Comparative study of microvascular function: Forearm blood flow versus dynamic retinal vessel analysis

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Objective: Recently, dynamic retinal vessel analysis (DVA) has gained interest for investigation of microvascular function but comparative measurements with standard methods like the forearm blood flow technique (FBF) are uncommon till now.

Methods: We recruited 23 high-risk cardiovascular patients (Risk) and 17 healthy persons (Ctrl). During the FBF experiment, postocclusive reactive hyperaemia (RH) as well as endothelium-dependent and independent vasodilation was measured by infusion of acetylcholine (ACh) and sodium nitroprusside (SNP) into the brachial artery. The dynamic vessel analyzer was applied for measurement of the retinal arterial and venous response to flickering light during DVA and for determination of the central retinal arterial (CRAE) and venous equivalent (CRVE).

Results: Forearm blood flow technique was significantly attenuated in the patient group during postocclusive RH ($p < .005$). The increase of FBF in response to SNP did not differ significantly between the two groups ($p = .09$). In contrast, the FBF response to ACh was significantly blunted in the patient group ($p < .05$), indicating endothelial dysfunction. DVA did not detect any difference of retinal arterial ($p = .68$) or retinal venous ($p = .93$) vasodilation between both groups. The CRAE ($p = .55$) and CRVE ($p = .83$) did not differ significantly in either group.

Conclusions: Forearm blood flow and DVA cannot be regarded as equivalent methods for testing of microvascular function. Possible explanations include differences in the vascular beds and vessel diameters examined as well as differences in the trigger mechanisms applied. Further studies are needed to define the role of DVA in this context.

Keywords: central retinal arterial equivalent, central retinal venous equivalent, endothelial function, microcirculation, nitric oxide, postocclusive reactive hyperaemia, retinal vascular function imaging, venous occlusion plethysmography
1 | INTRODUCTION

Endothelial dysfunction plays a key role in the pathogenesis of cardiovascular disease (Epstein et al., 1990), and retinal vasculature may provide a non-invasive approach to examine the microvascular endothelial function. An increase of human retinal vessel diameter in response to flickering light was initially investigated by Formaz et al. (1997). Subsequently, the commercially available dynamic vessel analyser system was launched (Garhofer et al., 2010; Nagel et al., 2001; Polak et al., 2002) and a decrease of flickering light-induced dilation of the retinal vasculature has been reported in several conditions that were at least partly associated with endothelial dysfunction like impaired glucose tolerance, diabetic retinopathy, untreated hypertension, hyperlipidaemia and obesity (Kotliar et al., 2011; Lim et al., 2014; Nagel et al., 2004; Petersen & Bek, 2017). However, these methods are very complex for routine clinical use and are also problematic for cardiovascular high-risk patients. Furthermore, static retinal vessel analysis revealed that patients with diabetes and a wider CRAE were more likely to have prevalent heart failure, while a wider CRVE was associated with the risk of ischaemic heart disease (Drobnjak et al., 2016; Phan et al., 2015). Endothelial function is closely linked to endothelial nitric oxide (NO) production (Furchgott & Zawadski, 1980), and flickering light-induced vasodilation was attenuated during inhibition of endothelial and neuronal NO synthase with L-NMMA (Dorner et al., 2003) but whether this result was of predominantly vascular or neuronal origin is still unclear. To the best of our knowledge, there has been no study up to now to investigate the relationship between retinal vascular calibre, ocular blood flow and retinal neuronal activity. Moreover, DVA has not been extensively compared to other accepted test methods of endothelial function. In a previous study, we found that acute changes in endothelial function caused by the intake of L-methionine and fat can be measured by DVA with comparable reproducibility as brachial flow-mediated dilation (FMD; Reimann et al., 2015). While in another study both DVA and FMD were reduced in patients with diabetes, hypertension and hyperlipidaemia, only a weak correlation was observed (Pemp et al., 2009). Accordingly, it is still unclear whether DVA is sufficient as a single approach for evaluation of systemic endothelial function and could serve as a biomarker of cardiovascular disease that could be adopted in clinical practice. Thus, the aim of our present study was to compare DVA with forearm venous occlusion plethysmography (FBF) as one of the “gold standards” in the assessment of endothelial function (Benjamin et al., 1995; Wilkinson & Webb, 2002). We would like to investigate whether both DVA and FBF could differentiate a group of cardiovascular high-risk patients from healthy persons and can therefore be equivalently used for the examination of endothelial function in patients.

2 | METHODS

2.1 | Subjects

We recruited 23 male patients with either a high-risk cardiovascular profile or manifest cardiovascular disease (Risk) and a control group (Ctrl) of 17 cardiovascular healthy male subjects. Exclusion criteria were eye disease, epilepsy, intolerance to nitrate vasodilators, intake of PDE5 inhibitors and arterial hypotension (<100 mmHg syst. BP). In healthy control subjects, hypertension or an underlying vascular disease was excluded by physical examination. Cigarettes, alcohol and all caffeine-containing beverages were withheld for 12 hr before the beginning of the study. All participants gave their written informed consent. The study protocol was approved by the University of Dresden Ethics Committee (Bearbeitungsnummer/Processing Number: EK 282092010). The investigation conforms to the principles outlined in the Declaration of Helsinki (1964).

2.2 | Forearm blood flow studies

All investigations were performed in a quiet room kept at a constant temperature of between 22 and 24°C. Each subject was in a supine position with both forearms resting slightly above heart level. FBF was measured simultaneously in both arms by venous occlusion plethysmography. Pressure of the congesting cuffs of both upper arms was set at 40 mmHg. Mercury-in-silastic strain gauges were wrapped around the widest parts of the forearms and connected to a calibrated venous occlusion plethysmograph (Gutmann Medizinelektronik, Eurausb, Germany). The blood flow of the hands was excluded by wrist cuffs inflated to a supra-systolic pressure (220 mmHg) during each measurement period. One FBF determination consisted of ten single FBF measurements, each lasting ten seconds, at a 15-s interval (except for the ten-second interval of FBF measurements during postocclusive reactive hyperaemia). The final five blood flow recordings were used to calculate the mean FBF. FBF is expressed as the millilitre of blood flow per 100 ml of forearm volume per minute. Blood pressure and heart rate were measured throughout each protocol at ten-minute intervals at the calf (using an automated device; Dinamap, Critikon, USA).

2.3 | Postocclusive reactive hyperaemia

First, the baseline FBF was measured in both forearms. To produce reactive hyperaemia (RH), blood flow to the forearm was prevented by inflation of the congesting cuffs of both upper arms to supra-systolic pressure (220 mmHg). The duration of arterial occlusion was 5 min. Subsequently, both cuffs were released automatically and ten single postischaemic FBF measurements were carried out in a shortened interval of ten seconds in both forearms (Figure 1a). The respective FBF of the non-dominant forearm (baseline and RH) was used as the test result.
2.4 | Arterial vascular access

After the RH experiment, the brachial artery of the non-dominant arm was cannulated with a 27-G steel needle (Coopers Needle Work, Birmingham, UK) for drug infusion (Figure 1a). The infusion rate of each substance was kept constant at 1 ml/min.

2.5 | Assessment of endothelium-dependent and endothelium-independent vasodilation

Saline solution was infused for 20 min to establish baseline conditions, and baseline FBF was measured again. For the analysis of endothelium-dependent vasodilation, acetylcholine (ACh, Miochol-E®, Ciba Vision, Germering, Germany) was infused at graded doses of 55, 110 and 220 nmol/min. Each dose was given over a period of 5 min. The FBF was measured during the last 2.5 min of each infusion period (Figure 1a). Subsequently, saline solution was infused for 20 min to reestablish baseline conditions. The baseline FBF was measured again. For the assessment of endothelium-independent vasodilation, sodium nitroprusside (SNP, Nipruss®, Schwarz Pharma, Monheim, Germany) was infused at graded doses of 2.5, 5 and 10 µg/min. SNP was dissolved in glucose 5% avoiding exposure to light. Each dose was given over 5 min. The FBF was measured during the last 2.5 min of each infusion period (Figure 1a). The order of ACh and SNP was changed with each subject to rule out an influence of the infusion sequence to the FBF.

2.6 | Retinal vessel analysis

After completion of the FBF studies and a short rest, static and dynamic retinal vessel analysis was performed in each subject using the dynamic vessel analyzer (DVA, IMEDOS, Jena, Germany). The dynamic vessel analyzer consisted of a retina camera with integrated optoelectronic shutter for optional interruption of fundus illumination (flickering light stimulation) during dynamic retinal vessel analysis (FF450plus, Carl Zeiss Meditec, Jena, Germany), a charge-coupled video camera for online imaging and computer units for system control, analysis and recording of the obtained data. Video sequences of each retinal vessel examination were digitized, which provided the possibility of off-line reassessment of the data. Following mydriasis of the right pupil by 1% tropicamide eye drops, the central retinal arterial (CRAE, scale unit: µm) and venous equivalents (CRVE, scale unit: µm) were determined using the equations of Parr and Hubbard (Hubbard et al., 1999). Subsequently, the diameter of an arterial and a venous retinal vessel segment was measured continuously during dynamic vessel analysis. The baseline diameter of each arterial and venous segment was measured after 30 s of steady fundus illumination (scale unit: µm), to which the subsequent diameter response was
normalized (change of diameter in %). The maximal vessel dilation was the largest vessel diameter averaged across three cycles of 20 s flickering light stimulation interrupted by 50 s of steady fundus illumination (Figure 1b). During DVA, blood pressure and heart rate were monitored with a non-invasive continuous blood pressure measurement system (Colin CBM-7000, Nihon Colin Co, Komaki, Japan).

2.7 | Statistical analysis

The SPSS software package (SPSS Inc., Chicago, IL, USA) was used for all statistical calculations. Results are presented as mean ± SEM. Values of $p < .05$ were considered to be statistically significant. The general group characteristics, the differences of the FBF baseline values, CRAE, CRVE, retinal baseline diameters as well as retinal arterial and retinal venous responses to flickering light between both groups were examined using a $t$ test after checking for normal distribution with the Shapiro–Wilk test. If no normal distribution was present, the Mann–Whitney $U$ test was used for significance analysis. The FBF of the patient group and the control group during postocclusive RH was analysed using split-plot ANOVA (dependent variable: FBF; within-subjects factor: time after ischaemia; between-subjects factor: Risk/Ctrl). Accordingly, differences in FBF during the ACh protocol and the SNP protocol were analysed by split-plot ANOVA (dependent variable: FBF; within-subjects factor: graded dose of ACh or SNP; between-subjects factor: Risk/Ctrl). Pearson's correlation coefficient was used for testing of linear correlation between the peak FBF value during postocclusive RH and the maximum FBF responses during the ACh and SNP protocols.

3 | RESULTS

Baseline characteristics of the subjects participating in each group and the cardiovascular profile of the patient group are given in Table 1. The high-risk subjects were older (56.9 ± 2.3 years in Risk; vs. 38.6 ± 2.3 years in Ctrl; $p < .005$) and smaller (176.4 ± 0.9 cm in Risk; vs. 181.5 ± 1.8 cm in Ctrl; $p < .02$) than the controls. Blood pressure and heart rate remained stable in each individual during the FBF studies and retinal vessel analysis, excluding systemic effects of the experiments.

3.1 | Results of FBF studies

The basal FBF did not differ between both groups in the RH, ACh and SNP protocol (Table 2).

3.2 | FBF during postocclusive RH

After 5 min of ischaemia, the FBF increased immediately to its peak value and subsequently decreased continuously during the period of RH. When compared to the controls, the FBF was significantly blunted in the patient group during postocclusive RH ($p < .005$; Figure 2a).

3.3 | Response of FBF to infusion of ACh

Acetylcholine increased FBF through a dose-dependent manner in both groups. However, there was a significantly attenuated
vascular response in the patient group when compared to the controls ($p < .05$; Figure 2b).

### 3.4 | Response of FBF to infusion of SNP

Sodium nitroprusside infusion resulted in a dose-dependent increase of FBF in both groups. There was no statistically significant difference of FBF between both groups ($p = .09$; Figure 2c).

### 3.5 | Testing of correlation

All subjects taken together (Risk and Ctrl), the peak FBF value during postocclusive RH correlated significantly with the maximum FBF response during the ACh protocol at 220 nmol/min ($r = .38$; $p < .05$; Figure 3a). But the peak FBF value during postocclusive RH did not correlate with the maximum FBF response during the SNP protocol at 10 $\mu$g/min ($r = .26$; $p = .11$; Figure 3b).

### 3.6 | Results of static retinal vessel analysis

Central retinal arterial amounted to 189.5 ± 5.39 $\mu$m in the patient group and to 188.9 ± 2.97 $\mu$m in the control group ($p = .55$; Figure 4a). CRVE amounted to 215.4 ± 4.46 $\mu$m in the patient group and to 214 ± 4.5 $\mu$m in the control group ($p = .83$, Figure 4b).

### 3.7 | Results of DVA

The retinal arterial baseline diameter amounted to 117.0 ± 4.1 $\mu$m in the patient group and to 122.5 ± 3.7 $\mu$m in the control group ($p = .34$). The retinal venous baseline diameter amounted to 152.2 ± 3.9 $\mu$m in the patient group and to 152.0 ± 5.0 $\mu$m in the control group ($p = 1$). There was also no statistically significant difference of retinal arterial vasodilation (3.4 ± 0.6%; vs. 3.1 ± 0.5%; $p = .68$; Figure 4c) and retinal venous vasodilation (4.0 ± 0.5%; vs. 4.1 ± 0.5%; $p = .93$; Figure 4d) after flickering light stimulation between the patient group and the control group.

### 4 | DISCUSSION

Up to now, it is still unknown whether results of DVA could be generalized to the whole-body vasculature in health and disease (Heitmar
Therefore, we aimed to compare DVA with FBF as one standard method for the assessment of microvascular endothelial function (Benjamin et al., 1995; Wilkinson & Webb, 2002). We asked whether DVA and FBF would be able to separate a group of high-risk cardiovascular patients from healthy persons and could therefore be used equivalently in clinical studies. The patient group was deliberately inhomogeneous in order to represent a wide range of cardiovascular diseases like diabetes, coronary heart disease and peripheral arterial occlusive disease. Interestingly, FBF was significantly attenuated in patients during postocclusive RH indicating vascular dysfunction. Iwatsubo et al. (1997) also reported on impaired FBF during postocclusive RH in subjects with essential hypertension according to the 96% incidence of arterial hypertension in the patient group. Although the mechanisms leading from ischaemia to vasodilation cannot be reconstructed completely, a significant contribution of endothelial NO to the mid- to late phase of RH was discovered (Joannides et al., 1995; Tagawa et al., 1994). This relationship has been further confirmed by the direct correlation of FBF during RH with FBF after infusion of ACh (Higashi et al., 2001) that was also evident in our subjects. Investigation of ACh-mediated endothelium-dependent vasodilation revealed a significantly blunted FBF response in the patient group demonstrating endothelial dysfunction. However, there was no difference in the increase of FBF during SNP-induced endothelium-independent vasodilation between both groups. These results are in compliance with other FBF studies that showed impaired ACh-mediated vasodilation on the one hand and preserved vascular response to SNP on the other hand in patients with diabetes mellitus, dyslipidaemia or essential hypertension (Casino et al., 1993; Linder et al., 1990; Mäkimattila et al., 1999; Panza et al., 1990). An important matter is that Heitzer et al. (2001) identified a blunted ACh-induced FBF response as an independent predictor of further cardiovascular events, highlighting the role of endothelial dysfunction. Against our expectations and although a significant vascular dysfunction was detected by FBF, DVA and static retinal vessel analysis were unable to detect any difference in retinal arterial or retinal venous parameters between both groups. This result was not consistent with that part of the literature reporting a diminished retinal vascular response during DVA in highly selected homogeneous patient groups with diabetes mellitus, dyslipidaemia, essential hypertension or coronary artery disease (Delles et al., 2004; Garhöfer et al., 2004; Heitmar et al., 2011; Mandecka et al., 2007, 2009; Nagel et al., 2004; Nägele et al., 2018; Nguyen et al., 2009). Therefore, one explanation could be that the respective incidence of diabetes mellitus, dyslipidaemia or arteriosclerosis was only about 50% in the patient group. However, 96% of the patients had a treated arterial hypertension. Other investigators reported a connection of untreated hypertension and reduced retinal flickering light response (Nagel et al., 2004; Pemp et al., 2009) but the influence of antihypertensive treatment on DVA is still controversial. Nagel et al. detected no change of retinal response to flickering light after 1.5 years of antihypertensive treatment (Nagel et al., 2006), whereas Delles et al. demonstrated restoration of central retinal artery blood flow velocity in response to flickering light after AT1-receptor blockade (Delles et al., 2004). In addition, our controls were significantly younger than our patients, so we cannot completely rule out that the convincing FBF result is partly due to the age difference between the patient and the control groups. Conversely, it can be assumed that the size of our study groups may not have been sufficient to reveal at least age-related differences of DVA. Furthermore, numerous large-sized studies have shown that arteriosclerosis, hypertension and diabetes have opposing influences on the static diameters of the retinal microvasculature (Dervenis et al., 2019; Drobnjak et al., 2016; Klein et al., 2018; Phan et al., 2015; Seidelmann et al., 2016; Triantafyllou et al., 2014). Possibly for this reason, and in line with our results, smaller studies without subgroup analysis found no changes of CRAE and CRVE in high-risk cardiovascular patients (Kreis et al., 2009;

**FIGURE 3** Correlation of the peak FBF during postocclusive RH with the maximum FBF response during the ACh and the SNP protocol. (a) The peak FBF values during postocclusive RH (FBFmax RH) of Risk and Ctrl correlated significantly with the respective maximum FBF responses during the ACh protocol (FBFmax ACh; at infusion of 220 nmol/min; *p < .05). (b) The peak FBF values during postocclusive RH (FBFmax RH) of Risk and Ctrl did not correlated with the respective maximum FBF responses during the SNP protocol (FBFmax SNP; at infusion of 10 µg/min; p = NS)

& Summers, 2012; Nagel et al., 2006).
Mandecka et al., 2009; Nägele et al., 2017). Measurements of FBF and retinal vessels also show subject-internal variability. The previously reported intra-subject variation coefficient of absolute FBF during postocclusive RH was 6.1% (three-hour examination interval) and 8.6% (one-week examination interval), and was 24%–27% under graded infusion of ACh (7, 5, 15, 30 µg/min; one-week examination interval; Thijssen et al., 2005; Walker et al., 2001). Based on a one-month examination interval, the previously reported DVA coefficient of variation was 0.9% for maximal arterial dilation and 1.17% for maximal venous dilation, whereas the correlation coefficient of CRAE and CRVE amounted to 0.86 and 0.87, respectively (McCanna et al., 2013; Nagel et al., 2005). Accordingly, regardless of the convincing results of FBF, the small and therefore unstratified patient group, the non-age-matched control group and only one examination in the trial course are noteworthy limitations of our study. Moreover, possible undetected cardiovascular risk factors in the controls as well as pharmaceutical treatment of the patients might have influenced the results of DVA and static retinal analysis. Thus, individual controls had high normal blood pressure (overall 137.8 mmHg systolic) and/or cholesterol values (overall 5.4 mmol/L) and exclusion of impaired glucose tolerance was not performed. Therefore, the control group was maybe not completely free of potential vascular pathology which, however, could be considered low compared to the patient group. The discrepant results of FBF and DVA could further be explained by the use of different triggers and by distinct mechanisms of vasodilation in the microvessels of the forearm and the retina as part of the central nervous vascular bed (Heitmar & Summers, 2012; Metea, 2006). It must also be mentioned that, according to the current literature, it cannot be completely ruled out that pupil dilation with tropicamide at least somewhat counteracted the endothelial NO-mediated retinal vasodilation in our study (Frost et al., 2019; Harazny et al., 2013; Özdemir & Şekeroğlu, 2020; Wang et al., 2018).

Our results show that FBF but not DVA or static retinal vessel analysis was able to segregate a heterogeneous group of high-risk cardiovascular patients from a group of healthy persons. We therefore conclude that FBF and DVA cannot be regarded as equivalent methods for testing of microvascular function. This outcome should encourage further trials to define the role of DVA in this context.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest, financial or otherwise.

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