

Assessing vascular function using dynamic retinal diameter measurements: A new insight on the endothelium

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Introduction

Reliable assessment of endothelial function is important for the diagnosis, risk prediction and treatment-monitoring in cardiovascular disease. Numerous techniques exist such as flow mediated dilation (FMD) (1, 2), venous occlusion plethysmography (3, 4), pulse-wave analysis (5) and laser Doppler iontophoresis (6). Individually, each technique provides some information on the state of endothelial function *in vivo* but reflects mainly large artery function; only limited information is conveyed about the body's microcirculatory state. Functional *in vivo* assessment of the microcirculation is largely limited to invasive technology – e.g. coronary angiography (7) – which is not suitable for routine screening. However, novel methods using non-invasive technology such as magnetic resonance imaging and positron emission tomography have shown notable improvements for the assessment of coronary microvasculature (8) but are relatively costly.

In a 2005 review on contemporary methods for assessing endothelial function (9) Alam et al. suggested the retinal vascular bed as suitable for non-invasive *in vivo* assessment. The ocular circulation is unique because it can be viewed directly and strongly resembles cerebral circulation. Measures of ocular circulation can thus be used as surrogate indices of cerebral circulatory mechanisms (10, 11). The focus of Alam's review, however, was on static

retinal vessel analysis and the use of parameters such as diameter measurements obtained by single retinal photographs. Since then, numerous groups have investigated dynamic retinal analysis (12) whereby continuous video recordings are made such that vessel parameters can be assessed over time.

Dynamic retinal analysis has the potential to quantify endothelial function non-invasively *in vivo* over short time periods (usually no more than 5 minutes) by means of continuous vessel diameter measurements (13, 14). In order to achieve optimum imaging quality the pupil must firstly be dilated. The retinal vasculature can then be measured many times a second. Non-invasive stimulation methods (e.g. isometric exercise [15–17] and flickering light [18]) can be used to provoke the retinal vessels to react allowing assessment of endothelial function in retinal arterioles and venules before, during and after stimulation. In contrast to most large artery assessments this is less time consuming, less invasive and more convenient for regular follow-ups. This technology enables both structural and dynamic assessment of microcirculation in humans and a plethora of measures have been introduced to quantify the structural and dynamic properties of retinal circulation (see below).

The importance of static retinal vessel analysis with respect to cardiovascular disease has been demonstrated in the results of large population studies (e.g. the Beaver Dam Eye Study [19], the Arteriosclerosis Risk in Community Study [20] and Rotterdam Study [21]). These studies have found that larger retinal venular calibre is associated with an increased risk for stroke (22, 23), reduced arterial wall compliance in large arterial beds is associated with retinal arteriolar narrowing and reduced arterial wall compliance in small arterial beds is associated with retinal venular widening (23). Despite its predictive value for evaluating risk of cardiovascular disease this

method only provides a snapshot of an individual's circulatory state. Assessment of the dynamic properties of the circulatory system could provide additional information about endothelial dysfunction and facilitate early diagnosis of related diseases. Hence, this review is focused on describing dynamic retinal diameter assessments and their potential for assessing endothelial function *in vivo* in humans.

Mechanisms underlying retinal vessel dilation to flicker light (FL) provocation

The reaction of retinal photoreceptors to different luminance levels alters their metabolic demand thus increasing blood flow in the inner retina. The increased metabolic demand due to the application of flickering light triggers a cascade of reactions. One such reaction is the release of nitric oxide (NO) which dilates retinal vessels. The role of NO and its role in retinal vasodilation have been studied in detail by changing lactate and glucose levels either by exogenous or endogenous routes (24, 25). Intravenous administration of the NO synthase inhibitor N_G -monomethyl-L-Arginine (26) reduces dilatory response of arteries and veins, following flicker-light stimulation, to approximately the level of normal vessel pulsation (~2–3%). Collectively these studies showed that blocking NO release blunts the retinal vessel dilatory response and demonstrates the role of NO in controlling retinal vascular tone.

Equipment and provocation protocols

The only commercially available system capable of continuous measurement of reti-

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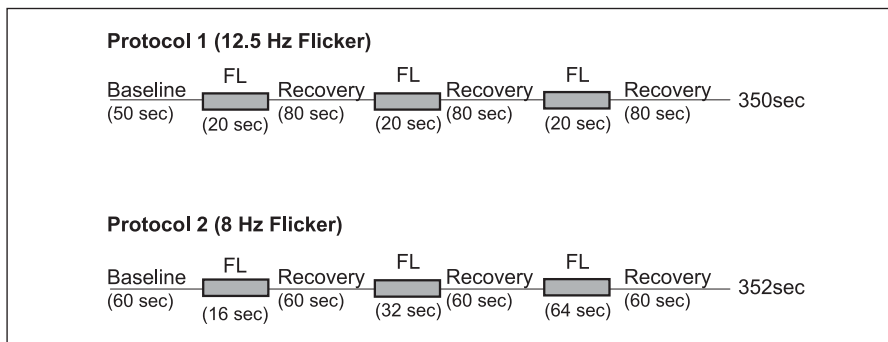


Figure 1: The two FL provocation protocols in common use. Top: 50 s baseline measurement followed by three cycles of 20 s flicker and 80 s recovery. Bottom: 60 s baseline measurement followed by three cycles of flicker provocation of increasing duration (16 s, 32 s and 64 s) and a fixed 60 s recovery per cycle.

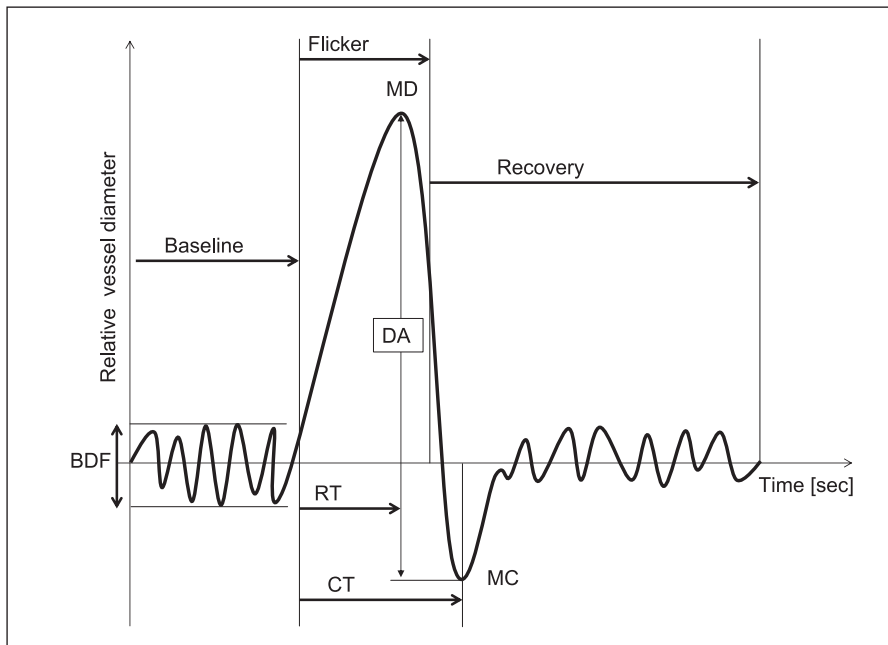


Figure 2: Schematic diagram of a single cycle of retinal vessel diameter recording, before, during and after FL-provocation. Calculation of various spine points is indicated: BDF: baseline diameter fluctuation; MD: maximum dilation; MC: maximum constriction; DA: dilation amplitude; RT: reaction time; CT: constriction time.

nal vessel diameters is the dynamic Retinal Vessel Analyser by IMEDOS (Imedos, Jena, Germany). This setup comprises the Zeiss FF450 fundus camera, a digital video camera, and a computer for real-time capture and analysis of the video signal. Flicker-light provocation is achieved by interrupting the fundus illumination using an optoelectronic shutter at a rate of 12.5 Hz. The video camera normally captures the fundus image at 25 Hz; during FL-provocation this drops to 12.5 Hz (though note Protocol 2

below). The video signal is digitised and can also be stored for later reanalysis. The accompanying software analyses the video signal and estimates the diameter of short sections (up to ~1 mm) of selected retinal vessels at a resolution of approximately 12.5 $\mu\text{m}/\text{pixel}$.

Currently there is a lack of standardisation in the collection and evaluation of data from dynamic RVA (Retinal Vessel Analyser), resulting in numerous provocation protocols and a bewildering array of data

analyses. Recently Garhofer et al. (12) have argued for the need to standardise both the protocols and data analysis to enable better study comparison. Fortunately, the underlying data acquisition has seen the use of only a few different protocols.

Flicker light provocation

Protocol 1 (or the Standard protocol)

Due to its incorporation into the Imedos system the most widely used protocol to date is a 350 second (s) continuous video recording of the retinal vasculature comprising 50 s of baseline measurement followed by three cycles of 12.5 Hz flicker light (FL) for 20 s and 80 s recovery. In the Imedos setup the 12.5 Hz flicker is generated by an optoelectronic shutter that interrupts the fundus illumination on alternate video frames. Consequently, during flicker, recording only takes place during half of the frames yielding a sampling rate of 12.5 Hz (27).

Protocol 2

This protocol uses 8 Hz flicker generated from a light source that is, additionally, spectrally separate from the fundus illumination. With suitable spectral filtering of the video camera to remove the flicker illumination this setup allows uninterrupted data acquisition (15). Although the length of recording is similar (352 s), studies carried out with this protocol tend to use shorter recovery times (duration between flicker cycles) and increasing flicker durations for each cycle (see ► Fig. 1).

Isometric exercise

Provocation of the vascular system using isometric exercise is usually achieved by the participants holding a weight resulting in an increase in systemic blood pressure. This technique has been used to assess the Bayliss effect (28) and also to examine regional differences in regulating vascular tone in diabetes (29).

Data analysis

The sheer volume of data from each recording session – up to 80 data points per vessel segment per video frame – has led most researchers to average the measurements over short time frames and along the vessel profile at different stages of the protocol (termed spine point analysis [30]). Recently, Fourier analysis has been used in an attempt to analyse the longitudinal vessel profiles averaged over time only (31). Both the spine point analysis and the Fourier analysis are detailed below.

Spine points and time course analysis

Usually the diameter readings are averaged over the vessel length and then averaged over 1-s intervals (32). The mean baseline diameter is then computed for 20–30 s prior to the first flicker and the whole time-series is normalised such that the diameter readings are expressed as percent of baseline. Several measures have been calculated from this normalised time-series. Initially researchers calculated maximum dilation (MD) and/or maximum constriction (MC) (30, 33). Then more complex computations were reported that attempted to characterise the vessel's elastic behaviour such as dilation amplitude (DA) – computed as MD-MC – and baseline diameter fluctuation (BDF) – the maximal range of diameter measurements during baseline (30, 32). The time from onset of flicker to the points of MD and MC, known as reaction time (RT) and constriction time (CT) respectively, have also been used to characterise provocation (32, 34–36). These spine points (see ► Fig. 2) can be analysed individually for each stimulation cycle or computed as the average over the number of cycles.

A recent additional parameter is area under the curve (AUC) (37, 38) at different time periods during the measurement cycle (see ► Fig. 3), e.g. baseline, flicker, post-flicker. This measure, in conjunction with the relevant spine points, e.g. MD and RT, indicates the amount, speed and longevity of the vessel's reaction.

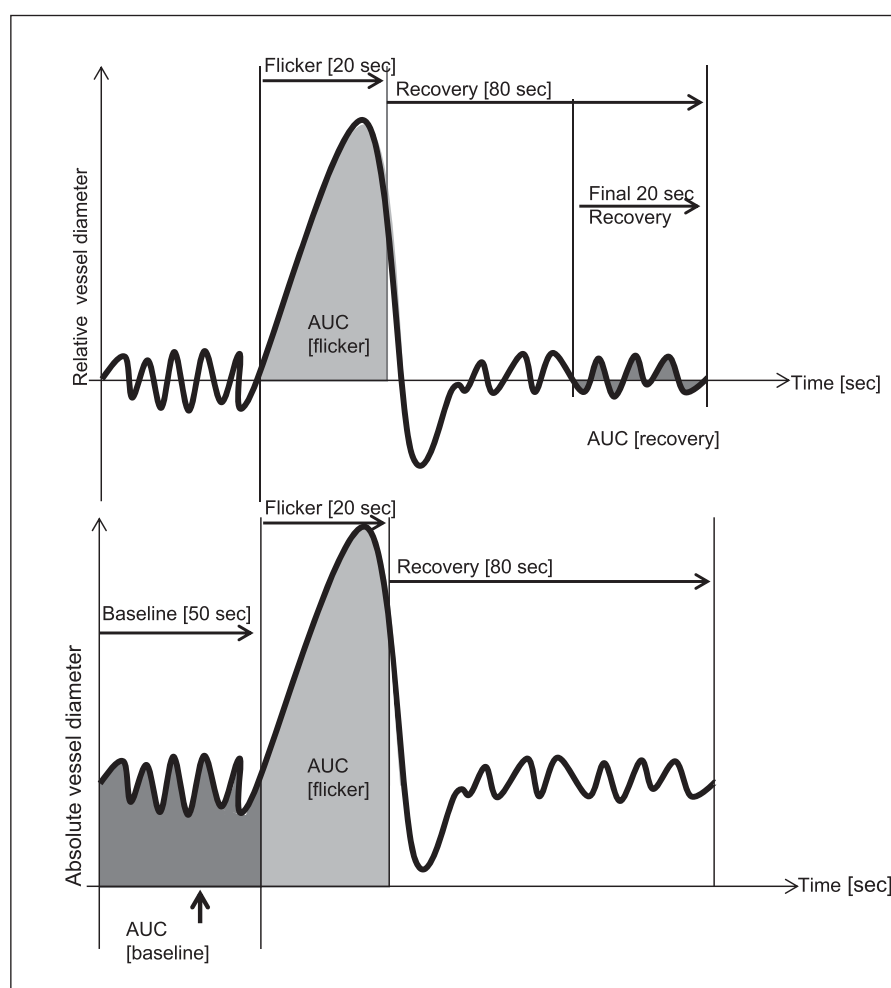


Figure 3: Schematic diagram of a single cycle of retinal vessel diameter recording, before, during and after FL-provocation. Calculation of area under the reaction curve (AUC) for (top) normalised vessel diameters (39) and (bottom) absolute vessel diameters (38) is shown.

Fourier analysis of longitudinal vessel profiles

Recent work (31) has used Fourier analysis to characterise the structure of vessel segments along their length, collapsed across various time periods. In short, Fourier analysis is used to decompose signals into a series of sine-waves of varying amplitude, frequency and phase. The power spectrum represents the energy ($amplitude^2$) in the original signal as a function of frequency. The spectral edge frequency (SEF) of the power spectrum of the longitudinal vessel profiles has been used to characterise the micro-structure of the vessel wall (31, 39). Increasing SEF indicates increasing energy in the higher-frequency parts of the signal,

which could be interpreted as reflecting the degree of roughness of the vessel wall. The SEF has been measured for longitudinal arterial vessel profiles associated with baseline, dilation, constriction and recovery periods of the stimulation cycle in both healthy subjects (31) and HT (39).

Reproducibility and repeatability of RVA measurements

Without applying any stimulation method, continuous retinal vessel diameter measurements appear to be highly repeatable in healthy subjects in the short term (up to a few days) (14, 40–42). Mean sub-

jects' coefficient of variation (CV) (either from three or four measurements taken over hours or days [14], or of the three cycles of a single measurement [40, 41]) is usually reported and CV values lying in the range of 1–5% are typical (14, 32, 40, 41). However, since each subject's CV is calculated from only three or four measurements the 95% confidence interval (CI) for CV is rather large (for four measurements 95% CI = $[0.57 \times CV, 3.73 \times CV]$). It is, therefore, almost impossible to infer the repeatability of RVA from these CV.

A more useful measure of reproducibility is the intra-class correlation coefficient R_1 or κ which has been reported for short-term (<1 day) and day-to-day (three days) readings (14). κ was very high for both short-term ($\kappa_{\text{arteries}}=0.96$ and $\kappa_{\text{veins}}=0.98$) and for day-to-day ($\kappa_{\text{arteries}}=0.87$ and $\kappa_{\text{veins}}=0.90$) measurements. This indicates

that, for healthy subjects before FL-provocation, the estimation of vessel diameter is highly reproducible in the short term.

Unfortunately, κ has not been computed for spine-points and we only have limited CV data from three cycles of a single recording session for a limited number of measures. From 21 healthy non-smokers (32), MC ($CV_{\text{arteries}}=1.2\%$, $CV_{\text{veins}}=0.6\%$) and MD ($CV_{\text{arteries}}=1.3\%$, $CV_{\text{veins}}=1.0\%$) are least variable whilst RT is very variable ($CV_{\text{arteries}}=30.6\%$, $CV_{\text{veins}}=18.6\%$). We can find no published data on the repeatability of AUC or spectral analysis.

For healthy patients MD relative to baseline for arteries and veins has been found to lie in the range ~3%–7% (33, 34, 43–48)¹ though veins usually dilate slightly more

¹ Note using protocol 2 (43) lower values of MD were found for healthy controls: 1.6% to 2.8%.

than arteries. In CVD patients MD of arteries is in the range 3.1% to 5.7% and for veins is 3.6% to 5% (34, 49). For Diabetes patients MD, which is generally significantly smaller compared with controls, for arteries lies in the range 0.1–2.9% (dependent on type and presence of retinopathy) and in veins 0.5–4.6% (29, 44–47). For HT patients MD of arteries values lie in the range 3.6–4.3 and veins 3.6–6.0% (find 33, 47, 48).

Links with well-established measures of vascular endothelial function

Significant correlations have been found between retinal vessel diameters obtained from static retinal photography and plasma

Table 1: Patients suffering from cardiovascular disease.

Author	Year	Subjects	Measurements	Main outcome
Garhöfer G (43)	2004	24 DM type1 24 Controls	Protocol 2	No significant arterial vessel dilation in response to flicker stimulus in DM. No association with DM duration.
Nagel E (33)	2006	9 HT patients 11 controls	Standard protocol (repeat: 1.5–2.5 years time interval)	No significant change in retinal vessel reactivity to flicker light in patients or controls after ~24 months. In HT patients arteriolar baseline diameter negatively correlated with change in MAP between visits.
Mandecka A (44)	2007	172 DM type 2 68 DM type 1 53 controls	Standard protocol	Reduced arterial dilation in DM (type 1 or type 2) compared to controls. Flicker response in retinal vessels diminished with increasing severity of diabetic retinopathy.
Mandecka A (45)	2009	18 DM type 1 19 controls	Standard protocol	Reduced arterial and venous dilation during flicker stimulation despite comparable static retinal parameters.
Nguyen TT (46)	2009	85 DM type 1 139 DM type 2 103 controls	Standard protocol	Reduced arterial and venous dilation during flicker stimulation. Reduced reactivity due to flicker was a significant indicator of DM.
Pemp B (47)	2009	20 DM type 1 40 HT 20 controls	60s baseline; 60s 12.5Hz flicker; 120s recovery.	Reduced dilatory response in retinal vessels in DM and HT compared to controls. Brachial artery FMD correlated negatively with FL-induced retinal dilatory parameters. No association between retinal dynamic parameters and age was found.
Pressler A (48)	2010	34 patients after isthmic coarctation 34 controls	Standard protocol	Retinal arterial diameter assessed by static photography after isthmic coarctation significantly reduced. No functional difference (MD, MC) between patients and controls measured with RVA.
Heitmar R (34)	2011	24 CAD patients 30 controls	Standard protocol	Reduced RT and CT in retinal arterioles to flicker stimulation in CAD patients.
Jensen PS (29)	2011	17 patients with diabetic maculopathy 17 patients with peripheral diabetic retinopathy 17 controls	3 protocols (isometric exercise, flicker, and isometric exercise with flicker)	Significant difference in arteriole diameter due to isometric exercise or FL-provocation between all three groups.

Table 2: Individuals at risk of developing cardiovascular disease.

Author	Year	Subjects	Measurements	Main outcome
Reimann M (49)	2009	21 hypercholesterolaemic patients	Standard protocol	Reduced retinal arteriolar and venular reactivity to flicker provocation. Increased venous reactivity after single LDL apheresis (attributed to larger baseline diameter in veins rather than LDL apheresis per se).
Heitmar R (32)	2010	78 healthy controls 21 otherwise healthy smokers	Standard protocol	Significantly increased level of arterial baseline diameter fluctuation before flicker stimulation in smokers. Significantly increased stiffness of retinal arterioles in smokers than non-smokers.
Kotliar KE (37)	2011	46 obese volunteers 46 controls	Standard protocol	Reduced retinal vessel dilatory response in obese patients. Increased RT of arterioles for obese patients. Correlation between AUC 50–80 s after FL and waist circumference.
Patel S (36)	2011	45 healthy White Europeans (WE) 45 healthy South Asians (SA)	Standard protocol	South Asians had higher fasting triglyceride and lower HDL levels than Western Europeans. Arterial RT positively correlated with measured plasma TG levels only in SA.

Table 3: Effect of age upon dynamic retinal vessel parameters.

Author	Year	Subjects	Measurements	Main outcome
Kotliar KE (31)	2008	33 healthy volunteers	Two cycles of: 100 s baseline; 60 s 12.5 Hz flicker; 150 s recovery.	Correlation between an index thought to be related to arterial-wall roughness and age of participants.
Kneser M (54)	2009	52 healthy volunteers Averaged spine point analysis	Standard protocol	Reduced arterial dilation to flicker provocation in older participants.
Heitmar R (32)	2010	78 healthy volunteers	Standard protocol	No relationship between MD, MC, DA, RT, CT (etc) of individual flicker cycles and age.

markers of endothelial function (20) and retinopathy (50, 51) but not FMD (52). Static retinal vessel analysis has also revealed a significant correlation between a reduction in FMD and the diameter of wider retinal veins but not arteries (53). The different results for arteries and veins were attributed to differences in regulation of macro- and micro-circulation and the uniqueness of the retinal circulation. However, the lack of an association could also be due to the comparison between a method that captures dynamic properties (FMD) with one that does not (retinal photography).

Correlations have been found between FMD and FL-induced arteriolar (but not venular) dilation in controls, diabetic patients and HT patients (47). The correlation was weak but significant (Pearson's $r=0.3$, $p=0.044$), perhaps due to the data being pooled over all participants that

limited the strength of the analysis. However, this is evidence that dynamic retinal vessel assessment is capturing functional properties of the vascular system.

So far, no firm conclusions can be drawn regarding a correlation between measurements of large arteries and retinal endothelial measurements. This does not mean, however, that there is no connection. The vessel morphology of micro- and macro-circulation differs along with their regulatory mechanisms. Systemic blood pressure regulation depends on both macro- and micro-circulation in order to function normally; an imbalance in one system could influence the other.

Overview of current studies using continuous retinal diameter measurements

The different studies relevant for assessing endothelial function in systemic disease have been summarised in ► Tables 1–4 and are described further in the following section.

Value of assessing micro-circulatory parameters of endothelial function

Continuous retinal vessel diameter measurements have been widely applied to assess endothelial function in diabetes mellitus (29, 43–45), hypertension (31, 33, 47, 48), obesity (37), smoking (32, 57), coron-

Table 4: Patients suffering from peripheral vasospasm and ocular vascular abnormalities.

Author	Year	Subjects	Measurements	Main outcome
Branca F (55)	2005	30 healthy volunteers (15 with peripheral vasospasm)	60 s baseline measurement only (2 visits: 1 week apart)	No difference in baseline measurements between groups. Good repeatability across visits of single baseline diameter measurements in arteries and veins.
Gugleta K (56)	2006	24 healthy volunteers (14 with peripheral vasospasm)	Standard protocol	Dilatory response augmentation seen in healthy but not vasospastic subjects. Reduced arteriole and venule dilation to FL (in distal and proximal vessels).
Lanzl IM (35)	2010	10 AMD patients 10 controls	Standard protocol	Diminished arteriolar and venular response to FL provocation in AMD patients compared to controls. No change in vessel reactivity after single dose of VEGF inhibitor.
Gugleta K (38)	2011	47 POAG 46 OHT 56 controls	Standard protocol	Differences in inferior and superior retinal vessel reactivity. POAG patients had lower reactivity.

ary artery disease (34) and ocular/vascular dysregulation (38, 55, 56). Many of these studies link a diminished retinal vascular response or delayed provocation reactivity with endothelial dysfunction. Although there is overwhelming evidence that the mechanisms underlying vascular dilation to FL provocation reflect endothelial function (27, 44, 58), thus far few studies have found a link between established markers of endothelial function and FL provocation (20, 47, 51–53). In these groups, the presence of an imbalance of vasoconstrictive and vasodilatory factors implies endothelial dysfunction. Further research is needed with larger sample sizes and proper control of confounding factors such as the use of consistent acquisition times.

The autonomic nervous system is also believed to be involved in the regulation of vascular reactivity in the eye (43, 59). Patients suffering from primary Raynaud's Syndrome have normal FMD values (60) but significant abnormalities in their microcirculation (60, 61); micro- and macro-vessel endothelial dysfunction need not occur simultaneously. Assessment of one vascular bed provides only limited and possibly misleading information about the whole vascular system.

Differences in the regulation of vascular tone between central and peripheral retinal arterioles have been shown in patients suffering from DM but presenting with regionally different diabetic eye disease (29). This local difference in DM highlights the

need for assessing different vascular beds and use methodologies capable of evaluating differences in regulatory mechanisms. Therefore, the assessment of endothelial function at multiple vascular beds can highlight differences in the underlying pathophysiology. Microalbuminuria, also a marker of endothelial function, has shown to correlate with arterial dilation in healthy obese adolescents, but not in DM type I (62). However, retinal vascular changes do not necessarily occur at the same time as vascular changes in the renal system (63).

In summary, dynamic retinal vessel diameter assessment using FL and/or isometric exercise provocation is most likely a surrogate measure of endothelial function at the microcirculatory level, though note that there is no long-term data concerning the value of RVA measures in predicting future CVD events. It is, however, essential to be aware of its test re-test variability (41, 42). A carefully devised measurement protocol in conjunction with large-artery endothelial assessment should provide a more comprehensive picture of vascular health.

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Conflicts of interest

None declared.

Appendix: Spine point terminology		
Parameter	Description	Calculation
BDF	Baseline Diameter Fluctuation	Maximum amplitude during baseline
BfR	Baseline corrected flicker Response	$BfR = DA - BDF$
DA	Dilation Amplitude	$MD - MC$
ΔD	Vessel diameter increase after flicker initiation to the point where MD is reached	$MD - 1 \text{ s mean baseline diameter (prior to flicker initiation)}$
MD	Maximum Dilation	Maximum diameter following onset of flicker light provocation
MC	Maximum Constriction	Minimum diameter after MD
RT	Reaction Time	Time (seconds) to reach MD after flicker initiation
CT	Constriction Time	Time (seconds) to reach MC after flicker initiation
AU(R)C	Area Under the (Reaction) Curve	Integration of diameter measurements over specific time period

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