffness was assessed by PWA using the validated SphygmoCor device according to an already published protocol (O'Rourke et al. 2001), and as detailed previously (Mroczkowska et al. 2012). The augmentation index (AIx) value calculated by the device software was used as a measure of arterial stiffness (Wilkinson et al. 1998).

Dynamic retinal vessel analysis (DVA)

Retinal vessel reactivity was measured with the dynamic retinal vessel analyser (IMEDOS GmbH, Germany) using a previously published protocol (Nagel et al. 2006). All measurements were performed in a quiet, temperature-controlled room (22°C) following full pupil dilation with 1% Tropicamide (Chauvin Pharmaceuticals Ltd, UK). For all participants, measurements were conducted in one unselected eye. A visual fixation target was used to control eye movements and to position the region of interest at the centre of the fundus image. Within this region, a segment approximately 0.5 to 1 mm and 1 to 2 disc diameters from the optic nerve head was selected for continual diameter recording, for both the inferior

temporal retinal artery and retinal vein. The automated 350-second flicker stimulation protocol included a 50-second baseline diameter measurement (under still illumination 25 Hz) followed by three successive cycles of flicker stimulation (opto-electronically generated at 12.5 Hz) distinguished as 20 seconds of stimulus interrupted by an 80-second recovery period. The dynamic nature of the vessel response profile was further explored by extracting the raw response data and applying a statistical polynomial regression algorithm (MATLAB; Mathworks, MA) (Mroczkowska et al. 2012). The following vessel reactivity and time course parameters, were determined for each flicker cycle and then averaged over the three cycles, with the artery and vein regarded separately: the average baseline diameter and range of maximum and minimum baseline vessel diameters (baseline diameter fluctuation, BDF); the maximum vessel dilation diameter during flicker stimulation expressed as a percentage change relative to baseline diameter (MD%) and the time taken in seconds to reach the maximum diameter (tMD); the maximum vessel constriction diameter during the post-flicker recovery period expressed as a percentage change relative to baseline diameter (MC%) and the time taken in seconds to reach the maximum vessel constriction diameter (tMC); the overall dilation amplitude (DA) calculated as the difference between MD and MC; and the baseline-corrected flicker response (BCFR) used to describe the overall dilation amplitude after normalizing for fluctuations in baseline diameters (DA-BDF). In addition, the arterial (A) and venous (V) dilation slopes ($\text{Slope}_{AD/VD} = (\text{MD} - \text{baseline diameter}) / \text{tMD}$) and constriction slopes ($\text{Slope}_{AC/VC} = (\text{MC} - \text{MD}) / \text{tMC}$) were also calculated.

Statistical Analysis

All data are reported as mean (SD) unless otherwise indicated. The Shapiro-Wilk test was used to determine the distribution of the data. Univariate associations were determined using Pearson's (normally distributed data) or Spearman's method (non-normally distributed data), and forward stepwise regression analyses were performed to test the influences of BMI, BP, circulating markers, c-IMT, and Aix on the measured variables. Differences between groups were subsequently assessed using one-way analysis of variance (ANOVA) or analysis of

covariance (ANCOVA), followed by Tukey's post-hoc analysis as appropriate. P-values of less than 0.05 were considered significant, except in certain cases where a stricter p-value of less than 0.01 was adopted in order to correct for multiple comparisons. All analyses were performed using the commercially available Statistica[®] software (StatSoft Inc.; Version 9, Tulsa, OK).

Results

A total of 236 volunteers were initially screened for study inclusion of which 42 individuals were excluded based on having moderate or high FRS (>10%). The remaining 194 participants with low FRS (<10%) were included in the final analysis and classified into one of three age groups (Group 1: 19 to 30yrs; Group 2: 31 to 50yrs; Group 3: 51 to 70yrs). The number of participants in each group was similar (Group 1: 57; Group 2; 75; Group 3; 62, Chi-square test: $p = 0.295$), as was the distribution of male (M) and female (F) participants within each group (Group 1: M = 27, F = 30; Group 2: M = 42, F = 33; Group 3: M = 33, F = 29, Chi-square test $p = 0.612$).

Clinical characteristics

Table 1 summarizes the clinical characteristics of the study population. There was a significant difference between groups in age ($p < 0.001$), BMI ($p = 0.002$), SBP ($p = 0.002$), DBP ($p = 0.001$), HR ($p < 0.001$), MAP ($p = 0.001$), IOP ($p < 0.001$), CHOL ($p = 0.002$), HDL-c ($p = 0.007$), LDL-c ($p < 0.001$), FRS ($p < 0.001$), c-IMT scores ($p < 0.001$), and Aix ($p < 0.001$), but not in OPP ($p = 0.089$), GLUC ($p = 0.102$), or TG levels ($p = 0.161$). Post-hoc comparisons revealed that FRS and c-IMT scores significantly increased with age, with each group differing significantly from the other (all $p < 0.001$). In addition, in comparison to the youngest group BMI, DBP, IOP, and LDL-c were higher in the middle-aged ($p = 0.023$, $p = 0.013$, $p = 0.019$, and $p = 0.006$, respectively) and older ($p = 0.002$, $p = 0.001$, $p < 0.001$, and $p = 0.001$, respectively) groups. SBP and Aix were also higher in the oldest group compared to the youngest ($p = 0.009$; and $p < 0.001$, respectively) and middle-aged groups

($p = 0.003$; and $p = 0.010$, respectively). Finally, with regards to HR, MAP, CHOL and HDL-c, the middle-aged group did not differ significantly from the youngest or oldest group (all $p > 0.05$), however, HR ($p < 0.001$) and HDL-c ($p = 0.009$) were lower, and MAP ($p < 0.001$) and CHOL ($p = 0.001$) were higher in the oldest age group in comparison to the youngest group.

Retinal vessel diameter

Group differences in flicker-induced retinal arterial and venous diameter changes (DVA) are summarized in Table 2. All reported values are based on data averaged across the three flicker cycles, with the artery and vein regarded separately.

Arterial response

After controlling for influential covariates identified in multivariate analysis, there were no significant group differences in baseline diameter, BDF, BCFR, MD%, tMD, tMC, and Slope_{AD}, (all ANCOVA $p > 0.01$, Table 2). There were, however, significant group differences in arterial DA ($p = 0.003$), MC% ($p < 0.001$) and Slope_{AC} ($p < 0.001$) (Table 2). Post-hoc comparisons showed DA and MC% to be significantly decreased in the oldest age group compared to the middle-aged ($p = 0.028$; $p = 0.021$, respectively) and youngest groups ($p = 0.003$; $p = 0.026$, respectively). Additionally, Slope_{AC} was decreased in the oldest age group compared to the youngest group ($p = 0.027$), with the middle-aged group not differing significantly from the youngest ($p = 0.525$) or oldest groups ($p = 0.216$) (Figure 1A).

Venous Response

There was an overall significant difference in venous MC% across groups (ANOVA $p = 0.002$) with post-hoc comparisons showing MC% to be similarly decreased in the middle-aged ($p = 0.015$) and older ($p = 0.010$) groups in comparison to the youngest group (Figure 1B). After controlling for influential covariates no significant group differences in any of the other measured venous DVA parameters were identified (ANCOVA, all $p > 0.05$, Table 2).

Gender comparisons

Arterial MD% was significantly higher in men compared to women with regards to the study population as a whole (M: 4.42 ± 2.51 vs. F: 3.84 ± 2.27 , $p = 0.011$, Figure 2). Within-group gender comparisons in the measured retinal arterial DVA parameters are displayed in Table 3. Arterial DA ($p = 0.007$) and MD% ($p < 0.001$) were significantly higher in men compared to women belonging to the youngest age group (Figure 3, Table 3) but not between men and women in the middle-aged and oldest groups (all $p > 0.01$). There were, no significant differences between men and women in any of the other measured arterial DVA parameters for the study population or within groups (all $p > 0.01$, Table 3). There were also no significant gender differences in any of the measured venous DVA parameters (all $p > 0.01$, data not shown).

Discussion

In the present study we used a specific computational model to evaluate the entire dynamic response of retinal microvessels after flicker stimulation in a sample of individuals with low CVD risk belonging to various age groups. Our results show that independent of systemic influences, older healthy individuals displayed abnormal dilatory and constrictory responses to flicker-light stimulation in retinal arteries and veins. Additionally, in younger individuals, gender had an influence on retinal arterial dilation. This effect was, however, lost in the middle-aged and oldest groups.

It is known that decreased vessel distensibility and focal narrowing occur in ageing vessels independently of other arteriosclerotic risk factors such as hypertension (Van Bortel & Spek 1998; Hubbard et al. 1999; Wong et al. 2003). Despite various adaptations to vascular structural remodelling and changes in viscoelastic properties that occur with ageing, there could still be individual limitations in functional vascular reserves that may only be evident as responses to provocative stressors. In line with previous research (Nagel et al. 2004; Kneser et al. 2009), this study shows an age-related decline in retinal vasoregulative capacity in

both dilatory and constrictory phases that was independent of any systemic influences. It was previously hypothesized that in ageing vessels this vascular adaptive response may be attributed to a re-setting of vessels' average working points within which the points of maximum dilation and constriction tend to occur (Kneser et al. 2009). The cause of this shift in vessel behaviour remains unclear; however, a possible contender is a high level of oxidative stress that occurs with ageing and is a known cause of senescent endothelial dysfunction (Heo et al. 2011). Indeed, we have already demonstrated that in otherwise healthy individuals with low to moderate cardiovascular risk, retinal microvascular dilation and constriction responses to stress levels are influenced by systemic antioxidant capacity (Seshadri et al. 2015). Although the levels of antioxidant molecules were not determined in this study, it can be hypothesized that similar interactions take place in individuals with similar CVD risk.

Other factors such as age-related vascular stiffness can also be involved. An understanding of microcirculatory responses with regards to systemic haemodynamic parameters is important as the combination of age-related arterial stiffening and ensuing hypertension (O'Rourke 2007) can offset the stiffness gradient between the heart and periphery and augment pressure pulsatility penetrating into the microvasculature. Indeed, in our study groups the Alx, a measure of peripheral arterial stiffness, was higher in the older group than in middle-aged or younger individuals. In normal microvessels, low resistance protects them from intense pulsations and flow fluctuations, nevertheless, it is well known that this balance can be modified by age-related stiffening of the vascular wall, resulting in microcirculatory damage (Barodka et al. 2011). Although the age-related decline in the retinal vessels' dilation and constriction phases demonstrated in our study occurred independently of systemic factors, it cannot, however, be excluded that local microvascular stiffness played some role in our findings.

In previous studies we have already documented increases in pre-flicker baseline diameter fluctuations (Mroczkowska et al. 2013), decreases in the arterial corrected flicker response

(Patel et al. 2012) and dilation capacity (Qin et al. 2013), as well as, venous dilation capacity (Mroczkowska et al. 2012) and enhanced post-flicker vasoconstrictions (Mroczkowska et al. 2012; Mroczkowska et al. 2013) in individuals with various levels of cardiovascular risk. In the present study, in a sample with low risk, decreases in the post-flicker constrictory phase of retinal veins were apparent in the middle-aged and older individuals. The role of the venular circulation in CVD has previously received limited attention until unexpected associations implicated retinal venular dilation, rather than arteriolar narrowing, as a stronger predictor of adverse vascular phenomena (Wong et al. 2006; Wang et al. 2007; Wong & Mitchell 2007). These studies have since stimulated an increased interest in retinal venular physiology although it remains unclear whether venous changes detected by the DVA represent separate causal pathways of endothelial dysfunction, or are epiphenomena of the arterial response. In such a context, it could be possible that the observed decrease in post-flicker venous diameters returns reflects a compensatory adaptation following sustained arterial dilation during flicker. Further investigation is required to understand the relevance of an impaired venous constriction post-flicker, however, it could be hypothesized that a change in venous caliber associated with either structural or endothelial irregularities could also be used as a marker for ageing and associative cardiovascular risk.

The dynamic behaviour of retinal microvessels also appears to be affected by more than just the ageing functional state of the endothelium. In the present study, gender differences in the retinal vasoregulative response were lost with ageing. Sex hormones influence both vascular tone and blood flow in various organs and tissues, including retinal vessels (Ogueta et al. 1999). Taking into consideration the above, gender differences in vascular tonus and blood flow are to be expected due to changes in hormonal status across the life span of individuals. Indeed, in the present study we have observed an overall gender difference in arterial MD%; however with younger men exhibiting higher MD% values than age-matched women. To our knowledge, this is the first study to observe gender differences in DVA measurements in healthy individuals and as oestrogens upregulate NO production and suppress the effect of vasoconstrictors such as endothelin-1 (Kauser & Rubanyi 1997), our

results are somewhat unexpected. As the retinal vascular response to flickering light is also a neurovascular coupling driven response (Riva et al. 2005) and sex hormones can exert effects on other cells in the neurovascular unit such as neurons and astrocytes (Yang et al. 2005), it is possible that the gender differences in flicker-induced retinal diameter changes do not truly match those assessed by other methods that measure resting blood flow and vascular tone. Nevertheless, the expected ageing-related blunt with regards to gender differences in vascular reactivity was still apparent in our results. More work is, however, necessary in order to clarify the mechanism of gender differences in flicker-induced retinal diameter changes as measured by DVA.

Conclusion

In conclusion this study demonstrates that age and gender are variables to be considered when assessing the retinal vascular response to flicker stimulation. In addition, the entire retinal microvascular response to flicker provocation, as well as, the age and gender of each individual should be considered when assessing possible pathological changes associated with vascular disease by means of the DVA.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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Figure Legends

Figure 1. Comparison of retinal (A) arterial and (B) venous response profiles across age groups. AU, arbitrary units; MD, maximum dilation diameter during flicker; DA, dilation amplitude (MD-MC), MC, maximum constriction diameter post-flicker; MC%, percentage constriction below baseline; Slope_{AC}, arterial constriction slope (MC-MD/time taken to reach MC).

Figure 2. Comparison of the retinal arterial response profile between men and women in the study population. AU, arbitrary units; MD, maximum dilation diameter; MD%, percentage change in diameter from baseline to maximum during flicker; MC, maximum constriction diameter post-flicker.

Figure 3. Comparison of the retinal arterial response profile between men and women in the youngest age group (19-30yrs). AU, arbitrary units; MD%, percentage change in diameter from baseline to maximum during flicker; DA, dilation amplitude; MD, maximum dilation diameter during flicker; MC, maximum constriction diameter post-flicker.

Table 1. Summary of systemic characteristics ^a.

Characteristic	Group (1) (19-30yrs)	Group (2) (31-50yrs)	Group (3) (51-70yrs)	p-value	Significant difference by group
No. of participants	57	75	62	0.295	-
Gender distribution	27M : 30F	42M : 33F	33M : 29F	0.612	-
Age (years)	26 (3)	40 (6)	56 (5)	<0.001 ^c	1 < 2 < 3
BMI ^b	24.11 (3.84)	26.00 (3.74)	26.69 (4.69)	0.002 ^c	1 < 2, 3; 2 = 3
SBP (mmHg)	116 (13)	117 (12)	123 (13)	0.002 ^c	1, 2 < 3; 1 = 2
DBP (mmHg)	71 (9)	76 (11)	77 (10)	0.001 ^c	1 < 2, 3; 2 = 3
HR (bpm)	71 (11)	67 (8)	64 (8)	<0.001 ^c	1 > 3; 2 = 1, 3
MAP	85.94 (9.33)	89.63 (10.67)	92.92 (10.26)	0.001 ^c	1 < 3; 2 = 1, 3
IOP (mmHg)	13 (2)	14 (3)	15 (2)	<0.001 ^c	1 < 2, 3; 2 = 3
OPP ^d	44.69 (6.06)	45.97 (7.08)	47.44 (7.09)	0.089	-
GLUC (mmol/L)	4.80 (0.74)	4.92 (0.68)	5.09 (0.78)	0.102	-
TG (mmol/L)	1.04 (0.47)	1.22 (0.65)	1.18 (0.50)	0.161	-
CHOL (mmol/L)	4.18 (0.77)	4.49 (0.89)	4.75 (0.97)	0.002 ^c	1 < 3; 2 = 1, 3
HDL-c (mmol/L)	1.44 (0.50)	1.38 (0.41)	1.22 (0.38)	0.007 ^c	1 > 3; 2 = 1, 3
LDL-c (mmol/L)	2.25 (0.75)	2.71 (0.86)	2.82 (0.91)	<0.001 ^c	1 < 2, 3; 2 = 3
FRS %	0.74 (0.48)	3.41 (2.41)	8.25 (2.71)	<0.001 ^c	1 < 2 < 3
c-IMT (mm)	0.46 (0.01)	0.56 (0.01)	0.63 (0.02)	<0.001 ^c	1 < 2 < 3
Alx	10 (9)	15 (12)	22 (12)	<0.001 ^c	1, 2 < 3; 1 = 2

Abbreviations: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; MAP, mean arterial pressure; IOP, intraocular pressure; OPP, ocular perfusion pressure; GLUC, glucose; TG, triglycerides; CHOL, total cholesterol; HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol; FRS, Framingham risk score; c-IMT, carotid intima-media thickness; Alx, augmentation index.

^a Data are presented as mean (SD) unless otherwise indicated.

^b Calculated as weight in kilograms divided by height in metres squared.

^c p < 0.05 was considered a significant difference.

^d Calculated as $MAP = 2/3 DBP + 1/3 SBP$.

^e Calculated as $OPP = 2/3 MAP - IOP$.

Table 2. Retinal arterial and venous vascular function parameters (DVA).

Parameter	Mean (SD)			p-value	Significant difference by group
	Group (1) (19-30yrs)	Group (2) (31-50yrs)	Group (3) (51-70yrs)		
Arteries:					
Baseline	99.89 (0.76)	99.97 (0.20)	99.98 (0.14)	0.488	-
BDF	6.06 (3.29)	5.93 (2.59)	5.54 (2.78)	0.093	-
DA ^b	7.04 (3.61)	6.59 (2.72)	5.36 (2.36)	0.003 ^a	1, 2 > 3; 1 = 2
BCFR ^c	1.05 (3.03)	0.90 (2.65)	0.03 (2.36)	0.083	-
MD%	4.38 (3.00)	4.09 (2.22)	3.82 (2.04)	0.036	-
MC%	-2.67 (2.32)	-2.41 (1.67)	-1.37 (1.77)	<0.001 ^a	1, 2 > 3; 1 = 2
tMD (sec)	22 (9)	20 (8)	21 (7)	0.105	-
tMC (sec)	24 (9)	28 (9)	29 (8)	0.041	-
Slope _{AD} ^d	0.23 (0.15)	0.27 (0.16)	0.28 (0.41)	0.063	-
Slope _{AC} ^e	-0.42 (0.35)	-0.27 (0.57)	-0.23 (0.20)	<0.001 ^a	1 > 3; 2 = 1, 3
Veins:					
Baseline	99.89 (0.76)	99.98 (0.13)	99.96 (0.20)	0.490	-
BDF	4.83 (2.78)	3.99 (1.63)	4.64 (2.82)	0.114	-
DA ^b	5.80 (3.33)	5.25 (2.53)	5.51 (2.78)	0.557	-
BCFR ^c	1.05 (2.67)	1.30 (2.29)	0.92 (2.55)	0.097	-
MD%	4.31 (2.19)	4.59 (2.43)	4.46 (2.74)	0.794	-
MC%	-1.61 (1.70)	-0.81 (1.10)	-0.75 (1.16)	0.002 ^a	1 > 2, 3; 2 = 3
tMD (sec)	23 (8)	21 (6)	22 (7)	0.129	-
tMC (sec)	28 (9)	30 (7)	29 (7)	0.390	-
Slope _{VD} ^d	0.23 (0.15)	0.25 (0.14)	0.26 (0.17)	0.391	-
Slope _{VC} ^e	-0.25 (0.17)	-0.19 (0.15)	-0.22 (0.16)	0.087	-

Abbreviations: DVA, dynamic retinal vessel analysis; baseline, baseline diameter; BDF, baseline diameter fluctuation; DA, dilation amplitude; BCFR, baseline corrected flicker response; MD%, percentage change in diameter from baseline to maximum; MC%, percentage constriction below baseline; tMD, reaction time to maximum dilation diameter; tMC, reaction time to maximum constriction diameter from maximum dilation diameter; Slope_{AD/VD}, slope of arterial/venous dilation; Slope_{AC/VC}, slope of arterial/venous constriction. Unless otherwise indicated, all values are expressed in arbitrary units, which approximately correspond to micrometres (μM) in a normal Gullstrand eye.

^a $p < 0.01$ was considered a significant difference.

^b Calculated as MD – MC.

^c Calculated as DA – BDF (Nagel et al. 2004).

^d Calculated as (MD – baseline)/tMD (Mroczkowska et al. 2012).

^e Calculated as (MC – MD) / tMC (Mroczkowska et al. 2012).

Table 3. Group comparisons of retinal arterial vascular function parameters (DVA).

Parameter ^a	Mean (SD)								
	Group 1 (19-30yrs)			Group 2 (31-50yrs)			Group 3 (51-70yrs)		
	M	F	p	M	F	p	M	F	p
Baseline	99.79 (1.07)	100.00 (0.01)	0.288	99.99 (0.07)	99.95 (0.27)	0.414	99.97 (0.19)	100.00 (0.01)	0.306
BDF	6.95 (3.50)	5.36 (2.77)	0.059	5.99 (2.40)	5.92 (2.48)	0.895	5.05 (1.57)	6.02 (3.09)	0.137
DA	8.21 (4.01)	5.72 (2.75)	0.007 ^b	6.38 (2.69)	7.01 (2.82)	0.298	5.53 (2.27)	5.43 (2.64)	0.872
BCFR	1.26 (3.44)	0.48 (2.64)	0.336	0.46 (2.94)	1.09 (2.16)	0.277	0.50 (2.15)	-0.37 (2.55)	0.120
MD%	5.85 (3.29)	3.06 (1.80)	<0.001 ^b	3.93 (2.08)	4.61 (2.53)	0.175	3.97 (1.96)	3.71 (2.14)	0.592
MC%	-2.37 (2.82)	-2.66 (1.88)	0.654	-2.45 (1.55)	-2.41 (1.68)	0.895	-1.56 (1.72)	-1.72 (1.73)	0.687
tMD (sec)	23 (10)	21 (7)	0.222	19 (7)	22 (8)	0.080	20 (7)	21 (8)	0.316
tMC (sec)	23 (10)	26 (8)	0.148	29 (7)	25 (9)	0.027	29 (8)	27 (8)	0.232
Slope _{AD}	0.29 (0.18)	0.20 (0.17)	0.071	0.27 (0.16)	0.27 (0.17)	0.991	0.28 (0.31)	0.31 (0.49)	0.757
Slope _{AC}	-0.50 (0.28)	-0.35 (0.37)	0.081	-0.26 (0.13)	-0.50 (0.72)	0.022	-0.24 (0.14)	-0.29 (0.19)	0.289

Abbreviations; DVA, dynamic retinal vessel analysis; M, men; F, women; Baseline, baseline diameter; BDF, baseline diameter fluctuation; DA, dilation amplitude; BCFR, baseline corrected flicker response; MD%, percentage change in diameter from baseline to maximum; MC%, percentage constriction below baseline; tMD, reaction time to maximum dilation diameter; tMC, reaction time to maximum constriction diameter from maximum dilation diameter; Slope_{AD/VD}, slope of arterial/venous dilation; Slope_{AC/VC}, slope of arterial/venous constriction. Unless otherwise indicated, all values are expressed in arbitrary units, which approximately correspond to micrometres (µM) in a normal Gullstrand eye.

^a See Table 2 footnotes b through e for calculations of DA, BCFR, Slope_{AD} and Slope_{AC}.

^b p < 0.01 was considered a significant difference.