Measurement of microcirculation in clinical research

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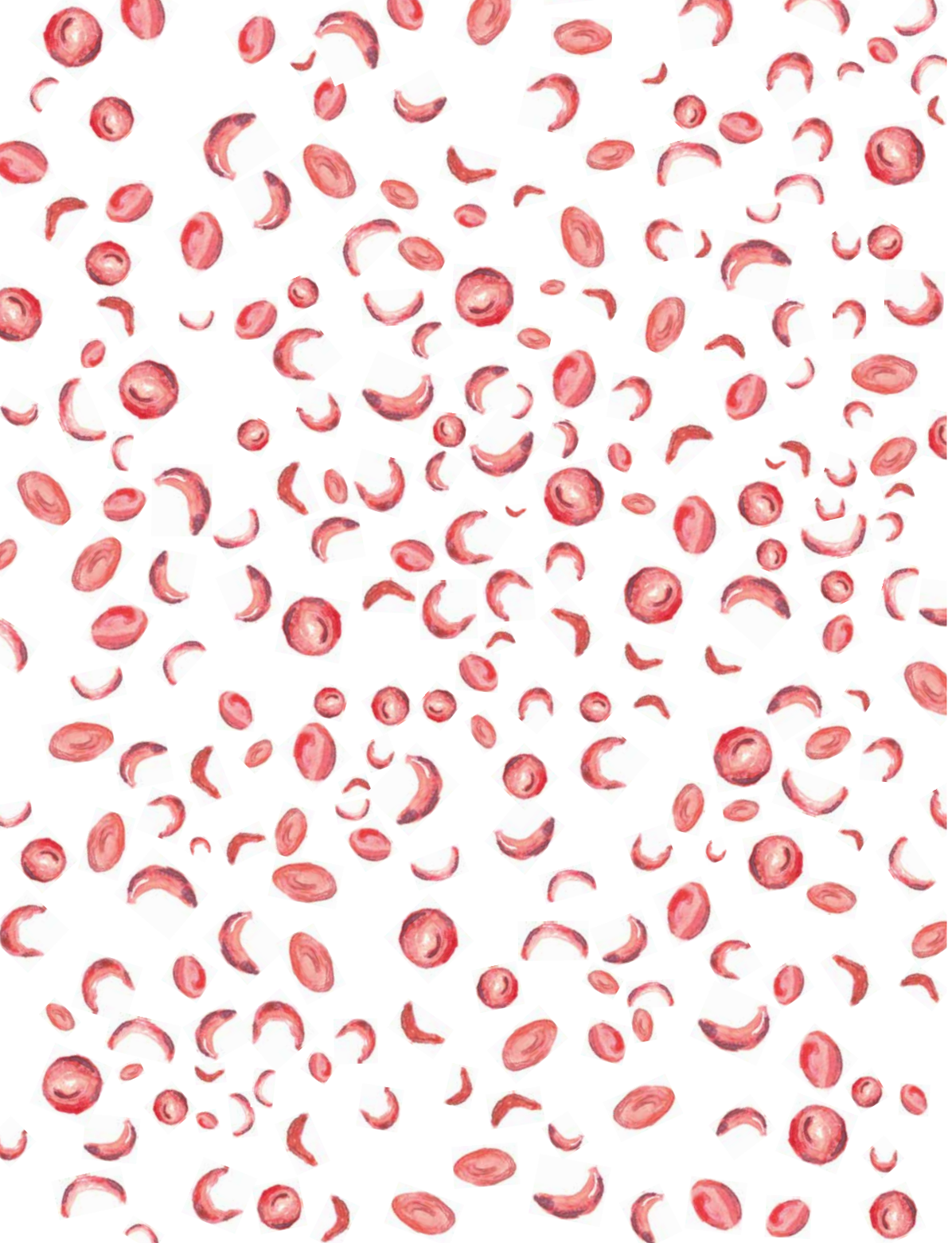

CHAPTER II

Microcirculation measurements in the skin and retina: review of non-invasive tools and their challenges

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In the last decade new and more advanced microvascular imaging techniques have become available for use in clinical research. As it became more evident that microvascular dysfunction might prelude (cardio) vascular events, microvascular measurements have gained increasing interest for their applicability in the clinic and research. In this narrative review we aim to provide an updated oversight of microvascular measurement techniques. We have made an outline of the most common and latest techniques for microvascular cutaneous and retinal measurements and included the most frequently used challenges used together with these measurements. Furthermore this review includes an updated evaluation of the mechanistic background of microcirculatory dysfunction. Hereby this narrative literature review aims to assist with the identification of the most adequate microvascular imaging technique(s) for clinical application and research

The microcirculation plays a major role in tissue perfusion, the exchange of nutrients, oxygen and carbon dioxide between blood and interstitial fluid and in vascular homeostasis. Endothelial tissue is a key regulator of the microcirculation. Abnormalities in the microcirculation are considered to be strong predictors of major adverse cardiovascular events and one of the first signs of atherosclerosis.[1] Adequate physiological measurement of the microcirculation is challenging because the vessel structure is spatially heterogeneous in most locations and perfusion shows high variability over time and in different environmental conditions, such as temperature.[2] Recently however, new devices have become available, such as dynamic optical coherence tomography (d-OCT) and laser speckle flowgraphy (LSFG). Moreover, image processing from existing devices improved due to technological advancements, for instance better lenses, higher resolution cameras and more sensitive sensors with improved noise performance.

There are several ways to evaluate the microcirculation, and, although the influence of different endothelial-derived factors, such as nitric oxide

(NO) and endothelium-derived hyperpolarizing factors, is variable in vascular beds of different organs, the pathological alterations in these signaling pathways are remarkably similar.[3] Due to the easy accessibility, the skin is an excellent location to investigate pharmacological effects on the microcirculation. In particular for healthy volunteer studies, challenge models have been developed that can be applied in healthy volunteers so that pharmacodynamic effects can be monitored in first into man studies. Another easily accessible site is the retina, where the microcirculation can be visualized and monitored repeatedly over time. Retinal microvascular abnormalities have been linked to several cerebrovascular, [4,5] cardiovascular, [5] renal [6] and metabolic outcomes. [7]

Few reviews on the applicability of microvascular measurements have been published, and these frequently focused on a limited number of imaging techniques or on measurements in a single organ system.[8–11] The most comprehensive technical review on cutaneous non-invasive methods for imaging of the microcirculation was published in 2014.[10] However, there have been new developments and as such, there is a need to provide an updated oversight of microvascular measurement techniques. This narrative literature review aims to assist with the identification of the most adequate microvascular imaging technique(s) for clinical application and research. In contrast to previous reviews on this subject, this review puts emphasis on the clinical utility of the non-invasive microvascular imaging techniques that are currently commercially available for skin and retinal microcirculation assessments. In this review we have made an outline of the most common and latest techniques for microvascular cutaneous and retinal measurements. Most microcirculation measurements are indirect and tend to give little information about microcirculatory function over the full range of adaptive response possible. Therefore, these measurements are usually combined with challenges to drive the regulatory system to a more extreme state. Over the years various challenges techniques have been developed which can give inside in different aspects of the microcirculatory function. However, most of them focus on the endothelial microcirculation function. In this review we also provide an overview of the most applied challenges with these techniques.

MECHANISTIC BACKGROUND OF MICROCIRCULATORY DYSFUNCTION

Endothelial dysfunction (ED) and abnormalities in the microcirculation are considered to be prelude development of atherosclerosis and are correlated with various cardiovascular risk factors as well as cardiovascular outcomes. Endothelial dysfunction is a vascular phenotype characterized by an imbalance between endothelial-derived contracting factors (EDCF), such as endothelin-1 and angiotensin II and endothelial-derived relaxing factors (EDRF), such as nitric oxide (NO), prostacyclin and bradykinin.

The ED phenotype can be induced in endothelial cells by a variety of stimuli, such as disturbed blood flow, bacterial lipopolysaccharides (LPS), pro-inflammatory cytokines and chemokines, pathogen- or damage associated molecular patterns and oxidized low-density lipoproteins (OXLDL).[12] These stimuli activate transcription factors such as NF-KB through various cell adhesion molecules (CAMs). This results in increased expression of these adhesion molecules, such as VCAM-1, ICAM-1, p- and e-selectin.[13] Additionally, monocytes, neutrophils and various types of T-cells are recruited to the vascular wall by these adhesion molecules, enhancing the inflammatory process by secreting various pro-inflammatory cytokines. The amount of reactive oxygen species (ROS) increases in this pro-inflammatory environment, leading to a reduction in availability of NO, one of the main EDRFs,[14] and increased pro-inflammatory signaling to the recruited leukocytes. This cellular environment also promotes the formation of OXLDL, creating an additional feedback loop of pro-inflammatory signaling through NF-KB in endothelial cells.[12,15] Production of EDCF is increased, while pro-dilatory signaling is reduced. All these processes combined lead to ED, promoting the development of atherosclerosis before any macroscopic atherosclerotic changes in vasculature can be detected.

Nitric Oxide as a main actor

One of the main and early indicators of the presence of the ED phenotype is the reduction in bioavailability of NO.[13,16] NO is a highly reactive soluble gas with an extravascular half-life of 0.09 to >2 seconds, decreasing with rising NO concentration.[17]

Since NO decays quickly, it has to be produced continuously in the vascular endothelium. Endothelial produced NO is a potent vasodilator responsible for endothelium-dependent vasodilation. NO reverses the constrictive effects of acetylcholine (ACH) on smooth muscle cells, mediating the potent endothelium-dependent vasodilatory effects of ACH. Similarly, endothelial NO release can reverse the vasoconstrictive effects of various vasoactive agents such as serotonin and norepinephrine.[18,19] Moreover, endothelial NO signaling inhibits inflammation, platelet activation, leukocyte adhesion and vascular smooth muscle cell proliferation, processes that are essential in the development of atherosclerosis.[12,20,21]

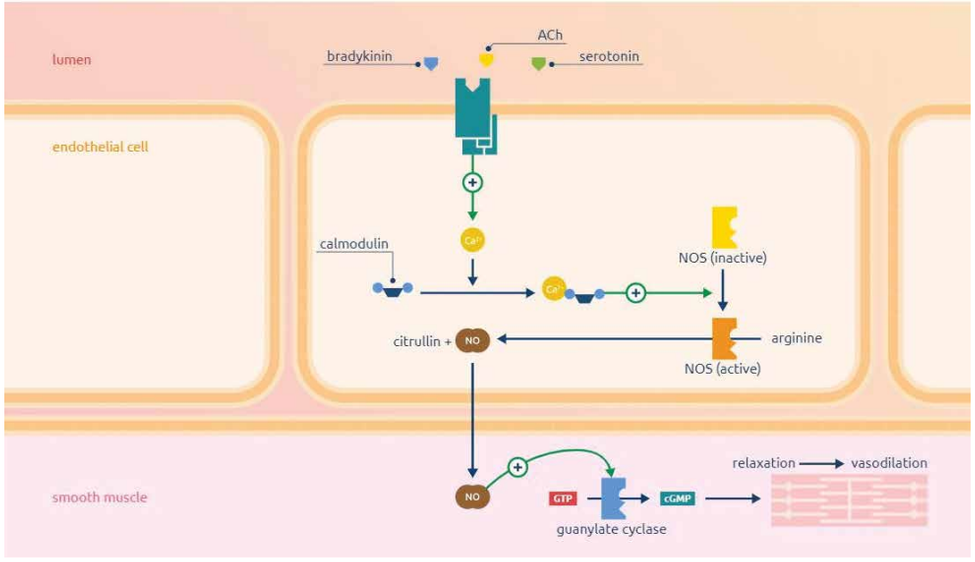
NO biosynthesis

Under physiological circumstances, NO is produced in endothelial tissue by endothelial nitric oxide synthase (ENOS) by metabolizing L-arginine, creating L-citrulline as a byproduct. It is also synthesized in various tissues by inducible nitric oxide synthase (INOS) and neuronal nitric oxide synthase (NNOS). In their monomeric (uncoupled) state ENOS, INOS and NNOS are incapable of binding L-arginine, instead producing the ROS $O_2^{\cdot-}$.[22] In the presence of calmodulin (CaM), heme and tetrahydrobiopterin (BH4) NOS proteins become coupled and produce NO. ENOS and NNOS are dependent on the presence of Ca^{2+} activated CaM to form dimers [23], whereas INOS is calcium-independent. Activity of ENOS in endothelial cells is thus increased significantly by raised intracellular Ca^{2+}levels, although various other cellular signals increase the activity of ENOS, including shear stress, bradykinin and insulin.[24]

Effects of endothelial NO

The effects of endothelial derived NO are mainly mediated through activation of soluble guanylate cyclase, which promotes the conversion of guanosine triphosphate to cyclic guanosine monophosphate (CGMP). CGMP then activates CGMP-dependent protein kinase (protein kinase G), which induces vasodilation in vascular smooth muscle cells[25] and inhibits platelet activation.[18] Additionally, S-nitrosylation, the addition of NO to a cysteine thiol, modifies the function of various cellular proteins, including inhibition of NF-KB,[26,27] producing the anti-inflammatory and anti-proliferative effects of NO. These effects are crucial to maintaining vascular homeostasis, since NF-KB itself upregulates ENOS in addition to its pro-inflammatory effects,

FIGURE 1 **Schematic overview of NO biosynthesis**



acting as its own inhibitor. Dysfunction of eNOS or reduction in NO availability can lead to unopposed NF-kB activation and vascular inflammation.[27]

NO in endothelial dysfunction

During the development of ED, production of NO is decreased, while consumption of NO is increased. ROS, in particular O_{2-}, react with NO to produce $ONOO^-$, an inactivating agent of NO itself, as well as a powerful oxidant inducing oxidative stress in the endothelium. $ONOO^-$ also oxidizes BH4 [28], inactivating it, leading to the uncoupling of eNOS dimers. eNOS in its uncoupled state produces more O_{2-}, creating a vicious feedback loop. The subsequent reduction of NO bioavailability and disappearance of its anti-inflammatory, anti-proliferative and vasodilatory effects creates imbalance between EDCF and EDRF and exacerbates the inflammatory environment and recruitment of leukocytes, ultimately causing the development of atherosclerotic plaques.[29]

Measurement of NO in pathophysiological conditions

In vivo, inhibition of NO synthesis raises baseline blood pressure in rats, increases coronary vascular resistance in dogs and promotes aortic atherosclerosis in rabbit hypercholesterolemia models. In healthy humans, inhibition of NO synthesis increases fore-arm vascular tone, decreases coronary artery diameter and blunts the response to ACH infusion.[18] Diminished NO synthesis is also associated with various pathological conditions promoting atherosclerosis, such as diabetes, hypertension, hypercholesterolemia, smoking and heart failure. Since reduction of NO bioavailability plays a pivotal role in the development of ED [25], measurement of NO bioavailability is an important diagnostic and investigative goal in the assessment of ED.

However, due to the instability of NO, direct measurement of eNOS activity in vivo has proven challenging. Nitrite and nitrate are stable metabolites produced by oxidation of NO, so their levels were considered surrogate levels of NO production and eNOS activation. However, nitrite and nitrate are commonly present in the human diet, and nitrite and nitrate are also endogenously reduced to form NO independently of eNOS activity.[24,30] Nitrite and nitrate are thus more reflective of an equilibrium between NO oxidation and reduction and dietary effects rather than in vivo eNOS activation. Most measurements of endothelial (dys) function and NO bioavailability therefore rely on measuring the physiological effects of local NO production, chiefly vasodilation.[21] By inducing NO production with challenges or inhibiting it with antagonists while simultaneously non-invasively measuring its effects on vasculature, a relatively accurate picture of eNOS activity and endothelial function can be acquired.

Evaluation of the endothelial system in man

The importance of measuring endothelial dysfunction as an early marker of atherosclerosis and cardiovascular risk is shown by its correlation to various proven cardiovascular risk factors, such as hypercholesterolemia, smoking, hypertension, obesity and diabetes.[31] Endothelial dysfunction is consistently documented in patients with established cardiovascular disease using a variety of methods.[32] Moreover, endothelial dysfunction, measured by flow-mediated dilation (FMD) or reactive hyperemia-peripheral arterial tonometry is significantly predictive of CV events, with a 1 SD decrease in endothelial function associated with double risk of CV events, and FMD itself being a significant predictor of myocardial infarction, angina, coronary revascularization, stroke, resuscitated cardiac arrest and CV related death.[33,34] Accurate assessment of endothelial function is therefore a promising method for early CV risk stratification and risk prevention.

MICROVASCULAR CUTAENEOUS IMAGING TECHNIQUES

Doppler flowmetry

Doppler flowmetry is a technique measuring the Doppler shift in ultrasonic waves induced by the movement of blood through arteries to quantify the velocity of flow through that artery. This, multiplied with the measured cross-sectional area of that artery results in a measurement of flow volume. Determination of flow by itself does not provide much information about the functional status of the vascular bed. However, once combined with a challenge like the passive leg movement (PLM), changes in flow before and after PLM can provide

insight in endothelial function. During PLM, the flow volume through the common femoral artery (CFA) is measured before and after passive 90 degree flexion of the knee.[35] This passive movement produces an increase in CFA blood flow thought to be due to vasodilation and hyperemia downstream from the CFA.[36] The hyperemic response measured in the CFA after PLM is shown to be 80-90% NO-dependent [37,38] and correlates with the established gold standard of endothelial functional measurements, flow-mediated dilation (FMD). Moreover, measurement of the PLM hyperemic response correlates strongly with an established invasive method of assessing NO-dependent vasodilation: intra-arterial ACH infusion.[38] Disadvantages of the technique include the requirement of a trained operator maintaining a constant angle of insonation, including during movement of the leg, familiarization of subjects with the technique before measurements and the relatively high cost of equipment needed.[39] Advantages include that the technique is relatively easy to master compared to FMD and minimally invasive. Moreover, changes in PLM response due to age or vascular dysfunction far exceed the intra subject variability (within-day and day-to-day coefficient of variation 15-20%), making it a reliable technique to detect inter-subject differences in vascular function.[39]

Laser Doppler flowmetry

Laser Doppler flowmetry (LDF) is based on the principle of measuring the Doppler shift[40] induced by erythrocyte movement, to the illuminating coherent light. LDF output, is in literature often expressed as flux and is close related to the blood flow. In practice, LDF has a reported coefficient of variation of ~35%,[41] mostly related to the spatial variability (variability caused by different measurement sites) of the measurements.[42] However the coefficient of variation of most challenges can be reduced to <10% in a controlled laboratory setting and when executed under the right protocol.[43] As in most microvascular measuring techniques, LDF flux measurements, without using any challenge, have little predictive value for microvascular function, therefore they are usually combined with challenges. LDF measurements, like most other microvascular function techniques, does not correlate well with macrovascular function measurements[44,45] except for post occlusion reactive hyperemia measurements (PORH) which shows a weak correlation with flow-mediated dilation FMD.[46]

Laser Doppler imaging

Laser Doppler imaging (LDI) was developed to overcome the spatial variability of LDF. LDI follows the same mechanism as LDF, also measuring the doppler shift of the movement of erythrocytes. However, the laser light scans over the skin surface, rather than measuring one single point, thereby measuring the flux over an area. Contrary to LDF, LDI is a non-contact measurement method and has been used with the same challenges as LDF.[47] Furthermore LDI can be used to make perfusion maps of the skin, to obtain information about wound healing, burn wounds, skin flap surgery and to evaluate pharmacological responses[48,49] LDI has an intra-subject variability of ±20%.[47] LDI has an image acquisition frequency ~1 Hz frequency (depending on the size of the measurement area) which makes it less suitable to measure acute microvascular changes such as obtained during PORH as these changes happen within a second.[47]

Laser speckle contrast imaging

Laser speckle contrast imaging (LSCI) is able to record the changes in speckle pattern that is returned from the skin (i.e. a flat surface) after illumination with a laser. From the changes in the speckle pattern the microcirculatory erythrocyte flow can be obtained.[50] Similar to LDI and LDF LSCI output is also expressed as flux,[47,51] but flux can be measured at a higher frequency and in a larger area than LDI and LDF. LSCI has an intra-subject variability of 6-12% CV for healthy volunteers [51–53] and patients,[54–57] Since there is a limited number of trials in cardiovascular disease, [56] the available evidence is not sufficient to use LSCI measurements as a diagnostic or prognostic biomarker in the clinic and therefore the method is currently mainly used in (pharmacological) research.[58] Disadvantages of LSCI are a high sensitivity to movement artefacts and indirect and superficial measurements.[59] However new techniques are developed to overcome these disadvantages.[60, 61]

Infrared thermography

Infrared thermography measures skin temperature by recording the infrared radiation that is being returned from the skin, from which dermal

blood flow can be estimated through an proprietary algorithm.[62] Besides being of diagnostic value in sSC/Raynaud's phenomenon patients[63,64] and burn wounds[65] it is used in experimental settings in dermatological conditions such as psoriasis[66] or eczema.[67] Reported reproducibility for healthy volunteers was moderate for most measurements ICC ~0.7-[62] and for Raynaud's phenomenon and sSC ICC ~0.55.[68] The predictive value of this methodology remains unknown due to lack of standardization of methods[63] which are currently under development.[69] Disadvantages are the extreme sensitivity to environmental temperature as a drop from three degrees C decreases baseline measurements by about 20%. Therefore measurements should be performed in strictly climate-controlled laboratories.[62] Thermography is a relatively simple measurement, however calibration and maintenance of the device requires experts.

Video capillaroscopy

Video capillaroscopy (VC) is the gold standard for morphological measurements of the microcirculation. VC allows for real-time 2D capillary network visualization. The preferred sites of measurement are the nailfolds or the sublingual mucosa, as capillaries at these sites are u-shaped and lie horizontally to the skin surface.[64] VC measures shape (torquation, microaneurysms), density, size (diameter, length), morphology (oedema, microhaemorrhages) and in experimental setting can measure flow dynamics.[70] Of all microvascular imaging techniques, VC is the most frequently applied technique in clinical practice.[71] In rheumatology it can be used to distinguish between primary and secondary Raynaud's phenomenon and is included as a standard assessment in the ACR-EULAR guidelines.[72,73] Several studies show an excellent intra-visit ICC of >0.9 and a moderate inter-visit ICC of ~0.65 distal vessel density.[74,75] Disadvantages of VC are spatial variability which can lead to high intra subject variability,[76] time required for the assessment and the high technical skills needed for the appropriate measurement.[77]

Optical coherence tomography

OCT allows visualization of tissue structures in a manner similar to ultrasound but instead uses infrared light. Reconstruction of cross-sectional images to en-face images of tissue can be performed, allowing visualization of the microvasculature. Recent development of dynamic OCT (D-OCT) also allows for the visualization of erythrocyte movement, through combining the speckle variance technique with OCT.[78] In dermatology, OCT is used for morphological assessments for the diagnosis of non-melanoma skin cancer, inflammatory skin disease[79] and burn wounds.[80] Furthermore D-OCT has been used to demonstrate the effect of brimonidine.[81] OCT showed a CV in the range of 5.5 – 8.5%.[82] Disadvantages of OCT are the relatively long time required to perform the scan (+- 3-4 minutes) and the limited area that can be scanned during a single measurement which may lead to variable results due to spatial variability. When performed using the same measurement site, and under the same environmental conditions, OCT measurements can be easily and rapidly performed and show a limited inter- and intra-observer variability of 4.1 and 7.1 (CV%)respectively.[82]

Costs, mechanism, time for assessment, measurement area, variability as well as the major disadvantages of the identified microvascular imaging techniques are summarized in *Table 1*.

CUTANEOUS CHALLENGES

This section covers different challenges that can be used in cutaneous microcirculation techniques. These challenges change or destabilize the cutaneous microcirculation, thereby allowing an induced (pharmacological) effect to be quantified. (*Table 2* for an overview).

Transdermal iontophoresis

Transdermal iontophoresis is the delivery of drugs through the dermal barrier using a weak electrical current. For microcirculation testing, acetylcholine (ACH) and sodium nitroprusside (SNP) are most frequently used. ACH causes a temporal increase in local vasodilation and subsequently increase blood flow through predominantly nitric oxide (NO) and prostaglandin release by the endothelium. Administration of exogenous NO through SNP is used as a control measure for general vasodilatory capacity.[83] Iontophoresis is mainly used to study endothelial NO release and can be used the investigate the effect of pharmacological

TABLE 1 Overview of skin and retinal microvascular measurements

Technique	Cost estimate	Measurements and challenges	Time for assessment	Repeatability
Laser Doppler flowmetry	€13.500	Flux, LTH, PORH, Local cooling, iontepheresis	± 30 s Challenges: 15-30 min	intra subject CV ~35% [41]
Laser Doppler imager	NA	Flux, LTH, PORH, Local cooling	± 30 s Challenges: 15-30 min	intra subject CV ±20% % [47]
LSCI	€60.000	Flux, LTH, PORH, Local cooling, iontepheresis	± 30 s Challenges: 15-30 min	intra subject CV 6-12% [51-53]
Thermography	€50.000	Skin temperature, PORH	± 30 s Chalenges: 15-30 min	Intra class correlation (ICC) 0.7-0.85 [62] [68]
Video capilairoscopy	€6.000	Flux, LTH, PORH, Local cooling	2 min	intra visit ICC 0.91 – 0.96, inter visit ICC of 0.56- 0.90[74]
OCT	€85.000	Vessel density / morphology. flow	2/3 minutes	Intra day (CV%) 5.5 – 8.5 [82]
OCT	€20.000	Retinal thickness, retinal vasculature	± 10 min	Measurements (CV% ≈ 1.8) between observers ICC ≈ 0.99 [100]
Laser Doppler flowmetry (ophthalmology)		Retinal venous and arterial flow	± 3 min	CV 12.7% - 13.8% [94]
LSFG	€85.000	Retinal venous and arterial flow		ICC of ≥ 0.937 [95]
Retinal function imager	€150.000	Retinal venous and arterial flow, retinal vasculature, retinal oximetry	±20 min	Intra subject CV 6-11% [90]
Fundus photography	€15.000	Retinal vasculature	±10 min	Measurement ICC 0.64-0.69 for, grader ICC 0.79 to 0.83 [110]

TABLE 2 Overview of suitable challenges for cutaneous microvascular measurements

	Laser Doppler flowmetry	Laser Doppler imaging	Laser speckle contrast imaging	Thermography	Video capilairoscopy	D-OCT
Transdermal iontophoresis	X	X	X	X		X
Local thermal hyperemia	X	X	X	X	X	
Local cooling	X	X	X	X	X	
Post-occlusive reactive hyperemia (PORH)	X	X	X	X		
Assessment of micro-oscillations	X		X			

compounds that influence the bioavailability of NO in the endothelium. Furthermore, iontophoresis can be also used to study other effects on the microvascular physiology, such as the effect of histamine on skin blood flow and thermoregulation capacity of the skin. An issue with iontophoresis is the variability which is especially large with laser Doppler imaging (34.4% to 42.0%)[84] and the nonspecific vasodilation caused by the electric current and ions in the vehicle, which is variable in effect. Although this can be minimalized by using deionized water as vehicle and using a control vehicle to estimate this effect, the effect remains substantial.[83] Moreover, there is currently no standard protocol which makes it difficult to compare from different studies.

Local thermal hyperemia

The skin is locally heated to a temperature of up to 42°C producing a local vasodilation which induces an increase in skin blood flow within 5 minutes after the start of heating and a prolonged secondary plateau is reached after 20–30 minutes of heating. The initial peak is caused by a sensory nerve axon reflex mainly mediated through endothelium-derived hyperpolarizing factors and transient receptor potential vanilloid type-1 (TRPV1) channels. The secondary peak is mostly mediated through the endothelial tissue, for a large part due to NO release.[85] Alternatively, local heating to 39° is thought to induce an isolated NO-dependent dilation without the sensory axon reflex.[86] Results of this challenge need to be performed in strictly climate controlled laboratory, and have an intermediate variability (CV% 17 for plateau and CV% 39 for peak measurements), however this is dependent on the peak temperature and the heating rate.[87]

Local cooling

Rapid local cooling of the skin leads to vasoconstriction and consequent decreased blood flow, which is followed by a transient vasodilation and subsequently a prolonged vasoconstriction. The initial vasoconstriction is mediated through the adrenergic system by upregulation of α-2c-receptors, where the sustained vasoconstriction is mostly mediated by inhibition of NO. The mechanism for the transient vasodilation is unknown.[85] Local cooling is mostly studied in patients with Raynaud phenomena, but can also be used to study endothelial function, however due to the complex and partly not understood physiology of this reaction, other methods are preferable.

Post-occlusive reactive hyperemia

PORH measurements are usually performed on the volar forearm, where occlusion is produced by inflating a blood pressure cuff on the upper arm. Releasing the occlusion results in an initial peak in blood flow, which returns to baseline several minutes after the occlusion. The initial peak is mediated by a mixture of sensory nerve involvement[88] and endothelium-derived hyperpolarizing factors, caused by the shear stress ischemia during the occlusion.[88] NO-release does not play a major role in occlusion-induced cutaneous hyperemia.[89] Endpoints of PORH include, time to hyperemia, maximal flow, time to return to baseline and AUC from time of maximal flow to time of return to baseline. PORH is frequently used as a test for microvascular endothelial function, as it is minimal invasive, easy to perform, shows good contrast between populations with different endothelial functions and has an excellent repeatability(CV 8-11%).[47,87,90]

Assessment of micro-oscillations

Frequency domain analysis of longitudinal microvascular blood flow measurements such as LDF and LSCI has been performed in various disease models.[91] Oscillations in different frequencies have been linked to vasomotion, neurogenic activity and endothelial function. NO release and EDHF have been attributed the main factors to the vasomotion. However, there is limited evidence for the use as a marker for endothelial or vascular function. Furthermore there is no standardization of this measurement between different studies.[91]

RETINAL MICROVASCULAR MEASUREMENTS

Laser Doppler flowmetry

Doppler/speckle imaging techniques have also been applied to monitor the retinal microcirculation. Through Doppler imaging, the Doppler shift

(i.e., the movement of the erythrocytes in the retina vessels) can be assessed, which is an indirect measurement of retinal blood flow. Measurements are expressed in arbitrary units. Besides the application in ophthalmologic research these measurements can be predictive for microvascular dysfunction and therefore can be applied in research for a wide range of diseases including cardiac and nephrological disease.[92,93] Laser Doppler flowmetry showed good inter session repeatability (CV of 12.7% - 13.8%) and intra observer (CV of 12.6%) in healthy volunteers. [94] Limitations are the use of arbitrary units and the requirement of clear media to obtain images of sufficient quality.

Laser speckle flowgraphy (LSFG)

Laser speckle flowgraphy (LSFG) is a relatively new technique and uses the speckle pattern to obtain information about microvascular blood flow and is an indirect measurement of retinal blood flow. Measurements are expressed in arbitrary units.

LSFG has showed excellent reproducibility with an ICC of ≥ 0.93[95] and also LSFG measurements are strongly correlated with age,[95] brachial–ankle pulse-wave velocity and intima–media thickness.[96] Measurements are relatively easy to perform, are non-invasive, and do not require pupil dilation.[97] Limitations are the use of arbitrary units, the requirement of subject fixation and clear ocular media to obtain images of sufficient quality.

Optical coherence tomography- angiography

The technology behind ophthalmologic OCT is similar to dermatological OCT and gives information about the retinal nerve fiber layer and retinal micro vasculature using OCT- angiography (OCT-A) measurements. Although experimental set-ups have been used to quantitatively measure retinal blood flow with D-OCT[98,99] currently no commercial OCT devices are available for the assessment of retinal blood flow. Most studies with OCT therefore include static measurements of the retinal vasculature and morphology. OCT has demonstrated an excellent CV of 1.8% between measurements and an excellent intra-observer correlation of 0.99.[100] However, results from devices from different manufacturers are not interchangeable, due to the high inter device variability, and therefore comparisons between these instruments is nearly impossible.[101] Besides its utility in several ocular diseases,[102] OCT has shown to be a useful biomarker measurement in several other specialisms for example neurology[103] Disadvantages of OCT-A are the small measurement area, measurement time (up to 10 minutes per eye) and the complex analysis.

Retinal function imager

Retinal function imager (RFI) is a technique that measures retinal blood flow velocity by detecting the movement of individual erythrocytes via stroboscopic illumination and high-speed digital imaging of the retina. With RFI, the velocity is assessed instead of the flow, while theoretically, this can be estimated through vessel diameter. Through an advanced algorithm, detailed capillary maps of the retina can be obtained. RFI has been applied in several patient groups, including sickle cell patients, multiple sclerosis patients, and several ocular diseases.[104–107] RFI has a CV of 6-11% in both patients and healthy volunteers.[90] However further development of the device has been halted. Acquirement of pictures requires well-trained specialists. Other disadvantages are the time required for acquisition and the time required and the complexity of the analysis.

Fundus photography

Fundus photography covers the imaging of the retina, fundus photography cannot be used to directly measure the retinal blood flow. Although fundus video with fluorescence agents can provide none quantitively information, such as vasospasm,[108] or venous increased pressure. Outcome measures such as vessel diameters, tortuosity, and fractal dimensions, fovea size and thickness have been intensively studied and related to several systemic diseases.[109] Fundus photography has a repeatability of 0.64 to 0.69 for intra individual variability and 0.79 to 0.83 for the inter-observer variability.[110] Fundus photography has been used for the diagnosis and long-term follow-up of eye disease in several mechanistic and epidemiologic studies, due to its widespread availability.[111] With the usage of deep learning algorithms it is expected that even more outcome measures can be obtained from fundus photography such as cardiovascular risk factors and gender.[112] Furthermore new devices have been developed with a dynamic imaging frequency which can be useful in combination with challenges.

Retinal challenges

Hyperoxic measurements

The breathing of 100% oxygen leads to an increase in pO_2 and subsequently vasoconstriction of vessels and a decreased blood flow. The exact mechanism of this vasoconstriction is not understood.[113] Measurements of hyperoxia-induced vasoconstriction are usually performed in the retina, however have been assessed in the skin as well.[114] Disadvantages of the challenge are the differences in results with different measurement techniques,[115] and the cutaneous measurements, showing little effect. To our knowledge, there is no literature available about the variability.

Retinal Flickering light stimulation

Stimulation with flickering light leads to an increased neural activity and metabolic activity subsequently leading to vasodilation of the retinal vasculature. This response has been shown to be mediated by the endothelial release of NO.[116] There is a large body of studies that links retinal abnormalities to cardiac and metabolic diseases.[117] Advantages of the technique are that responses are fast, can be measured in real time, show a good reproducibility [118] are noninvasive and the technique is widely available. Although some studies have issues regarding repeatability the intra subject repeatability has been reported up to ICC 0.819.[119]

CURRENT PRACTICE

For cutaneous functional microvascular assessments, LSCI is becoming the gold standard, due to the low variability, full field imaging and high acquisition speed compared to other methods. Moreover, most challenges can be performed using LSCI, meaning most endothelial effects can be assessed, measurements are relatively simple and therefore can be performed in a clinical setting. However, drawbacks are the high costs and limited mobility.

For morphological and anatomical assessments of the skin, video capilaroscopy is still the primary choice for morphological assessments due to the excellent measurement accuracy and limited variability. Downsides however include relatively complex execution of the measurement. Furthermore, D-OCT has great potential to surpass video capilaroscopy, although standardization between device specifications and homogenization of measurement protocols is necessary in addition to clinical validation.

For ophthalmologic measurements there is no gold standard and several measurements can be used complementary with each other as they measure different outcomes. For LSFG, there is a great potential for functional measurements although larger standardized studies in which the outcomes are related to clinical outcomes are required to further establish this methodology. For morphological assessments, there is a large body of evidence for using fundus photography outcomes as a biomarker.[109] However OCT-A is able to measure the microvasculature in higher detail, but has not been applied in large cross sectional studies.

Future implications

For wider acceptation and application of these methodologies in the clinical setting, standardized protocols are essential. These standardized protocols should be clinically validated including variability of the measurements and comparison of the microvascular measurements with clinical outcomes.[120]

Future development in microvascular imaging is expected to be heading towards low cost handheld devices which will be easy to apply and with low variability in clinical practice. The recent development of infrared cameras attached to mobile devices is one example. [121] Although these devices might show higher variability, their low costs and high portability will make them more usable in a clinical setting since measurements can be repeated within the same subject frequently. Also this will allow for microvascular home monitoring, which will more closely mimic a real life situation.

Furthermore, work is done for imaging devices to directly measure flow speed, opposed to indirect measurements.[122] For dermal imaging multispectral imaging devices might become commercial available, which will allow outcomes based on different techniques such as oxygenation and microvascular flow to be obtained during a single measurement. Automated analysis of measurements will be of great value for the comparison of results between different studies with different methods as this will decrease inter-observer variability. There

are currently a few devices available that are able to do this such as the periflux. [123] Furthermore analysis with artificial intelligence and deep learning will have a great impact as it might be able to predict events and outcome measurements that we were previously not able to predict by manual analysis. [112]

Microvascular (retinal) abnormalities may be a useful marker for disease in the traditional (cardio) vascular specialisms such as cardiology and nephrology, but intriguingly have also be used as a predictor for events in psychiatry (schizophrenia)[124] and neurology (multiple sclerosis).[125]

Despite the wide-spread use of some of the techniques, there is a lack of measurement standardization between different studies. Therefore, it is difficult to compare variability and sensitivity of different studies and to relate them to physiological or pathological processes. Moreover, it is difficult to compare the results from different methodologies. A few studies have been performed comparing measurements of different techniques, which showed that results from laser based methods are exchangeable,[47] however there is only a weak to none correlation between microvascular and macrovascular measurements for endothelial function.[44,126,127] Although, for clinical application it is useful that outcomes of different methodologies are exchangeable.

These techniques could not only be useful for research but also on a daily basis in clinical practice. VC and OCT are already established tools for the diagnosis of certain diseases and also LDI and thermography are used in the monitoring of wound healing. Intra-operative imaging with LSCI to predict clinical outcome such as flap survival.[128,129] Future larger multicenter studies, might help in validating these methodologies and thereby establishing their clinical use.

CONCLUSION

In conclusion, various methods for microvascular imaging are currently available and selection of the applied methodology should be based on pathology and clinical setting of the different techniques. LSCI and LSFG show great potential for functional measurements however different challenges cannot be related to particular pathways. The skin and retinal measurements described in this article are an example of how these techniques can be used to assist in research and even clinical monitoring of patients. Validation of all current techniques is at best partial and more work has to be done to perform such standardized validation tests.[120]

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