Continuous Retinal Vessel Diameter Measurements: The Future in Retinal Vessel Assessment?

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Purpose. To establish an alternative method, sequential and diameter response analysis (SDRA), to determine dynamic retinal vessel responses and their time course in serial stimulation compared with the established method of averaged diameter responses and standard static assessment.

Methods. SDRA focuses on individual time and diameter responses, taking into account the fluctuation in baseline diameter, providing improved insight into reaction patterns when compared with established methods as delivered by retinal vessel analyzer (RVA) software. SDRA patterns were developed with measurements from 78 healthy nonsmokers and subsequently validated in a group of 21 otherwise healthy smokers. Fundus photography and retinal vessel responses were assessed by RVA, intraocular pressure by contact tonometry, and blood pressure by sphygmomanometry.

Results. Compared with the RVA software method, SDRA demonstrated a marked difference in retinal vessel responses to flickering light ($P < 0.05$). As a validation of that finding, SDRA showed a strong relation between baseline retinal vessel diameter and subsequent dilatory response in both healthy subjects and smokers ($P = 0.001$). The RVA software was unable to detect this difference or to find a difference in retinal vessel arteriovenous ratio between smokers and nonsmokers ($P = 0.243$). However, SDRA revealed that smokers' vessels showed both an increased level of arterial baseline diameter fluctuation before flicker stimulation ($P = 0.005$) and an increased stiffness of retinal arterioles ($P = 0.035$) compared with those in nonsmokers. These differences were unrelated to intraocular pressure or systemic blood pressure.

Conclusions. SDRA shows promise as a tool for the assessment of vessel physiology. Further studies are needed to explore its application in patients with vascular diseases. (*Invest Ophthalmol Vis Sci.* 2010;51:5833–5839) DOI:10.1167/iovs.09-5136

The measurement of retinal vessel diameters is useful in the assessment and risk stratification of several cardiovascular disorders.[1,2] However, structural assessment offered by fundus photography has several limitations. For example, static assessment captures only a single image of the vascular system, and so is unable to explore dynamic relationships and vascular function. Furthermore, parameters commonly used for assessment (such as the arteriolar narrowing) vary with age,[3] and so it is possible that other factors would also fluctuate. Alternatively, dynamic retinal vessel assessment (also known as continuous retinal vessel diameter measurement) has been clinically useful in several conditions, such as diabetes mellitus[4,5] glaucoma,[6] and in evaluating potential pathophysiological mechanisms in the regulation of ocular blood flow.[7–9]

Several approaches are available for assessing dynamic vessel diameter responses, as are different stimulation techniques. The latter include flickering light,[10] carbogen and oxygen inhalation,[8,11] and intravenous vasoactive substance infusions.[12,13] After stimulation with flickering light, retinal vessel analyzer (RVA) software calculates maximum retinal vessel response to 20 seconds of flickering light over three stimulation cycles. The average responses within a 17- to 23-second window after the start of the stimulation is taken to be the maximum diameter response.[4] This analysis generates a maximum artery dilatory response index (A_{max}), as well as similar outputs for minimum response (A_{min}), peak response (A_{peak}), and maximum venous dilatory response (V_{max}) to flicker.

Analysis based on RVA software currently in practice analyses retinal vascular responses to flicker in patients who have various diseases. However, a major limitation of this averaging approach is that it incorporates both time and diameter responses. For example, time responses fixed at 20 seconds may be open to error, as subjects reaching maximum dilation before 17 seconds or later than 23 seconds after flicker initiation may have their maximum dilatory response underestimated. In addition, when results from all three stimulation cycles are merged, differences in the reaction pattern or time course of each cycle may result in lost significance. To assess differences between cycles, others have incorporated an analysis of each flicker cycle.[14] A further problem in assessing maximum dilatory response to flicker light is of the baseline diameter fluctuation (BDF) due to vascular tone and arterial pulsation. Nagel et al.[15] introduced the concept of baseline corrected flicker response (BFR), where BDF is adjusted for the dilation amplitude (DA) reached by flicker stimulation (i.e., BFR = DA − BDF). However, this analysis does not examine each flicker cycle separately. An additional problem is that of defining a reaction pattern. To do so, the point of maximum dilation (MD), the point of maximum constriction (MC), the DA, and the time course of the reaction must all be determined. A recently published report discussing these problems highlighted the need for a more advanced and in-depth analysis incorporating both diameter and dilatory response.[16]

We propose a novel approach, which we have designated sequential and diameter response analysis (SDRA), for the investigation of retinal vascular function by seeking to establish a more cogent analytical methodology. SDRA was developed after a comparison of the established retinal diameter assessment method of static (AVR) and dynamic (A_{max}, A_{min}, A_{peak}, and V_{max}) measurements with a new group of measures (BFR, DA, MD, and MC) and time course responses (see Fig. 1). We

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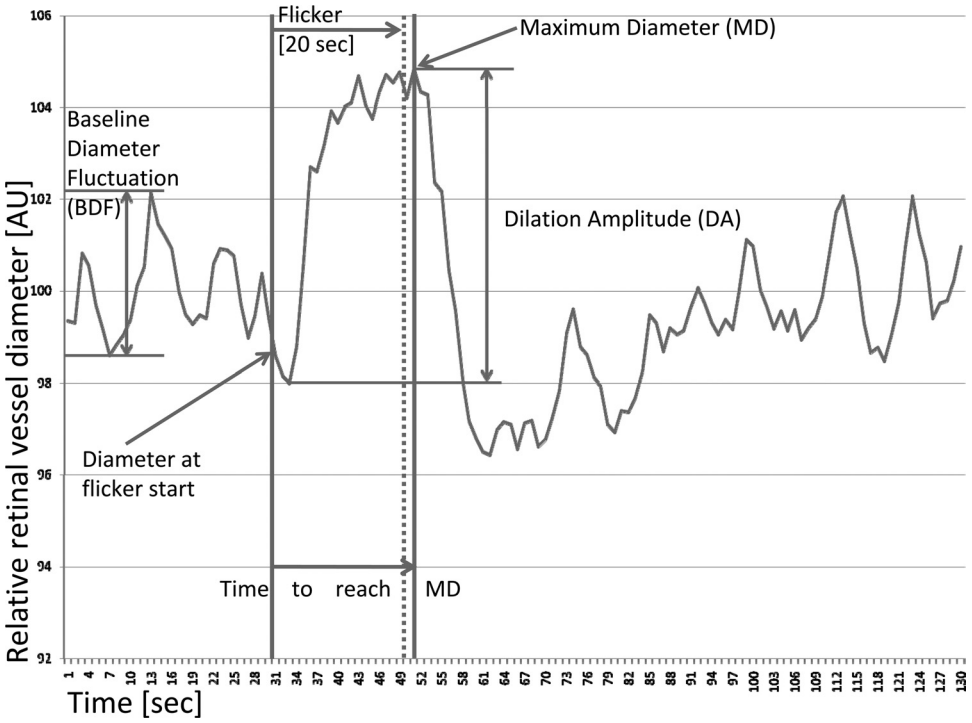

FIGURE 1. The retinal diameter response to flicker provocation. Illustrated are changes in a retinal vessel as it is stimulated by flickering light. On the far *left*, BDF defines the fluctuation in the baseline diameter. The *central section* shows an increase in relative vessel diameter due to flickering light. This response provides the MD, the DA, and the time to reach MD. The *right* side shows the responses of the vessel as it recovers (no data collected).

tested and validated SDRA in healthy volunteers and a group of otherwise healthy but smoking individuals, who were likely to exhibit vascular disturbances.

METHODS

Subjects

One hundred healthy smoking and nonsmoking individuals were recruited. Smoking was identified by self-report (smoking on a regular basis for at least 6 months before the study). Subjects were excluded if they had an ocular refractive error of more than ± 3 D spherical power and more than ±1 D cylindrical power.[16] This criterion was necessary to address the magnification or minification that may cause over- or underestimation of retinal diameter measured. Other exclusion criteria were intraocular pressure (IOP) >24 mm Hg, the presence of cataract or any other media opacities, a history of intraocular surgery, and any form of retinal or neuro-ophthalmic disease affecting the ocular vascular system. Systolic and diastolic blood pressure (SBP/DBP) was measured by automated sphygmomanometry. Other exclusion criteria were age <18 years, any history of systemic disease such as diabetes mellitus, hypertension, or any vascular abnormalities such as Raynaud's syndrome, and any use of vasoactive drugs, anticoagulants, and lipid-lowering agents. The study was approved by the Aston University Ethics Committee. Written informed consent was received from all subjects participating in the study. The study was designed and conducted in accordance with the tenets of the Declaration of Helsinki. All subjects were instructed to refrain from consuming caffeinated products, smoking, and drinking alcohol on the study day. Measurements were performed between 12 noon and 2 PM on all subjects.

Procedures

For IOP determination, the subjects were seated for contact tonometry according to standard practice, as per the manufacturer's instructions (TonopenXL; Medtronic Solan, PMS Instruments, Maidenhead, UK). The probe was placed on the central aspect of the cornea. All measurements were taken after topical instillation of 1 drop of 0.4% benoxinate hydrochloride (Minims; Chauvin Pharmaceuticals, Bausch and Lomb Ltd., Kingston-upon-Thames, UK). After an acclimatization period of 15 to 20 minutes in a temperature-controlled room (22–25°C), SBP, DBP, and heart rate were obtained by a validated medical device (Digital BP Monitor UA-767EX-C; PMS Instruments). SBP and DBP were measured three times at baseline (good clinical practice, as recommended by the British Hypertension Society)[17] before the start of retinal vessel measurements and at 1-minute intervals during retinal vessel assessment, to determine its potential influence on the parameters measured. All ocular and blood pressure measurements were performed by a single operator (RH) registered with the General Optical Council of the United Kingdom.

For retinal vessel diameter assessment, all measurements were performed in one unselected eye from each subject. Continuous retinal diameter assessment and fundus photography were obtained by RVA and its inbuilt fundus camera (FF450; Carl Zeiss Meditec, GmbH, Jena, Germany). Photography was performed on each patient (after full dilation) with the angle set at 30° in black-and-white mode for highest contrast. AVR was measured semiautomatically (VesselMap software; Imedos GmbH, Jena, Germany).[18]

For RVA flicker measurement, the assessment of retinal vessel diameter reaction to flickering light an optoelectronic shutter is inserted in the optical pathway of the camera, illuminating the retina. Flicker is generated with the shutter by interrupting the observation illumination to the fundus, producing a bright-to-dark contrast ratio of at least 25:1. Rectangular light interruption of 12.5 Hz has been shown to be in the range of maximum exciting flicker frequency.[10] As video frequency is set at 25 Hz, the flicker frequency will give one dark image every second frame, translating into a sampling rate of 12.5 Hz during flicker provocation. In the present study, we applied the protocol of Nagel et al.[19] After full pupil dilation was achieved (topical 0.5% tropicamide; Minims; Chauvin Pharmaceuticals, Bausch & Lomb Ltd.) flickering was commenced according to the pattern of a baseline measurement of 50 seconds followed by three cycles of 20 seconds of flickering and 80 seconds of recovery. Thus, total retinal assessment lasted 350 seconds.

Sequential and Diameter Response Analysis

We collected standard AVR analysis data and the RVA software-generated parameters A_{max}, A_{min}, A_{peak}, and V_{max} and used the raw data to gener-

ate an SDRA taking each of the three individual flicker cycles into account. Our approach to SDRA focuses on three steps. First, to more accurately assess dilatory responses, in addition to the calculation of MD, MC, and DA, we investigated the time course of retinal vascular dilation by calculating the time needed to reach MD for each flicker cycle separately in both the selected artery and vein. Second, to determine any effect that the baseline variability in vessel diameter might have, we derived a new index, ΔD, representing the degree of vessel dilation occurring *between* the flicker initiation *and* vascular MD (i.e., ΔD = MD − the mean diameter of the particular vessel assessed in a 1-second time period before flickering commences). In this way, ΔD shows the remaining dilatory capacity after flicker initiation. Third, to assess the relative stiffness of the measured retinal arteriole, we defined a second new index by calculating the ratio between the BDF *before* stimulation with flickering light and the DA *after* provocation; we term this index the average peak ratio (APR) and use it as a measure of retinal arteriolar elasticity (i.e., APR = DA/BDF).

Plan of Validation

To validate the SDRA, we assessed basic demographic (age and sex in all participants) and anatomic (left or right eye in a representative subgroup) indices so as to address these possible confounders in subsequent analyses (e.g., smoking).

Statistical Analyses

Differences between two groups were analyzed either by using the Mann-Whitney U test or *t*-test, depending on distribution. Differences within a group were assessed by paired *t* tests. If non-normally distributed, the data were log transformed. In other cases, differences were sought by repeated measurements ANOVA or Friedman's method, followed by Tukey's post hoc test. Data correlations were determined according to Spearman's method. Relationships between age, dynamic response values to flicker light provocation, and changes in vessel diameter were sought using multivariate regression analyses. Statistical significance was defined at the level of $P < 0.05$. When computing multiple comparisons, we set the significance level at $P < 0.01$ to minimize bias (all analyses performed with Statistica ver. 6.0; Statsoft, Tusla, OK).

Results

Patient Demographics and Basic Vessel Indices

Age, sex, and blood pressure indices of the nonsmokers and smokers are shown in Table 1. In the smokers, cigarette usage per day ranged from 5 to 15 (mean ± SD, 9 ± 3), and the duration of smoking varied between 3 and 20 years (smoking history: 10 ± 5 years). There were no statistically significant differences between the 21 smokers and the 21 nonsmokers (selected to be age and sex matched to the smokers) at baseline with regard to IOP, SBP, DBP, mean arterial pressure (MAP) and retinal arteriolar vascular diameter, although venous diameter was higher in smokers. In a regression analysis, age, sex, and BP indices had no impact on the retinal vessel parameters in the nonsmokers.

Table 1. Demographic and Vascular Data

Parameter	Nonsmoker (n = 78)	Nonsmoker Subset (n = 21)	Smoker Subset (n = 21)	P
Age, y	36 (14)	27 (6)	31 (10)	0.202
Sex, M/F	45/33	8/13	8/13	1.000*
IOP, mmHg	14 (3)	14 (3)	15 (2)	0.618
SBP, mmHg	120 (12)	117 (12)	112 (10)	0.165
DBP, mmHg	74 (9)	72 (9)	71 (9)	0.561
MAP, mmHg	89 (9)	87 (9)	85 (9)	0.282
Size, A (μm)	120 (20)	120 (20)	122 (15)	0.842
Size, V (μm)	150 (18)	150 (16)	161 (16)	**0.018**

Data presented as mean (standard deviation) or actual number. *P* values by *t*-test or $^*\chi^2$ test. SBP, systemic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; Size (A), arterial diameter, Size (V), venous diameter.

SDRA: Impact of Age, Sex, and Left/Right Eye

We developed SDRA using data from 78 healthy, nonsmoking individuals. Coefficients of variation (CV) of the effect of flickers were determined by analyzing data from three flicker cycles in each of the 78 subjects. In the arteries, mean (SD) CVs were 1.3% (1.1%) for MD, 1.2% (0.9%) for MC, and 31.5% (19.3%) for reaction time (RT). In the veins, parallel data were 1.0% (0.8%), 0.8% (0.6%), and 17.9% (12.7%). In regard to vessel responses to flickering light, the mean (SD) MD, MC, and RT of 78 arteries were 4.9% (2.2%), −3.2% (1.8%%), and 16.1 (4.1) seconds, respectively. In veins, parallel data were 5.9% (2.2%) (P = 0.008 vs. arteries), −1.3% (1.0) ($P < 0.001$), and 19.4 (2.8) seconds ($P < 0.001$), respectively.

Correlation coefficients of age with MD, MC, and RT in arteries were $r = 0.1$ ($P = 0.393$), $r = 0.31$ ($P = 0.008$), and $r = 0.12$ ($P = 0.301$), respectively. In veins, parallel data were $r = -0.12$ ($P = 0.300$), $r = 0.02$ ($P = 0.854$), and $r = -0.13$ ($P = 0.320$). The correlation coefficients of age with CVs of MD, MC, and RT of arteries were $r = 0.16$ ($P = 0.162$), $r = -0.08$ ($P = 0.478$), and $r = 0.15$ ($P = 0.199$). In veins, parallel data were $r = -0.12$ ($P = 0.316$), $r = -0.04$ ($P = 0.723$), and $r = 0.05$ ($P = 0.637$), respectively. We conclude that, using SDRA, the MD, MC, or RT of arteries or veins, or their CVs, are not influenced markedly by age, with the exception of the arterial MC.

The SRDA parameters BDF, MD, MC, DA, and the reaction time to reach MD in arteries and veins between 45 male and 33 female subjects are as follows: Arterial BDF (%) was 4.04 (1.66) and 4.30 (2.18), respectively ($P = 0.890$). Similarly, MD (%) was 5.23 (2.59) and 4.64 (2.22) ($P = 0.432$), MC (%) was −3.07 (1.68) and −3.38 (2.07; $P = 0.607$), DA (%) was 8.29 (2.84) and 8.01 (3.37; $P = 0.636$), and RT (seconds) was 16.3 (4.0) and 15.8 (4.2) ($P = 0.559$). Venous responses were MD (%) 5.57 (1.94) and 6.22 (2.28) ($P = 0.238$), MC (%) −1.15 (0.86) and −1.36 (1.26) ($P = 0.755$), DA (%) 6.71 (2.27) and 7.58 (3.13) ($P = 0.334$), and RT (seconds) was 19.2 (2.8) and 19.4 (3.1) ($P = 0.607$). Paired right and left eye measurements (n = 33) were compared in a subset of subjects as follows: Arterial BDF (%) was 4.03 (1.63) and 4.35 (2.24) ($P = 0.706$), MD (%) was 4.97 (2.23) and 4.84 (2.32) ($P = 0.540$), MC (%) was −2.75 (1.78) and −3.55 (1.85) ($P = 0.074$), DA (%) was 7.72 (2.86) and 8.68 (3.19) ($P = 0.244$), and RT (seconds) was 15.8 (3.9) and 16.5 (4.2) ($P = 0.487$). Venous responses were MD (%) 5.77 (2.03) and 6.04 (2.33) ($P = 0.704$), MC (%) was −0.99 (0.93) and −1.5 (1.20) ($P = 0.083$), DA (%) was 6.76 (2.35) and 7.57 (3.13) ($P = 0.259$), and RT (seconds) was 19.0 (2.9) and 19.7 (2.8) ($P = 0.652$).

A strength of the SDRA is that it can obtain improved insight into the mechanisms that determine the amount of vascular dilation due to flickering-light stimulation in retinal vessels. For example, SDRA can be used to assess the relationship between the mean diameter measured in the 1-second period directly before the start of flicker stimulation with ΔD. The arterial MD reached due to flicker stimulation correlated inversely with its prestimulation diameter, as assessed for each of the three flicker cycles (cycle one: $r = -0.40$, $P = 0.001$; cycle two: $r = -0.31$, $P = 0.009$; cycle three: $r = -0.26$, $P = 0.029$). Similarly, there was a significant relationship between the individual venous baseline diameters and ΔD for each cycle, although this change was weaker and reached significance only in the

TABLE 2. Retinal Artery and Vein Dilatory Responses: Comparison between Analysis Methods

Parameter (*n*)	RVA Software	SDRA	*P*
Arterial response			
A_{max} (%)			
Group 1 (52)	2.83 (2.00)	4.66 (2.20)	<0.0001
Group 2 (23)	4.30 (2.18)	5.40 (2.49)	<0.0001
Group 3 (3)	2.13 (0.57)	5.30 (0.71)	0.108*
A_{min} (%)			
Group 1 (52)	−0.77 (1.43)	−3.59 (1.92)	<0.0001
Group 2 (23)	−0.66 (1.45)	−2.53 (1.46)	<0.0001
Group 3 (3)	−0.67 (0.40)	−2.62 (1.43)	0.109*
A_{peak} (%)			
Group 1 (45)	3.58 (2.55)	8.25 (3.17)	<0.0001
Group 2 (23)	4.94 (2.56)	7.93 (2.79)	<0.0001
Group 3 (3)	2.86 (0.84)	7.92 (2.13)	0.109*
Venous response			
V_{max} (%)			
Group 1 (16)	4.32 (1.98)	5.56 (2.08)	0.0001
Group 2 (54)	4.93 (1.84)	6.00 (2.12)	<0.0001
Group 3 (8)	2.65 (2.37)	4.76 (1.91)	0.0277*

Data are expressed as the mean (standard deviation). Comparison of retinal arterial and venous dilatory responses as calculated by RVA software and SDRA. Values are expressed as % changes. Group 1, reaction time <17 seconds; group 2, reaction time 17 < RT <23 seconds; group 3, reaction time >23 seconds.

* Data acknowledged to be nonsignificant as a result only of small sample size.

first two cycles (cycle one: $r = -0.26$, $P = 0.032$; cycle two: $r = -0.34$, $P = 0.004$; cycle three: $r = -0.19$, $P = 0.112$).

Comparing SDRA and Dynamic Retinal Diameter Evaluation

To support our hypothesis that inbuilt RVA software underestimates retinal artery and vein dilation in subjects reacting faster than 17 seconds or slower than 23 seconds, we compared both established (RVA) and novel (SDRA) parameters in healthy nonsmokers. Subjects were divided into three groups: Group 1 reached MD within 17 seconds after flicker initiation, group 2 reached MD within 17 to 23 seconds, and group 3 reached MD after 23 seconds[14] (Table 2).

Of the 78 subjects, 52 reached arterial MD before 17 seconds (mean (SD) arterial reaction time: 13.7 (2.2) seconds), 23 subjects reached MD within 17 to 23 seconds (mean reaction time of 19.7 (1.2) seconds), whereas the remaining three reached MD beyond 23 seconds (average arterial reaction time: 27.3 (1.2) seconds). These data were markedly different from the presumption that all 78 subjects would have reached MD within 17 to 23 seconds ($P < 0.001$). Similarly, the frequency of subjects whose venous MD were in one of the three groups was $n = 16$, $n = 54$, and $n = 8$, respectively, for <17 seconds, 17 to 23 seconds, and >23 seconds. This frequency also differed markedly from a presumed distribution of 0, 78, and 0, respectively, according to the traditional definition ($P < 0.001$).

The frequency of arterial responses (45, 23, and 7) differed markedly from the frequency of venous responses (16, 54, and 8) ($P < 0.001$): Actual mean (SD) reaction times in arteries and veins were 16.1 (4.1) and 19.4 (2.8) seconds, respectively ($P < 0.001$). Other analyses for A_{max}, A_{min}, A_{peak}, and V_{max} for arterial and venous responses are shown in Table 2 and once more, there are several significant differences in both vessel responses between the data delivered by the RVA software and data delivered by SDRA. We conclude from this section that the underlying assumption that both arteries and veins reach MD within 17 to 23 seconds is fundamentally flawed.

Validating SDRA in a Pathologic Setting

We validated SDRA by comparing retinal vessel responses to flicker light in 21 healthy subjects with 21 age- and sex-matched subjects likely to have minor vascular disease by virtue of smoking (Table 1). Table 3 shows that there are no significant differences between arterial and venous MD, MC, and RT, or their CVs and age, except a weak relationship between age and arterial MD in smokers. Table 4 shows dilatory responses of retinal arteries and veins in smokers and nonsmokers. Significant differences in arterial responses were present in regard to DA and for the BFR in the first flicker cycle alone: Responses were higher in smokers. In health, the BFR did not change with repeated stimulation. However, in smokers, arterial BFR (a measurement that takes into account the arterial pulse) was markedly lower at the second and third stimulation cycles when compared with the first cycle ($P = 0.048$). There were no significant differences in the venous parameters.

Table 5 shows that there were no significant differences between smokers and nonsmokers in mean reaction time in either retinal arteries or veins, according to the SRDA. Table 6 shows traditional retinal vessel analysis using AVR along with

TABLE 3. Correlation between Dynamic Retinal Vessel Parameters and Age Using SRDA

	Nonsmokers (*n* = 21)		Smokers (*n* = 21)	
Parameter	**Raw Data**	**Correlation (*r*, *P*)**	**Raw Data**	**Correlation (*r*, *P*)**
Arteries				
MD (%)	4.94 (2.42)	(0.07, 0.752)	6.14 (2.78)	(0.43, **0.048**)
MD (CV)	1.3 (0.7)	(−0.22, 0.316)	1.7 (1.9)	(0.06, 0.806)
MC (%)	−3.23 (1.87)	(0.37, 0.098)	−4.22 (2.16)	(0.30, 0.183)
MC (CV)	1.2 (0.6)	(−0.08, 0.716)	1.4 (1.5)	0.04, 0.861
RT (s)	15.1 (3.5)	(−0.13, 0.569)	15.4 (4.9)	(−0.02, 0.945)
RT (CV)	30.6 (19.1)	(0.27, 0.231)	37.8 (20.8)	(−0.02, 0.930)
Veins				
MD (%)	5.89 (2.05)	(0.08, 0.688)	6.81 (2.49)	(0.41, 0.062)
MD (CV)	1.0 (0.6)	(0.12, 0.578)	1.4 (0.9)	(−0.24, 0.290)
MC (%)	−1.26 (1.53)	(0.05, 0.798)	−1.50 (0.87)	(0.06, 0.804)
MC (CV)	0.6 (0.3)	(−0.19, 0.399)	0.8 (0.4)	(−0.16, 0.487)
RT (s)	19.5 (2.8)	(−0.42, 0.057)	19.2 (3.4)	(0.11, 0.646)
RT (CV)	18.6 (9.4)	(0.50, 0.018)	18.4 (12.3)	(−0.41, 0.060)

Data are the retinal artery and venous responses and their relationship with age (bold denotes significant results).

TABLE 4. Retinal Arterial and Venous Dilatory Parameters to Flickering-Light Stimulation

Parameter	Nonsmoker (n = 21)	Smoker (n = 21)	P
Arterial response			
BDF (%)			
Flicker 1	4.79 (2.66)	5.04 (2.63)	0.769
Flicker 2	4.69 (2.75)	6.05 (3.10)	0.141
Flicker 3	4.65 (3.25)	5.95 (2.21)	0.142
Friedman ANOVA (within group)	0.879	0.067	
DA (%)			
Flicker 1	8.17 (3.77)	10.69 (2.94)	**0.020**
Flicker 2	8.25 (3.57)	10.26 (3.96)	0.092
Flicker 3	8.08 (3.73)	10.14 (4.04)	0.098
Friedman ANOVA (within group)	0.915	0.467	
BFR (%)			
Flicker 1	3.37 (2.74)	5.65 (2.11)	**0.004**
Flicker 2	3.55 (2.96)	4.21 (3.22)	0.501
Flicker 3	3.52 (2.74)	4.20 (3.54)	0.501
Friedman ANOVA (within group)	0.818	**0.048**	
Venous response			
MD (%)			
Flicker 1	5.80 (2.57)	6.60 (3.15)	0.382
Flicker 2	6.09 (2.11)	7.08 (2.60)	0.184
Flicker 3	5.86 (2.25)	6.56 (2.84)	0.398
Friedman ANOVA (within group)	0.691	0.580	
MC (%)			
Flicker 1	−1.66 (1.85)	−1.37 (1.24)	0.709
Flicker 2	−1.10 (1.54)	−1.71 (1.29)	0.173
Flicker 3	−1.30 (1.50)	−1.32 (0.86)	0.944
Friedman ANOVA (within group)	0.513	0.861	
DA (%)			
Flicker 1	7.41 (3.98)	7.98 (3.04)	0.609
Flicker 2	7.19 (3.25)	8.79 (2.84)	0.097
Flicker 3	7.16 (3.46)	7.88 (3.19)	0.498
Friedman ANOVA (within group)	0.945	0.212	

Data are expressed as the mean ± SD retinal arterial and venous dilatory parameters for all three flicker cycles (bold denotes significant results). All relative values are expressed as percent changes in baseline diameter.

the software's own data analysis of dynamic retinal vessel response to flickering light in arteries and veins. There were no differences between smokers and nonsmokers.

Examination of retinal arteriolar elasticity (as defined by the ratio of DA divided by BDF) in healthy eyes showed no significant difference in the mean (SD) ratio between the three flicker light cycles: cycle 1, 2.21 (1.10); cycle 2, 2.18 (0.92); and cycle 3, 2.43 (1.22) (P = 0.343). However, in smokers, the ratio decreased from the first to the third cycle: cycle 1, 2.43 (0.94); cycle 2, 1.92 (0.83); and cycle 3, 1.81 (0.69) (P = 0.022).

DISCUSSION

The current approach to the analysis of intraocular blood vessel responses to flicker light is attractive as it is technologically simple and noninvasive, but it has several drawbacks. In the present study, we addressed these questions by assessing the retinal vasculature at baseline and during flickering-light stimulation. The response of retinal vessels to flickering-light stimulation and its relationship to age and sex and to right and left eye and baseline retinal diameter was evaluated in healthy nonsmokers. Testing the applicability and importance of clinical assessment in vascular disease was determined in a sample of regular cigarette smokers who otherwise had no apparent ocular and vascular disease.

TABLE 5. Retinal Arterial and Venous Reaction Time to Flickering-Light Stimulation, according to SDRA

Parameter	Nonsmoker (n = 21)	Smoker (n = 21)	P
Arterial response, s			
Flicker 1	15.0 (5.3)	13.8 (7.7)	0.570
Flicker 2	15.5 (4.3)	17.1 (7.0)	0.387
Flicker 3	14.8 (5.7)	15.6 (5.2)	0.526
Friedman ANOVA (within group)	0.906	0.559	
Venous response, s			
Flicker 1	19.6 (3.4)	19.7 (4.1)	0.955
Flicker 2	19.1 (4.3)	18.5 (5.1)	0.697
Flicker 3	20.1 (4.6)	19.3 (4.3)	0.526
Friedman ANOVA (within group)	0.681	0.434	

Data are expressed as the mean (standard deviation), reaction time for all three flicker cycles of retinal veins and arteries in nonsmokers and smokers.

We have presented technical and validation data of our novel analytical method, SDRA, which has good coefficients of variation. Although response time is variable in arteries and veins, this is less relevant as we have analyzed each flicker response individually. We have demonstrated that dynamic retinal responses in arteries and veins are independent of age, sex, and right/left eye, and that arterial reaction to flicker light stimulation is faster but less pronounced than venous responses. The assumption of the RVA software analysis that arteries and veins react at the same speed[10] therefore should be re-evaluated. SDRA showed marked differences in individual reaction times (based on the 17–23 second model) which the traditional method is unable to address. We find that the traditional method underestimates retinal arterial and venous dilatory responses—that is, that SDRA is more sensitive.

The vascular system degrades with age, and there can be increased development of vessel disease.[20,21] At the retinal level, age-related changes such as arteriolar narrowing and loss of reflex can be readily observed noninvasively by ophthalmoscopy and fundus photography.[22,23] Previous studies using static assessments have reported age to be associated with structural retinal venular dilation in smokers,[24] possibly relating chronic smoking to premature aging of the vascular system. Using SDRA, we were unable find an association between dynamic retinal parameters (and their CVs) and the age of the subject. However, we found that MC increased with age. The loss of reflex with age could explain this finding, since all dimension measurements are based on contrast levels, which

TABLE 6. AVR and RVA Software-Generated Analysis

Parameter	Nonsmoker (n = 21)	Smoker (n = 21)	P
AVR	0.81 (0.12)	0.77 (0.12)	0.243
A_{max}, %	3.47 (2.34)	3.51 (2.21)	0.921
A_{min}, %	−0.32 (1.48)	−0.66 (1.91)	0.485
A_{peak}, %	3.81 (2.82)	4.37 (2.56)	0.330
V_{max}, %	4.65 (1.72)	4.90 (2.60)	0.881

Data are expressed as the mean ± SD. Static retinal vessel data and the software- generated output. All relative values expressed as percent changes compared with baseline diameters.

can be expected to be decreased with age. However, if MC is age dependent, one would expect the CV of MC to be increased in parallel, which was not the case.

In the large sample of 78 nonsmokers, SDRA showed a significant correlation between each baseline diameter and its corresponding dilatory response in all three flicker stimulation cycles in retinal arteries and the first two stimulation cycles in retinal veins. On the basis of this result, we conclude that retinal vessels have a fixed dilatory capacity: the baseline diameter is a strong predictor of the ultimate vessel dilation and must therefore be considered in analysis.

Smoking is the primary risk factor for both CAD and peripheral artery disease (PAD) and causes vascular disease.[25–27] The traditional methods of static retinal imaging and the RVA software analysis were unable to detect any differences in retinal vessel responses in smokers and nonsmokers. However, despite the small sample (n = 21/group), SDRA detected differences in arterial DA, BFR, and elasticity between these groups; there was no difference in venous responses. In the present study, we confirm and extend findings in other studies,[28,29] as we found that smokers had significantly larger retinal venular diameter compared with healthy, nonsmoking control individuals, and we presume that this finding is due to smoking. However, as there were no differences in functional vein responses, the pathophysiological significance of our finding that smokers' venules have a larger diameter (perhaps reflecting structural changes) is unclear. Our data also confirm a report[30] that neither SBP nor DBP has any influence on retinal vascular reactivity.

In arteries, when the BDF was taken into account and the pure reaction to flicker light stimulation was compared between smokers and healthy nonsmokers, a significant difference was apparent only for the first stimulation cycle. It could be hypothesized that diffuse endothelial impairment and decreased bioavailability of NO associated with chronic smoking may have had an exhaustive effect on vessels reactivity and elasticity and may reflect a change in microvascular function at the retinal level in smokers that could be the result of a disequilibrium between endothelial vasodilatory and vasoconstrictive molecules.[31,32]

The static assessment of retinal vessels is a powerful technique for assessing vascular physiology and disease.[1–3] SDRA extends these techniques by examining dynamic changes and indicates that the diameter analysis, as provided by the RVA software, has the tendency to underestimate the dilatory response released by flicker stimulation. In addition, unlike the dynamic method, static retinal vessel evaluation was not able to detect differences between healthy subjects and cigarette smokers. We speculate that SDRA will also detect differences in vascular responses in other conditions such as diabetes and cardiovascular disease.

References

1. Klein R, Klein BE, Knudtson MD. Subclinical atherosclerotic cardiovascular disease and early age-related macular degeneration in a multiracial cohort: the multiethnic study of atherosclerosis. *Arch Ophthalmol.* 2007;125(4):534–543.
2. Wong TY, Klein R, Sharrett AR, et al. The prevalence and risk factors of retinal microvascular abnormalities in older persons: the cardiovascular health study. *Ophthalmology.* 2003;110(4):658–666.
3. Hughes AD, Wong TY, Witt N, et al. Determinants of retinal microvascular architecture in normal subjects. *Microcirculation.* 2009;16(2):159–166.
4. Mandecka A, Dawczynski J, Blum M, et al. Influence of flickering light on the retinal vessels in diabetic patients. *Diabetes Care.* 2007;30(12):3048–3052.
5. Bek T, Hajari J, Jeppesen P. Interaction between flicker-induced vasodilatation and pressure autoregulation in early retinopathy of type 2 diabetes. *Graefes Arch Clin Exp Ophthalmol.* 2008;246(5):763–769.
6. Nagel E, Vilser W, Lanzl IM. Retinal vessel reaction to short-term IOP elevation in ocular hypertensive and glaucoma patients (published correction in *Eur J Ophthalmol.* 2002;12(1):73. Lanzi IM [corrected to Lanzl IM]). *Eur J Ophthalmol.* 2001;11(4):338–344.
7. Lanzl IM, Witta B, Kotliar K, Vilser W. Retinal vessel reaction to 100% O2-breathing: functional imaging using the retinal vessel analyzer with 10 volunteers (in German). *Klin Monbl Augenheilkd.* 2000;217(4):231–235.
8. Wimpissinger B, Resch H, Berisha F, Weigert G, Schmetterer L, Polak K. Response of choroidal blood flow to carbogen breathing in smokers and non-smokers. *Br J Ophthalmol.* 2004;88(6):776–781.
9. Luksch A, Garhöfer G, Imhof A, et al. Effect of inhalation of different mixtures of O(2) and CO(2) on retinal blood flow. *Br J Ophthalmol.* 2002;86(10):1143–1147.
10. Nagel E, Vilser W. Flicker observation light induces diameter response in retinal arterioles: a clinical methodological study. *Br J Ophthalmol.* 2004;88(1):54–56.
11. Luksch A, Garhöfer G, Imhof A, et al. Effect of inhalation of different mixtures of O(2) and CO(2) on retinal blood flow. *Br J Ophthalmol.* 2002;86(10):1143–1147.
12. Jeppesen P, Knudsen ST, Poulsen PL, Mogensen CE, Schmitz O, Bek T. Response of retinal arteriole diameter to increased blood pressure during acute hyperglycaemia. *Acta Ophthalmol Scand.* 2007;85(3):280–286.
13. Dorner GT, Garhöfer G, Huemer KH, Riva CE, Wolzt M, Schmetterer L. Hyperglycemia affects flicker-induced vasodilation in the retina of healthy subjects. *Vision Res.* 2003;43(13):1495–1500.
14. Gugleta K, Zawinka C, Rickenbacher I, et al. Analysis of retinal vasodilation after flicker light stimulation in relation to vasospastic propensity. *Invest Ophthalmol Vis Sci.* 2006;47(9):4034–4041.
15. Nagel E, Vilser W, Lanzl I. Age, blood pressure, and vessel diameter as factors influencing the arterial retinal flicker response. *Invest Ophthalmol Vis Sci.* 2004;45(5):1486–1492.
16. Garhofer G, Bek T, Boehm AG, et al. Use of the retinal vessel analyzer in ocular blood flow research. *Acta Ophthalmol.* Published online August 14, 2009.
17. Williams B, Poulter NR, Brown MJ, et al.; British Hypertension Society. Guidelines for management of hypertension: report of the fourth working party of the British Hypertension Society, 2004-BHS IV. *J Hum Hypertens.* 2004;18:139–185.
18. Nagel E, Vilser W, Fink A, Riemer T. Static vessel analysis in nonmydriatic and mydriatic images. *Klin Monbl Augenheilkd.* 2007;224(5):411–416.
19. Nagel E, Vilser W, Lanzl I. Vergleich der durchmesserreaktion retinaler arterien und venen auf flickerlicht. *Ophthalmologe.* 2005;102(8):787–793.
20. O'Rourke MF. Arterial aging: pathophysiological principles. *Vasc Med.* 2007;12(4):329–341.
21. O'Rourke MF, Hashimoto J. Mechanical factors in arterial aging: a clinical perspective. *J Am Coll Cardiol.* 2007;50(1):1–13.
22. Klein R, Klein BE, Knudtson MD, et al. Subclinical atherosclerotic cardiovascular disease and early age-related macular degeneration in a multiracial cohort: the multiethnic study of atherosclerosis. *Arch Ophthalmol.* 2007;125(4):534–543.
23. Wong TY, Klein R, Sharrett AR, et al. The prevalence and risk factors of retinal microvascular abnormalities in older persons: the cardiovascular health study. *Ophthalmology.* 2003;110(4):658–666.
24. Wang JJ, Taylor B, Wong TY, Retinal vessel diameters and obesity: a population-based study in older persons. *Obesity* (*Silver Spring*). 2006;14(2):206–214.
25. Blann AD, McCollum CN. Adverse influence of cigarette smoking on the endothelium. *Thrombosis Haemostasis.* 1993:70;707–711.
26. Klein R, Sharrett AR, Klein BE, et al. Are retinal arteriolar abnormalities related to atherosclerosis?—the atherosclerosis risk in communities study. *Arterioscler Thromb Vasc Biol.* 2000;20(6):1644–1650.

27. Rahman MM, Laher I. Structural and functional alteration of blood vessels caused by cigarette smoking: an overview of molecular mechanisms. *Curr Vasc Pharmacol.* 2007;5(4):276–292.
28. Wong TY, Islam FM, Klein R, et al. Retinal vascular caliber, cardiovascular risk factors, and inflammation: the multi-ethnic study of atherosclerosis (MESA). *Invest Ophthalmol Vis Sci.* 2006;47(6):2341–2350.
29. Liew G, Sharrett AR, Wang JJ, et al. Relative importance of systemic determinants of retinal arteriolar and venular caliber: the atherosclerosis risk in communities study. *Arch Ophthalmol.* 2008;126(10):1404–1410.
30. Garhofer G, Zawinka C, Huemer KH, Schmetterer L, Dorner GT. Flicker light-induced vasodilatation in the human retina: effect of lactate and changes in mean arterial pressure. *Invest Ophthalmol Vis Sci.* 2003;44(12):5309–5314.
31. Wimpissinger B, Resch H, Berisha F, Weigert G, Schmetterer L, Polak K. Response of choroidal blood flow to carbogen breathing in smokers and nonsmokers. *Br J Ophthalmol.* 2004;88(6):776–781.
32. Wimpissinger B, Resch H, Berisha F, Weigert G, Schmetterer L, Polak K. Response of retinal blood flow to systemic hyperoxia in smokers and nonsmokers. *Graefes Arch Clin Exp Ophthalmol.* 2005;243(7):646–652.