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Measurement of microcirculation in clinical research

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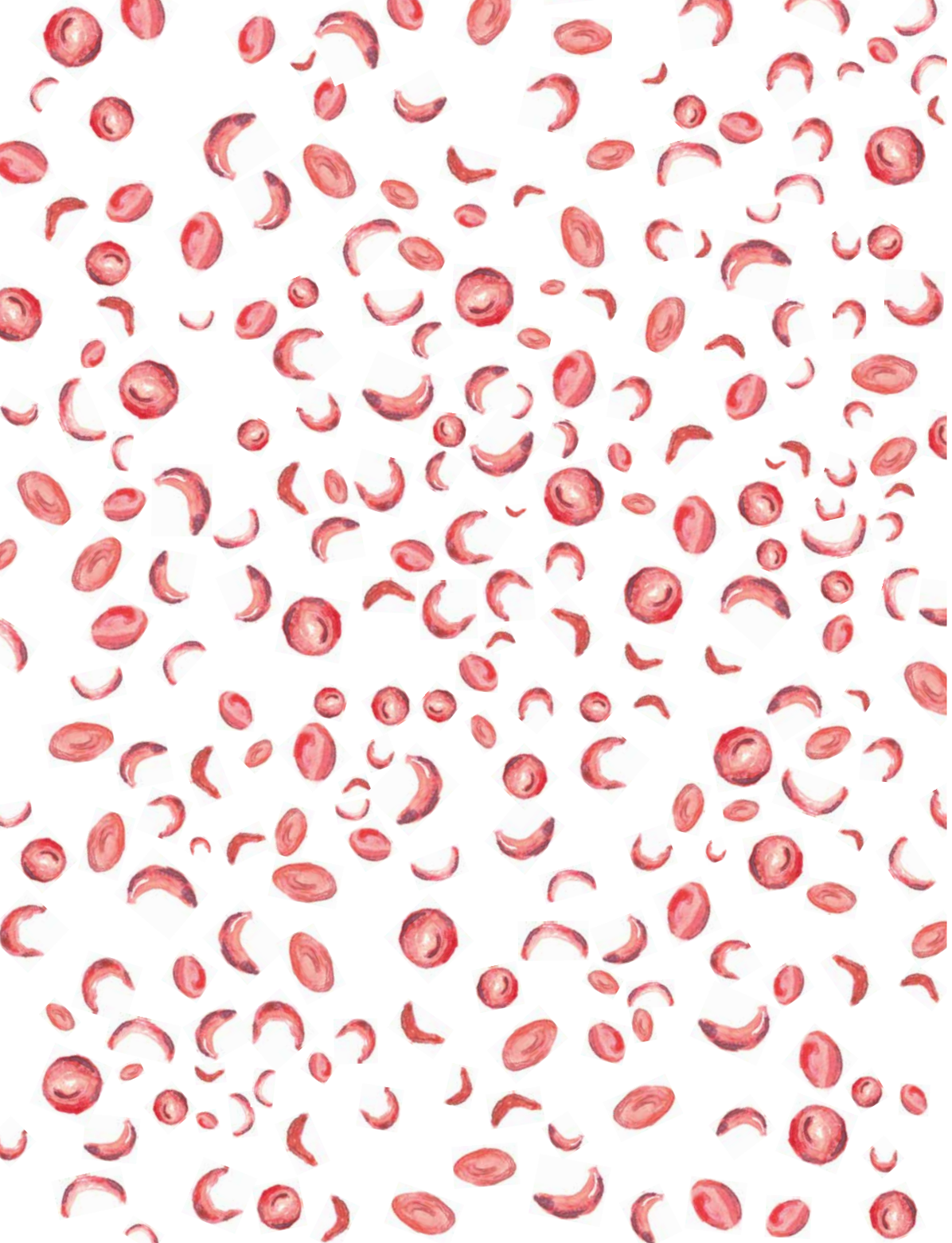

CHAPTER III

Retinal microcirculation imaging in sickle cell disease patients

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OBJECTIVES: To explore the feasibility of a new quantitative method for microvascular function: noninvasive retinal function imaging (RFI). in sickle cell disease (SCD) patients and healthy controls and have it benchmarked against Laser Speckle Contrast Imaging (LSCI) measurements. METHODS: The variability of Microvascular measurements was assessed in 8 SCD patients and 8 healthy matched controls. Measurements were conducted twice on two different study days. RFI was performed for assessment of arterial and venous retinal blood flow. LSCI measurements included post occlusive reactive hyperemia and IBH challenges. Measured variables included basal flow, flow upon occlusion-reperfusion and flow during an IBH. RESULTS: RFI arterial flow and venous flow and LSCI basal flow and peak flow showed excellent intra subject repeatability between days (CVC of 8.5% 9.5%, 7.6% and 7.7% respectively) and between measurements on one day (CVC of 7.0%, 7.7%, 7.6% and 4.7% respectively). RFI arterial flow (P<0.002), and RFI venous flow (P=0.007) differed significantly between SCD patients and controls in as did LSCI basal flow, maximal flow and delta flow during IBH (P <0.0001) CONCLUSIONS: RFI showed low variability for all readout measures, comparable with most microvascular measures from LSCI. The discriminating power of the RFI between SCD patients and controls demonstrate the feasibility of this device for quantitative assessment of the microcirculation in clinical research.

Sickle cell disease (SCD) is an inherited genetic disorder that affects approximately 100.000 people in the United States of America. One out of 500 African American newborns is affected by this disease and in some western European countries SCD is the most common hereditary disorder.[1–3] The disease is caused by a single amino acid substitution in the hemoglobin molecule,[3] which leads to a rigid, sickle-like shape of red blood cells which polymerize when deoxygenated.[4] The polymerized sickle cells form heterocellular aggregates that alters blood flow and tissue perfusion. These acute vascular obstructions results clinically in severe painful episodes known as vaso-occlusive crisis, which often requires hospitalization and are a hallmark of SCD.[5]

Hydroxyurea (HU) is the only approved drug for SCD. It increases the expression of fetal hemoglobin

in the erythrocyte, which inhibits the polymerization of hemoglobin S.[6] HU may also exhibit nitric oxide (NO) donor properties and induce NO-synthase activity and production in endothelial cells [7]. Several studies show that HU reduces the occurrence of SCD-related acute complications as well as survival.[8–11] However, not all SCD patients respond to HU, and the exact individual factors contributing to treatment success are unclear.[12] Moreover, HU therapy has side effects such as constipation, nausea, drowsiness, hair loss, and inflammation of the mouth, or even more severely neutropenia or thrombocytopenia. Hence, for some patients the risks of untreated SCD may outweigh the risks of HU's side effects.[10] Thus, there is a need for alternative pharmacological therapies for SCD.[13]

Drug development in SCD often uses clinical measures such as vaso-occlusive crises, which are not practical for proof-of-pharmacology experiments and drug development in general. The availability of quantitative biomarkers, which report on the underlying pathophysiology leading to clinical insults, could be of great assistance. Since vaso-occlusive phenomena in SCD originate from the impact of sickled cells on the integrity and functionality of the microvasculature,[14–16] validated quantitative measures of microvascular function in SCD patients may provide useful pharmacodynamics biomarkers. Abnormal hemodynamics in SCD patients has been reported for several organs.[17–23] Because of easy accessibility, earlier studies examined cutaneous and conjunctival microcirculation as a surrogate to investigate microvascular mechanisms in SCD. These studies demonstrated an abnormal cutaneous microvascular blood flow with periodic oscillations and a prolonged reactive hyperemic response compared to control subjects.[17] Conjunctival red blood cell velocity is also slower in SCD patients than in control subjects.[18,19,23,24]

New methodologies may allow more precise and quantitative assessments in SCD microcirculation. An interesting new non-invasive technique for quantitative assessment of microvascular function is retinal function imaging (RFI).[25,26] RFI measures retinal blood flow velocity by detecting the movement of individual erythrocytes via stroboscopic illumination and high-speed digital imaging of the retina. By calculating the distance that an erythrocyte moves over a series of pictures, blood velocity is quantified.[27] Using RFI, early changes in retinal blood flow in diabetes,[28] MS patients,[29] age-related macular degeneration and retinitis pigmentosa[30,31] have been demonstrated. As such, RFI could be a valuable technique for quantification of the effects of drugs targeting endothelial integrity in sickle cell disease. However, data on retinal function in sickle cell disease patients are not available. Moreover, performance of RFI in healthy volunteers or patient populations in terms of measurement variability over day and over a longer period of time is lacking. Since these factors are crucial for rational clinical application of the technique, a series of clinical experiments was performed to assess the performance of the RFI in sickle cell disease. The variability of the measurements was assessed in SCD patients and matched controls, and retinal function was compared between moderate to severe sickle cell disease patients and healthy age-, ethnicity-, skin tone- and gender-matched controls. As reference, laser speckle contrast imaging (LSCI)[32,33] measurements were included. LSCI measures cutaneous microvascular blood flow by detecting the movement of laser-illuminated circulating red blood cells in dermal capillaries. Although LSCI has not been used for assessment of vascular function in SCD patients before, it has been shown to be superior to laser Doppler flowmetry in terms of reduced variability and higher discriminating power when assessing microvascular function.[34,35] Laser Doppler has been commonly used in studies with SCD patients.[36,37] LSCI measurements included acute microvascular changes in response to a physiological challenge (post-occlusive reactive hyperemia and inspiratory breath holding).

MATERIALS AND METHODS

Study population

Variability in microvascular measures was assessed in SCD patients, aged 18-65 (n=8), and healthy controls matched for age, ethnicity, gender, and body mass index (n=8). SCD patients had a minimum of 4 vaso-occlusive crises in the past, and at least one vaso-occlusive crisis in the last year. SCD patients who underwent transfusion therapy within 3 weeks

prior to the measurements and experienced a vaso-occlusive crisis within 1 week prior to the measurements were excluded. SCD patients continued their usual medication, including, but not limited to, hydroxyurea, vitamin D, folic acid and (prophylactic) antibiotics. For all study participants, the incidental use of acetaminophen (up to 4 g/day) was allowed.

Study design

This was an observational study, carried out at the Centre of Human Drug Research (CHDR) in Leiden, the Netherlands. Sequential RFI and LSCI were conducted twice on two study days separated by one week. On both study days, subjects arrived at the clinical unit fasted for at least 4 hours. Subjects were asked to abstain from the use of alcohol from 12 hours prior to each study visit, and from tobacco-or nicotine-containing products for at least 2 hours prior to the each study visit, until discharge from the clinical unit. Upon arrival at the clinical unit, the subjects received a standardised breakfast. Two hours after arrival, the first block of RFI and LSCI measurements was started. The second block measurements started two hours after start of the first measurements. All measurements were performed in climate-controlled rooms, at 20–24°C, after a 30minute acclimatization period with the subject in supine position. During the study days, subjects were encouraged to drink sufficient water to avoid dehydration (which is was especially relevant for SCD patients).

The study protocol was approved by the ethics committee of Leiden University and performed according the Dutch law on medical research.

RFI

The retinal microcirculation was quantified using the Retinal Function Imager 3005 (Optical Imaging, Rehovot, Israel). Measurements were performed as described previously.[38] In brief, one pupil was dilated using tropicamide. The subject remained seated quietly with the head in a headrest, which allowed collection of 10-15 series of 8 retinal images over a period of 25 minutes. The images were analyzed using Odian browse software (Optical Imaging, Rehovot, Israel). RFI endpoints included average arterial and average venous retinal blood flow velocity (mm/sec).

LSCI

LSCI (PSI; Perimed, Järfälla, Sweden) was performed on the ventral side of the upper forearm, on a surface of 3 x 10 centimeters. The laser head was placed 20 cm above the skin. The frame was positioned more than 5 cm from the elbow and beginning of the wrist, avoiding visible veins. A vacuum pillow was used to limit arm movements. The cutaneous blood flow was measured continuously. After the basal flow was recorded for at least 5 min, the brachial artery was occluded by inflating a pressure cuff placed around the upper arm to 200 mm/Hg for 5 min. Subsequently, the cuff was deflated, inducing a hyperemic reaction. Six minutes after the occlusion, subjects were asked to hold their breath for at least 20 seconds. This was repeated two more times, with inspiratory breath holding (IBH) periods separated by three minutes.

Obtained data were analyzed using PIMSOFT oftware (Perimed, Järfälla, Sweden). LSCI endpoints included basal flow (arbitrary units (AU)), peak flow after occlusion (AU), ratio peak flow/basal flow (%),time to return to basal flow (seconds), post-occlusive index as previously described [39] (AUC peak flow/basal flow; %), time to recovery after IBH (seconds), and the delta flow before - during IBH (arbitrary units).

Statistical analysis

For repeatedly assessed endpoints, contrasts between groups were estimated with a mixed model analysis of variance with fixed factors group (SCD versus controls), day (day 1 and day 8), measurement (0 and 2 hour), group by day, group by measurement and group by day by measurement, and subject, subject by day and subject by time as random factors. LSCI time to maximum flow (sec), LSCI time to return to basal flow (sec) and LSCI time to recovery after IBH (sec) were log- transformed before analysis. For both groups, the estimated intra-subject variability within one day and between days, defined as the short- and long-term repeatability, was calculated. For all endpoints the minimal detectable effect size (MDES) was calculated as a combined measure of the effect size and the estimated variability, assuming a parallel comparison of two groups of 8 subjects. All calculations were performed using SAS for windows V9.4 (SAS Institute, Inc., Cary, NC, USA).

RESULTS

Study population

Microvascular function was assessed in SCD patients (n=8), and healthy controls matched for age, BMI, and ethnicity (n=8). Subject demographics are provided in *Table 1*. All included subjects were female and had normal resting blood pressure and heart rate. There was 1 light smoker in the SCD group, and no smokers in the matched control volunteer group.

Six sickle cell patients had a self-reported SCD type HbSS, one subject had HbSE, and one subject was uncertain about the SCD subtype. Medication included folic acid, hydroxycarbamide, vitamin D, deferasirox, deferiprone, and acetaminophen. One SCD patient was on hydroxyurea therapy. Most SCD patients and healthy controls had a Fitzpatrick skin tone of VI (black or African-American).

TABLE 1 Demographics

	SCD patients	Controls
Age (years)	34 ± 7.5	31 ± 7.6
BMI kg/m²	25.4 ± 4.8	26.4 ± 4.2
Male:female	0:8	0:8
Systolic blood pressure (mmHg)	109 ± 8	116 ± 18
Diastolic blood pressure(mmHg)	65 ± 6	73 ± 12

Variability

Sequential RFI and LSCI were conducted twice on two study days separated by one week. Measurements blocks performed on one study day were separated by 2 hours.

The variability for retinal blood flow velocity by RFI was limited between days (coefficient of variation (CV) 8.5% for arterial flow and 9.5% for venous flow) and between measurements on one day (CV: 7.0% for arterial flow and 7.7% for venous flow). Also LSCI parameters showed limited variability for basal flow and peak flow after occlusion between days (CV 7.6% and 7.7%, respectively) and between measurements on one day (CV: 7.6% and 4.7%, respectively). The variability for the time to recovery after IBH was larger (CV: 23.9% between days and 22.7% within day). *Table 2* provides the variability of microvascular parameters for the two groups separately.

Based on the calculated variances, MDES were assessed for the main microvascular measures, assuming a parallel study design with 2 groups of 8 subjects. The MDES for arterial blood flow velocity was 0.91 mm/sec, for venous blood flow velocity 0.66 mm/sec. The MDES for LSCI basal flow was 8.04 AU, for peak flow after occlusion 21.31 AU, and for the effect of inspiratory breath holding (as assessed by the difference between LSCI blood flow before and during breath holding) 4.51 AU.

Microvascular parameters and the effect of brachial artery occlusion and IBH

In SCD patients, arterial and venous blood flow velocity, quantified by RFI, were estimated to be 3.87 (95%CI 3.51, 4.23) mm/sec and 3.00 (95%CI 2.73, 3.26) mm/sec. The basal flow assessed by LSCI was 33.4 (95%CI 29.9-36.9) AU. Blood flow increased during the hyperemic reaction following occlusion of the brachial artery, with a peak flow of 93.1 (95%CI 83.9-102.3) AU, returning to basal flow in 129 (95%CI 117, 142) seconds after release of the occlusion. LSCI was also able to detect changes in blood flow upon inspiratory breath holding. This physiological challenge resulted in a temporary reduction in blood flow: delta flow before versus during IBH was estimated to be 9.8 (95%CI 7.8, 11.9) AU in SCD patients. Return to basal flow after IBH occurred within 54 (95%CI 48, 61) seconds. In controls comparable effects of brachial artery occlusion and IBH, quantified by LSCI were observed.

Contrasts between populations

Statistically significant differences were observed between SCD patients and controls in microvascular function as assessed by the RFI: retinal arterial blood flow velocity 3.87 vs 3.42 mm/sec, p=0.002; retinal venous blood flow velocity 3.00 vs 2.68 mm/sec, p=0.007. Also microvascular function as assessed by the LSCI was significantly different between SCD patients and matched healthy controls: basal flow 33.4 vs 24.5 AU, p<0.0001; peak flow after occlusion 93.1 vs 76.8 AU, p<0.0001; and ratio peak flow/basal flow 286.3 vs 318.6 %, p=0.002. No statistically significant differences were observed between both populations in time to peak flow or

TABLE 2 CV for LSCI and RFI parameters per population

Parameter	Group	Intra-subject cv (between days)	Intra-subject cv (within day)	Inter-subject cv
LSCI basal flow (AU)	Sickle cell patients	9.9%	6.4%	21.4%
	Controls	8.1%	8.1%	17.2%
LSCI peak flow after occlusion (AU)	Sickle cell patients	7.3%	4.5%	16.0%
	Controls	8.8%	5.8%	17.1%
LSCI ratio peak flow/basal flow (%)	Sickle cell patients	9.5%	6.9%	14.8%
	Controls	6.1%	11.1%	13.0%
LSCI time to return to basal flow (sec)	Sickle cell patients	14.4%	8.6%	12.8%
	Controls	10.8%	7.9%	15.7%
LSCI delta flow before- during IBH (AU)	Sickle cell patients	31.1%	25.8%	27.8%
	Controls	24.7%	35.0%	54.5%
RFI average arterial blood flow velocity (mm/sec)	Sickle cell patients	5.9%	5.9%	22.4%
	Controls	10.9%	6.8%	13.1%
RFI average venous blood flow velocity (mm/sec)	Sickle cell patients	7.2%	6.8%	22.3%
	Controls	7.5%	7.5%	7.3%

TABLE 3 LSCI and RFI parameters per population (least square means with 95% CI) and contrasts between populations

parameter	SCD patients	Controls	SCD patients versus controls
LSCI basal flow (AU)	33.4 (29.9 - 36.9)	24.5 (21.0 - 28.0)	8.9 (6.7, 11.1) p=<.0001
LSCI peak flow after occlusion (AU)	93.1 (83.9 - 102.3)	76.8 (67.6 - 86.0)	16.3 (10.2, 22.3) p=<.0001
LSCI ratio peak flow/basal flow (%)	286.3 (257.4 - 315.2)	318.6 (289.6 - 347.6)	-32.3 (-52.7, -11.8) p=0.002
LSCI time to return to basal flow (sec)	129 (117 - 142)	122 (111 - 134)	5.7% (-1.6%,13.7%) p=0.13
LSCI post-occlusive index (%)	213.1 (192.2 - 234.0)	210.6 (189.7 - 231.5)	2.5 (-10.5, 15.4) p=0.7
LSCI time to recovery after IBH (sec)	54 (48-61)	45 (41-51)	18.9% (9.5%, 29.2%) p=<.0001
LSCI delta flow before-during IBH (AU)	9.8 (7.8 - 11.9)	4.7 (2.7 - 6.3)	5.1 (3.8, 6.4) p=<.0001
RFI average arterial blood flow velocity (mm/sec)	3.87 (3.51 - 4.23)	3.42 (3.06 - 3.78)	0.45 (0.17, 0.73) p=0.002
RFI average venous blood flow velocity (mm/sec)	3.00 (2.73 - 3.26)	2.68 (2.42 - 2.95)	0.31 (0.09, 0.54) p=0.007

time to return to basal flow, or in post-occlusive index. The effect of inspiratory breath holding, as assessed by LSCI as the difference between blood flow before and during breath holding, differed significantly between both populations: 9.8 vs 4.7 AU, p<0.0001. Whereas the time to return to basal flow after occlusion of the brachial artery did not discriminate between populations, the time to recover after IBH did, 54 vs 45 sec p<0.0001. *Table 3* provides the point estimates of all microvascular parameters (least square means with 95%CI) and contrasts for both populations (SCD patients and controls).

DISCUSSION

We explored the performance of RFI in SCD patients, in comparison with a group of age- ethnicity- and gender-matched controls. Hereby, we aimed to qualify RFI-derived measures for microvascular function as biomarkers of vascular dysfunction in SCD. As reference, LSCI measurements were performed, also for quantification of acute microvascular changes in response to a physiological challenge. Sequential measurements were performed twice on two study days, separated by one week.

RFI and LSCI showed comparable limited variability for the various microvascular measures (coefficient of variation in the range of 4-10%), both between study days and between measurements performed on one day for all groups. The observed variability for RFI-derived measures is well comparable with the variability reported for retinal blood flow when assessed by trans-cranial colour Doppler[40] and the variability observed for LSCI-derived measures are in line with the variability and reproducibility of LSCI reported in literature.[13,25,33] Using the LSCI as a benchmark, these data suggest that in terms of variability RFI is equivalent to standard microvascular measurement methods in clinical research. A recent studied showed that there is a strong correlation between laser speckle flowgraphy measurements and RFI measurements for retinal blood flow, suggesting that the results are valid for use in clinical research.[41]

Microvascular function as assessed by RFI differed significantly between SCD patients and matched controls. SCD patients had an elevated arterial and venous blood flow velocity. Statistically significant differences were also observed between SCD patients and matched controls for LSCI-assessed microvascular function measures: SCD patients had an increased basal and peak flow after occlusion, and reduced ratio peak flow/basal flow. An increased cutaneous microvascular flow has been demonstrated previously in SCD by other techniques.[37,42,43] This increased flow is believed to compensate the effects of anaemia and insufficient tissue oxygenation, and may relate to low haematocrit levels and reduced erythrocyte aggregation.[44,45] The reduced ratio peak flow/basal flow in SCD patients is also in line with reports in literature, and may be explained by a reduced capacity of hyper-activated endothelial cells to secrete vasodilatory substances in response to shear stress.[46,47]

RFI assesses the blood flow velocity in the smallest arterioles and venules in the retina, whereas LSCI assesses the blood flow in the capillaries of the skin. It is unknown how the microvascular function in the two different vascular beds relate to each other, and how the estimated blood flow or blood flow velocity relate to clinical outcomes. Previous studies have shown that the cutaneous microvascular function could mirror the microvascular function in other organs.[48]

Visual loss has been observed in 10% to 20% of eyes of SCD patients, strongly associated with proliferative retinopathy.[49] Symptoms include vitreous hemorrhages, neovascularization, retinal detachment and anastomoses.[50] The observed increased retinal blood velocity in SCD patients may relate to these symptoms. Abnormalities in the retinal microvasculature may also relate to cerebral diseases such as dementia and decreased cognitive function, and retinopathy and brain abnormalities as shown by MRI or CT.[51] Although the RFI technique in this study allowed visualization of capillary maps without contrast agents, quantification of retinopathy was not possible since automated and validated software is not available yet. Another limitation of RFI is the fact that image acquisition requires highly experienced operators, since the technique is from an operational point-of-view more difficult than LSCI. As such, the introduction of operator-related measurement variability may be a risk for RFI. Also, a drawback of RFI is that is not possible yet to assess the effects of a physiological challenge on microvascular function (e.g. IBH), whereas LSCI does allow such interventions.

One limitation of our study is the fact that mainly female subjects were enrolled. We cannot exclude the possibility that gender is a factor of influence on microvascular function in SCD patients. Gender differences in NO availability have been reported for SCD patients, possibly related to the effects of oestrogens.[47] However, since all SCD patients in our study were matched to a healthy control subject, this does not affect our main conclusions.

In summary, RFI is a robust technique to assess microvascular dysfunction in SCD, even though the correlation between RFI-derived measures and clinical outcome is unknown. We showed that arterial and venous blood flow velocity by RFI are highly stable measures over time, and differentiate between SCD patients and controls.

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