Non-invasive assessment of skin microvascular function in humans: an insight into methods.

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Non-invasive assessment of skin microvascular function in humans: an insight into methods

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Running head: Methods to assess skin microvascular function

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Abstract

For more than two decades, methods for the noninvasive exploration of cutaneous microcirculation have been mainly based on optical microscopy and laser Doppler techniques. In this review we discuss the advantages and drawbacks of these techniques. Although optical microscopy-derived techniques, such as nailfold videocapillaroscopy, have found clinical applications they mainly provide morphological information about the microvessels. Laser Doppler techniques coupled to reactivity tests are widespread in the field of microvascular function research, but many technical issues need to be taken into account when performing these tests. Post-occlusive reactive hyperemia and local thermal hyperemia have been shown to be reliable tests, although their underlying mechanisms are not yet fully understood. Acetylcholine and sodium nitroprusside iontophoresis, despite their wide use as specific tests of endothelium-dependent and independent function, respectively, show limitations. The influence of the skin site, recording conditions and the way of expressing data are also reviewed. Finally, we focus on promising tools such as laser speckle contrast imaging.

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Keywords: microcirculation; capillaroscopy; laser Doppler; laser speckle; iontophoresis; local thermal hyperemia; post-occlusive hyperemia.

Why and how to assess skin microvascular function?

Since the development of methods allowing the study of microcirculation, microvascular dysfunction has been associated with several vascular diseases as well as in aging [1]. The role of generalized microvascular dysfunction in the pathophysiology or as a consequence of these diseases has also been questioned. Indeed, patients with impaired coronary microvascular function also have evidence of impaired peripheral microvascular function, suggesting a generalized disorder in the regulation of the microvasculature [2]. Similar findings have been reported of correlated abnormalities between cutaneous and retinal microvasculature in diabetic patients [3].

As the skin is readily accessible, it provides an appropriate site to assess peripheral microvascular reactivity. Moreover, recent technological advances have provided simple and non-invasive methods to assess skin microvascular function. Therefore, human cutaneous circulation could be used as a surrogate marker of systemic microvascular function in various diseases. However, this raises the issue of how representative the microcirculation in the skin is to the microcirculation in other organs. To date, the skin has been used as a model of microcirculation to investigate vascular mechanisms in a variety of diseases, including hypertension and other cardiovascular risk factors [4-6], diabetes [3, 7], or end-stage kidney disease [8]. Skin microcirculation has also been used as a model of microvascular function in experimental shock [9]. The issue of human cutaneous circulation as a model of generalized microvascular function has been discussed in a recent viewpoint by Holowatz et al [10].

In other cases, skin microvasculature is specifically affected, e.g. systemic sclerosis [11, 12], burns, flaps or wounds. Altered skin microvascular function could therefore be a surrogate marker in these pathologies.

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Finally, noninvasive and reliable tests would be useful to evaluate the effect of drugs on the peripheral microvascular system.

For more than two decades, methods for the noninvasive exploration of cutaneous microcirculation have been mainly based on optical microscopy and laser Doppler techniques [13], as well as the evaluation of tissue oxygenation. Capillaroscopy is an optical *in vivo* microscopy technique allowing direct visualization of superficial skin microvessels, which has been mostly used in the study of rheumatic diseases, especially systemic sclerosis [14]. More sophisticated techniques have recently been developed, including orthogonal polarization spectral (OPS) imaging [15] and most recently sidestream dark field (SDF) imaging [16]. Besides microscopy techniques, laser Doppler provides an index of skin perfusion by measuring the Doppler shift induced by coherent light scattering by moving red blood cells [17]. Laser Doppler techniques offer a simple and non-invasive estimate of skin perfusion. However, despite extensive use over the past thirty years, they still suffer from lack of standardization.

In this review we will describe recent advances in methods and discuss the issue of data expression. An evaluation of tissue oxygenation is beyond the scope of this review; the different techniques including venous oxygen saturation, PO_2 electrodes, reflectance spectroscopy, near-infrared spectroscopy and PCO_2-derived measurements, have been expertly reviewed by De Backer *et al* [18].

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Optical microscopy-derived techniques

Videocapillaroscopy

Videocapillaroscopy consists of the direct *in vivo* observation of skin capillaries using a microscope with an epi-illumination system and image transmission to a video camera [19].

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Recently available digital systems have made the technique more reliable and user-friendly [20].

The skin site most studied using videocapillaroscopy is the periungueal region. Indeed, nailfold capillaries are parallel to the surface of the skin which facilitates their observation. Nailfold videocapillaroscopy (NVC) allows the visualization of erythrocytes but not vessel walls. As a consequence, only microvessels with circulating erythrocytes at the time of the examination are visible [13]. The normal NVC pattern is characterized by a homogeneous distribution of parallel capillary loops from 6 to 15 µm in diameter [13] (Figure 1A).

Abnormal patterns are observed in diseases affecting digital skin microvasculature (e.g. systemic sclerosis, Figure 1B), showing morphological abnormalities of the capillaries (enlarged loops, giant capillaries, ramifications, capillary disorganization), micro-hemorrhages and lower density (capillary loss) [20]. Capillary abnormalities in systemic sclerosis have been classified into early, active or late patterns by Cutolo et al [21]. Since the first description of abnormal finger capillary patterns in connective tissue diseases using capillaroscopy [22], the technique has played an increasing role in the early diagnosis of scleroderma spectrum disorder [20], and when used significantly improves the sensitivity of the American College of Rheumatology (ACR) criteria in the diagnosis of patients with limited systemic sclerosis [23]. Finally, a prognostic capillaroscopic index has been proposed to identify patients with Raynaud's phenomenon in whom the risk of developing scleroderma spectrum disorders is high [24].

Although less widely used than in the diagnosis and follow-up of systemic sclerosis, several other applications of NVC in autoimmune diseases have been suggested. Indeed, capillary abnormalities have been described in some patients with systemic lupus erythematosus [25] or rheumatoid arthritis [26], although no specific patterns have been identified.

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Elsewhere to the periungueal region capillaries are perpendicular to the skin's surface and using videocapillaroscopy only the top of perfused loops are visible, which appear as red spots. This technique does not allow morphological observation of capillaries but provides the density of functioning capillaries per field. Reactivity tests, including venous occlusion and arterial post-occlusive reactive hyperemia (PORH), have been proposed to enhance capillary recruitment. They allow the assessment of total maximal density with good reproducibility [27]. When performed on the dorsum of the finger, venous congestion showed better results than brachial PORH [28]. Using such methods, both baseline and maximal capillary recruitment were significantly lower in patients with essential hypertension than in normotensive controls [4]. We note that some authors have described a reversion of both functional and structural capillary rarefaction in patients under effective antihypertensive treatment [29, 30]. Similar studies have shown impaired capillary recruitment (i.e. an absolute difference or percentage increase between functional and maximal densities) in patients with type 1 diabetes compared to controls, although the baseline density was higher in these patients [31]. Chang et al did not observe any difference in capillary density between patients with diabetes mellitus (with or without retinopathy) but morphological capillary abnormalities in patients with retinopathy compared to patients without retinopathy and controls [3].

The injection of a dye (e.g. fluorescein) coupled to capillaroscopy has been used to assess transcapillary and interstitial diffusion patterns. Indeed, fluorescein-enhanced capillaroscopy improves contrast and provides an index of capillary permeability. This technique has been used to study the influence of age on microcirculation [32] and in various diseases including diabetes [33], systemic sclerosis [34], psoriasis [35], or to evaluate the vascular integrity of skin flaps [36, 37]. This technique however is increasingly replaced by orthogonal polarization spectral (OPS) and sidestream dark field (SDF) imaging (see below), which are safer, non-invasive and provide better contrast.

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In conclusion, nailfold videocapillaroscopy has found clinical applications in diseases affecting digital skin microcirculation (e.g. systemic sclerosis). Otherwise, skin capillaroscopy provides low-contrast images and only allows capillary density to be quantified. A morphological study of the microvessels in areas other than the periungueal region has not found any clinical application. Indeed, it would require transillumination or fluorescent dyes, which, *in vivo*, is hardly compatible with a non-invasive exploration.

Orthogonal polarization spectral (OPS) and *sidestream dark field (SDF) imaging*

In OPS imaging the tissue is illuminated with linearly polarized green light and the remitted light is provided by depolarized photons scattered by the deeper layers of the tissue, imitating transillumination of the superficial layer [15]. SDF imaging is a closely related technique, but illumination is provided by concentrically placed light emitting diodes surrounding a central light guide [16]. The green light is scattered by the deeper layers of the tissue while it is absorbed by hemoglobin, providing an image in which RBCs appear as dark moving spots against a white/grayish background [16].

OPS imaging is a relatively inexpensive technique and has the advantage of being portable [38]. It provides optimal image resolution on organs covered by a thin epithelial layer and does not require the injection of fluorescein to obtain an excellent level of contrast [38].

OPS and SDF have been used during surgery to assess the microcirculation of several organs including the brain [39, 40], the kidney [41] or the liver [42]. The most studied site however is the sublingual region, where the density of perfused capillaries can be non-invasively assessed [18]. Semi-quantitative analysis of the microcirculation has been proposed with OPS, based on a scoring including both the measurement of perfused capillary density and the flow heterogeneity between the different areas [43]. The main applications of OPS

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and SDF concern critical care medicine. De Backer *et al* showed that microcirculation assessed with OPS on the sublingual mucosa was impaired in severe sepsis [44]. In the same way, SDF allowed identifying significant abnormalities in microvascular density during early post-resuscitation phase, which returned to baseline within 48h after cardiac arrest [45]. Although the image quality is not as good as on mucosa, OPS has also been used on lower limb skin to evaluate microcirculation in chronic venous insufficiency [46]. Other applications of skin OPS imaging include the assessment of microcirculation in burn wounds [47, 48]. Nonetheless, OPS use in burn wound severity is still predominantly used for research [38].

Application of pressure with OPS or SDF probes during examination modifies the flow velocity in vessels under investigation [49] and therefore induces artifacts. Moreover, motion-induced image blurring is another limitation of OPS, attenuated in SDF imaging. Finally, they cannot be used in individuals with phototypes IV, V and VI according to Fitzpatrick classification because melanin absorbs light of a similar wavelength than hemoglobin [50].

In conclusion, OPS and SDF imaging are semi-quantitative techniques implemented in small devices that can be used at the bedside. They provide good quality images of microvessels on thin epithelial layers. The most studied site is the sublingual region, and has been used mainly in critically ill patients. The main limitations of OPS and SDF imaging are the artifacts induced by movement and pressure. Finally, quantitative assessment of skin blood flow is not fully automatized yet, although this could be achieved by the development of new software [18].

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Laser Doppler

Techniques

Laser Doppler is based on the backscattering of a beam of laser light. The light undergoes changes in wavelength (Doppler shift) when it hits moving blood cells. The magnitude and frequency distribution of these changes in wavelength are related to the number and velocity of red blood cells [17]. Laser Doppler does not directly measure skin blood flow but provides an index of skin perfusion, quantified as the product of average red blood cell velocity and their concentration, often referred to as flux. Most of the current devices use a wavelength of 780 nm, which provides good skin penetration independently of skin color and oxygen saturation [51].

The first laser Doppler technique developed is called flowmetry (LDF), also referred to as laser Doppler perfusion monitoring (LDPM). Single point LDF assesses blood flow over a small volume (1 mm^3 or smaller) with a high sampling frequency (often 32 Hz) and is accurate at detecting and quantifying relative changes in skin blood flow in response to a given stimulus [52]. However, the regional heterogeneity of skin perfusion [53] leads to spatial variability, which contributes to the relatively poor reproducibility of the technique [54].

In contrast, the more recently developed laser Doppler imaging (LDI), or laser Doppler perfusion imaging (LDPI), provides 2D images using the same physical principle as LDF [52]. In LDI, the laser beam is reflected by a computer-driven mirror to progressively scan the area of interest. A fraction of the backscattered light is detected and used to map tissue blood flux, each pixel representing a perfusion value. LDI decreases spatial variability but it is much slower than LDF making rapid changes in skin blood flow over the larger areas more difficult to record. Nevertheless, more recent imagers use a multi channel laser Doppler line permitting faster scanning.

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A linear relationship between the laser Doppler signal and microvascular flow has been demonstrated in the range from 0 to 300 mL.min^{-1} per 100 g tissue [55]. However, it does not provide an exact measure of flow (i.e. mL.min^{-1}) as can be extrapolated when using strain gauge plethysmography. Therefore, laser Doppler is mostly used to assess microvascular reactivity, by challenging microvessels with various tests. Among the different tests used in combination with laser Doppler, the most common are iontophoresis of vasoactive drugs, post-occlusive reactive hyperemia (PORH) and thermal challenges. Results are often expressed as arbitrary perfusion units (PU; 1 PU = 10 mV) or as cutaneous vascular conductance [CVC, i.e. flux divided by arterial pressure (in mV/mm Hg)] [52].

Microdialysis is a technique consisting of the intradermal insertion of small fibers with semi-permeable membranes and is mostly used for the continuous sampling of small water-soluble molecules within the extracellular fluid space in vivo [56]. Nonetheless, it can also be used to deliver drugs to a small area of tissue, avoiding confounding systemic effects [52]. Although minimally invasive, microdialysis offers the advantage of a controlled drug infusion rate and the absence of current-induced vasodilation, compared with iontophoresis. However it is painful and justifies the use of local anesthesia. Both local inflammation and anesthetic drugs may interfere with the response. This approach coupled with LDF has been used to assess the role of NO in skin post-occlusive and thermal hyperemia [57, 58].

Acetylcholine and sodium nitroprusside iontophoresis

Iontophoresis is a method for non-invasive transdermal drug delivery based on the transfer of charged molecules using a low-intensity electric current (Figure 2). Among the factors involved in iontophoretic drug transfer, the concentration and the pH of the solution, the intensity of the current applied, the duration of iontophoresis, and the nature of the skin surface (thickness, glabrous or not) play a key role [59]. Combined with laser Doppler,

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acetylcholine (Ach) and sodium nitroprusside (SNP) iontophoresis have been widely used to assess microvascular endothelial dependent and independent vasodilation, respectively [52, 60]. It is of note that vasodilator iontophoresis has been proposed as a new therapy in diseases affecting skin microcirculation of the digits, such as systemic sclerosis [61, 62]. This is particularly interesting but must be distinguished from iontophoresis as a tool to explore microvascular function, and is beyond the scope of this review.

The mechanisms by which Ach iontophoresis induces vasodilation of the microvessels remain unclear [52, 60]. A Cyclooxygenase (COX)-dependent pathway seems to be predominant [63-65], although data are conflicting [66, 67]. On the other hand, nitric oxide (NO) does not extensively contribute to the response [63, 64]. Interactions between prostaglandin and NO pathways could explain the discrepancies between the results of these different studies [60]. Besides the endothelium-dependent vasodilation, iontophoresis of Ach induces C-fiber (axon reflex)-mediated vasodilation [66]. The variable effect of COX inhibition and local anesthesia [66, 67] on Ach-induced vasodilation may be attributed to these different components of the response to Ach iontophoresis.

One of the main issues to be taken into account with iontophoresis is the non specific effect of the current itself, which interferes with the vasodilation potency of administered drugs. Indeed, current-induced vasodilation is observed when pure water alone is used in iontophoresis (sometimes referred to as "galvanic response"); the reaction is more pronounced at the cathode and delayed at the anode [68, 69]. The amplitude of current-induced vasodilation depends on the delivered electrical charge (i.e. the product of current intensity by duration of application) [69] (Figure 3) and the current delivery pattern. For a similar charge, repeated applications induce more non specific effects than continuous iontophoresis [70]. Durand et al showed that current-induced vasodilation was abolished by local anesthesia and largely reduced after desensitization of C-nociceptive fibers by capsaicin [69], suggesting a

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role of neural axon reflex. Moreover, prostaglandins are likely to be essential for this axon reflex-related vasodilatation [71], mainly through the COX-1 pathway [72]. Nonetheless, the exact underlying mechanisms of the interference of current with vasodilation remain unclear.

Different vehicles have been used to dilute drugs (e.g. tap water, deionized water and saline) with various galvanic responses [60]. In the excellent paper by Ferrell et al [73], the authors have shown that distilled water alone induces a more pronounced current-induced vasodilation than saline [73]. However, it is interesting to note that Ach or SNP iontophoresis induced comparable increases in skin blood flow whether diluted in distilled water or saline [73]. This is probably due to the presence of ions which reduce the resistance of the solutions after drug dilution, whereas deionized solutions show higher resistance. The authors further showed a threshold (between 60 and 70 V.min) of the integral of voltage over time beyond which current-induced vasodilation is triggered. Although the choice between NaCl and deionized water as vehicle has little influence on Ach and SNP iontophoresis, one should bear in mind the difference between these vehicles when they are used as controls.

Besides the resistance of the solution, skin resistance also influences drug delivery [74]. Skin resistance is variable between individuals and between different skin areas, depending on the density of sweat ducts or hair follicles [60]. Ramsay et al showed a significant linear inverse correlation between skin resistance and the response to Ach or SNP iontophoresis [74]. Monitoring voltage across the iontophoretic circuit seems useful in order to take into account resistance, although it is rarely done today. General good practice however includes mild epidermal stripping with adhesive tape and an alcohol swap [60].

The reproducibility of Ach and SNP iontophoresis is good when assessed with LDI, especially when the perfusion is corrected by the resistance time integral [75]. Seven-day reproducibility of the peak SNP iontophoresis assessed with LDI has provided a within subject coefficient of variation (CV) of 22% and an intra-class coefficient of correlation (ICC)

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Ferrell et al therefore highlight the relevance of monitoring voltage during iontophoresis: the integral of voltage over time showed a threshold (between 60 and 70 V.min) beyond which current-induced vasodilation is triggered [64]. The same authors have shown
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of 0.72 [76]. When using LDF, the reproducibility of Ach iontophoresis was poorer (ranging from 25% to 35% depending on the way of expressing data) [77]. Some authors have recently proposed the use of methacholine chloride instead of Ach. Indeed, iontophoresis of methacholine exhibited less inter-site and interday variability than Ach [78]. The reproducibility of SNP iontophoresis assessed with LDF is extremely poor. In 14 healthy subjects, the CV ranged from 69% to 160% on the dorsum of the finger (according to the way of expressing data) whereas it ranged from 63% to 95% on the forearm (personal unpublished data). This suggests that the spatial variability of Ach and SNP iontophoresis is high; although this can be overcome by using large study areas assessed with LDI.

Another limitation is the site of iontophoresis. Indeed, on the finger pad, we did not observe any vasodilation on SNP iontophoresis in patients with SSc and in controls [79]. This could be due to rapid dermal clearance of the drug on the finger pad. In contrast, vasodilation has been reported on the dorsum of the finger [61].

In conclusion, iontophoresis of Ach and SNP have been used extensively to assess microvascular endothelium dependent and independent vasodilation, respectively. However, the complexity of the underlying mechanism of the reaction to the iontophoresis of Ach makes its use as a specific test of endothelial function debatable [1]. Moreover, other limitations must be acknowledged, including non-specific effects and poor reproducibility when LDF is used [80]. Therefore, studies using iontophoresis must be carefully designed to reduce these and LDI rather than LDF is recommended to assess perfusion. Provided that a low intensity current is used (i.e. <100 µA), saline should be preferred as the control (Figure 3). Pre-treatment with a local anesthetic is a way to limit axon reflex-induced vasodilation [76]. Limiting current density (<0.01 mA/cm²) and charge density (<7.8 mC/cm²) also decreases current-induced vasodilation [81]. Finally, skin resistance may be reported and can be readily approximated by connecting a voltmeter in parallel [75]. Perfusion data may then

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be normalized to skin resistance, or resistance can be standardized by adjusting the distance between the electrodes.

Post-occlusive reactive hyperemia

Post-occlusive reactive hyperemia (PORH) refers to the increase in skin blood flow above baseline levels following release from brief arterial occlusion [52]. Many mediators contribute to PORH. Sensory nerves are partially involved through an axon reflex response [82, 83]. Local mediators include large-conductance calcium activated potassium (BKCa) channels that seem to play a major role [82], suggesting that endothelium-derived hyperpolarizing factor (EDHF) is involved; while results are conflicting concerning the implication of prostaglandins [67, 84, 85]. The inhibition of NO synthesis does not alter PORH on the forearm [58], but recent work suggests that COX inhibition unmasks the NO dependence of reactive hyperemia in human cutaneous circulation [85]. On the finger pad however, the response seems to be partly NO-dependent [86]. In summary, PORH should not be considered as a test for microvascular endothelial function itself, but could be used as a tool to detect overall changes in microvascular function.

Various parameters can be quantified from the flux response after arterial occlusion (Figure 4). One of the most commonly used is peak hyperemia, whether expressed as a raw value or as a function of baseline, i.e. area under the curve, peak minus baseline or relative change between peak and baseline expressed as a percentage, calculated from [(peak − baseline)/baseline] × 100. Peak perfusion may also be scaled to the so-called maximum vasodilation achieved when the skin is heated to 42°C or higher [87]. Time to peak perfusion is another parameter quantified when performing PORH, but its physiological significance as a marker of skin microvascular reactivity remains to be established.

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When assessed with single-point LDF the inter-day reproducibility of PORH is variable, depending both on the skin site, the way of expressing data and the baseline skin temperature. On the finger pad, the reproducibility is acceptable when PORH is expressed as raw peak perfusion or scaled to maximum vasodilation (CV around 25%) [54]. However, reproducibility is poor (CV are 45% or higher) when peak perfusion is expressed as a function of baseline [54, 80]. Most of the studies exploring PORH reproducibility have been performed on the volar surface of the forearm, and results are conflicting. Reproducibility was excellent (CV from 6% to 22%) when the locations of the laser probes were marked so that exactly the same sites were studied from one day to another [88]. However, reproducibility was only fair to good (CV around 20%) when the position of the probe was recorded with less precision [77] and decidedly poor when the skin sites were randomly chosen (CV were 40% or higher) [54].

As temperature plays a key role in baseline flux it is not surprising that homogenizing skin temperature when performing PORH assessed with single-point LDF improved reproducibility on the forearm, especially when data were expressed as a function of baseline. Maintaining skin temperature at 33 °C throughout the recording provided acceptable 1-week reproducibility, whether expressed as peak CVC or as a function of baseline (CV were 33% or lower) [89]. However, skin temperature homogenization only partially compensates for spatial variability, as the inter-site reproducibility of simultaneous PORH measurements on the forearm was poor compared to that of full-field techniques [89]. Therefore, it is likely that the variation in capillary density between different skin sites is the major source of variability when using single-point LDF. The use of full-field techniques such as LDI could lessen this variability.

However, LDI is not fast enough to accurately assess the kinetics of PORH (which lasts only a few seconds) over large areas, resulting in a potential shift of the recorded peak

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compared to the peak measured with LDF. However, some groups have successfully used LDI to assess PORH by studying very small areas, scanning up to 20 images/min with good reproducibility (CV ranging between 10 and 15%) [90]. Nevertheless, the major advantage of LDI (spatial resolution over large areas) is lost. Line scanning LDI may be another way of overcoming this issue. Moreover, the recently developed high frame rate laser speckle contrast imaging (LSCI) technique allows continuous assessment of skin perfusion over wide areas, and could combine the advantages of both LDF and LDI [89].

Another issue when comparing protocols that use PORH is the heterogeneity of study designs. Indeed, there is no consensus about the optimum protocol and a wide variety in the duration of brachial artery occlusion exists, from 1 to 15 min, with a positive relationship between post-occlusive hyperemic response and the duration of arterial occlusion [58, 90, 91]. Occlusion lasting 5 min has been extensively used, probably from analogy with brachial artery flow-mediated dilation (FMD) methods, a standardized tool used to investigate endothelial function in conduit arteries [92]. Such standardization of methods is lacking for the evaluation of microvascular function. Nonetheless, different cuff pressures ranging between 160 and 220 mmHg did not significantly influence PORH, provided that the applied cuff pressure exceeded systolic blood pressure [90].

In conclusion, PORH is a widely used test of microvascular function when coupled with laser Doppler and provides an overall index of microvascular function, combining axon reflex, COX-dependent pathways and probably EDHF effects. All the same, special care should be taken to avoid methodological bias. Indeed, the duration of occlusion, baseline skin temperature and site of measurement (i.e. glabrous or nonglabrous skin) can influence PORH amplitude and reproducibility. Full-field techniques partly overcome these difficulties, but LDI is too slow to accurately assess the kinetics of the response over large areas, which limits

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its interest. Finally, LSCI has shown excellent reproducibility but more data are needed to assess the linearity between the LSCI signal and skin blood flow.

Local thermal hyperemia (LTH)

Among thermal challenges, local heating, also referred to as local thermal hyperemia (LTH), provides an integrated index of neurovascular and nitric oxide-dependent cutaneous blood flow regulation [52]. In healthy subjects, LTH is characterized by an initial peak within the first 5 min, a subsequent nadir followed by a sustained plateau (Figure 5). The initial peak mainly depends on sensory nerves as it is significantly attenuated by local anesthesia [57]. Although to date, there has been no positive evidence to support this claim, it has been suggested that calcitonin gene-related peptide (CGRP) [93], possibly co-released with substance P, is responsible for this initial peak [94]. Recent work has shown that transient receptor potential vanilloid type-1 (TRPV-1) channels contribute to the initial axon reflex and, to a lesser extent, to the late plateau [95]. The late plateau phase however is insensitive to local anesthesia and is mostly NO-dependent [57]. The binding of heat shock protein 90 (HSP90) to endothelial NO synthase (eNOS) may be involved in the late plateau as geldanamycin (a HSP90-specific inhibitor) decreased CVC during local heating [96]. As NO synthase inhibition does not completely abolish the response, other contributors are thought to be involved, including norepinephrine and neuropeptide Y [1]. Recently, reactive oxygen species have been shown to play a role in plateau hyperemia by limiting the availability of NO [97].

The two independent phases of LTH imply a dichotomized analysis of the recording. Figure 5 shows the parameters that are frequently used to assess the response, i.e. peak perfusion (“axon reflex-dependent vasodilation”) and plateau perfusion (“NO-dependent vasodilation”). The issue of data expression is similar to that discussed above for PORH.

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Indeed, data may be expressed as raw perfusion units or CVC, as a function of baseline or scaled to maximal vasodilation. The latter form of expression may be useful when studying the initial peak [98]. Interestingly, although the area under the curve of the whole tracing has the drawback of masking the existence of these two mechanisms, it has been used as a general indicator of endothelial dysfunction [8].

The reproducibility of LTH is strongly dependent on the way of expressing data and the technique used to record skin blood flux. When using single-point LDF, we found the inter-day reproducibility of both peak and plateau expressed as raw CVC to be acceptable for finger pad measurements (CV were 17 and 25%, respectively) but not for measurements on the forearm (CV were 57 and 40%) [54]. Normalizing baseline skin temperature to 33°C before heating did not improve the inter-day reproducibility of LTH on the forearm, whatever the way of expressing data [89]. Other groups have found better reproducibility of LTH on the forearm by using integrating probes (which process an integrated signal taken as the average flow value from seven or eight different scattering volumes). Agarwal et al found CV ranging from 9 to 38%, depending on the method of data expression [77]; however, the heating conditions were different from ours; the heating rate was 10-fold lower and the maximum temperature was 41°C. Moreover, Agarwal et al used local anesthesia to avoid axon reflex vasodilation, thus providing data only for the plateau [77]. Tew et al, using a similar protocol and form of data expression to ours, showed better reproducibility of LTH on the forearm expressed as raw CVC, %CVC_{max} or %CVC_{BL}, both for the initial peak (CV were 19, 11 and 32%, respectively) and the plateau (CV were 19, 4 and 30%, respectively) [99].

The inter-day reproducibility of LTH on the forearm when using full-field techniques such as LDI was good for the plateau (CV was 17% when expressed as raw CVC) [89]. However, LDI was not as accurate when used to assess the LTH peak on the forearm, probably because of its slow kinetics over wide areas (CV for peak was 39% when expressed

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as raw CVC). The good inter-site reproducibility of peak CVC simultaneously assessed at two sites on the same forearm strengthens this hypothesis [89]. As such, lower resolution over smaller areas would probably increase peak reproducibility using LDI, but to the detriment of the main advantage of LDI, i.e. recording flux over wide areas. We found that the recently marketed high frame rate LSCI offers excellent inter-day reproducibility of the LTH peak and plateau on the forearm (see below). These results suggest that lowering inter-site variability (by using integrating LDF probes or full-field techniques) could be decisive in improving the inter-day reproducibility of LTH on the forearm (Table 1).

Although many heating protocols have been proposed, local warming to 42-43°C is usually sufficient to induce maximal vasodilation [100]. In our experience, heating to 44°C is well tolerated in healthy subjects but may lead to pain or a burning sensation in patients with abnormal microvascular function (e.g. systemic sclerosis). The plateau appears 20-30 min after starting heating [1] and when heating is prolonged a "die away" phenomenon (i.e. slow reversal towards baseline) is observed. Although this "die away" is most noticeable beyond 60 min [100], it starts at around the 45th-50th min [101], thus justifying heating protocols restricted to between 30 and 45 min.

Finally, the nature of the device used to heat the skin plays a key role. Indeed, all the studies showing that maximal vasodilation was reached by heating the skin to 42°C or higher have used LDF probes and metallic heaters that were directly applied on the skin. In contrast the heating devices used with full-field techniques are water-filled chambers which the laser beam traverses. To study the influence of the water within the chamber, we compared the LTH plateau induced with a water-filled heating probe (Moor SHP3) before and immediately after probe removal in 12 healthy subjects. The mean (SD) LTH plateau assessed with LSCI at the end of heating for 30-min at 43° on the forearm (before probe removal) was 109.7 (18.2) PU compared to 153.9 (30.1) PU immediately after probe removal (data were averaged

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over 3 min; P<0.001, Wilcoxon rank test), suggesting a 30% decrease in signal when recorded across the chamber (personal unpublished data). Therefore, one should be extremely careful as to the methods used when comparing data expressed as %CVC_{max} between different experiments.

In conclusion, under routine conditions (i.e. unanesthetized skin and inter-day sites of the probes not precisely marked), integrating LDF and full-field techniques show better inter-day reproducibility of LTH on the forearm than single-point LDF. In all cases, data should preferentially be expressed as raw CVC or, for the initial peak, as %CVC_{max}.

Local cooling

Although local heating is by far the most common thermal challenge, local cooling has also been used, particularly in the study of Raynaud's phenomenon (RP). Several cooling methods coupled to LDF have been described, such as immersion of the hand or a finger in cold water [102], flexible cold packs [103] or use of a stream of carbon dioxide [104]. Due to its relative ease of use, immersion in cold water has been extensively used, including in patients with RP [105]. However, this technique induces a systemic sympathetic activation [106], which interferes with the local microvascular response. Custom-designed metal LDF probes coupled with a Peltier element allow local cooling while recording skin blood flux [107], without inducing any effect on ipsilateral and contralateral controls [108], enabling the physiology of skin microvascular reactivity to local cooling to be studied.

Local cooling of the skin induces an initial vasoconstriction followed by transient vasodilation and finally prolonged vasoconstriction [100] (Figure 6). The initial vasoconstriction depends on norepinephrine, and would be mainly mediated by the RhoA-Rho kinase (ROCK) pathway (by translocating α_{2c}-adrenoreceptors) whereas the prolonged vasoconstriction probably involves both the ROCK pathway [109] and inhibition of the NO

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system [100]. Sensory nerves could play a role in the transient vasodilation, which is less well understood [100]. Such transient vasodilation is more obvious when the cooling is rapid [110], making the rate of cooling an important parameter to consider when studying microvascular reactivity to local cooling.

We recently assessed the reproducibility of skin blood flux measurements while cooling locally to 15°C or to 24°C on the forearm. The best seven-day reproducibility of a 30-min cooling protocol was obtained at 15°C when data were expressed as percentage decrease from baseline flux (CV=23%) [108]. This test has been recently used to characterize increased vasoconstriction and blunted vasodilation on the finger pad of patients with primary RP compared to matched controls [111].

Laser Speckle Contrast Imaging

Laser speckle contrast imaging (LSCI) is a recently marketed technique based on speckle contrast analysis that provides an index of blood flow [112, 113]. High frame rate LSCI allows continuous assessment of skin perfusion over wide areas, thus theoretically combining the advantages of LDF and LDI, with very good inter-day reproducibility of PORH and LTH measurements, whether data were expressed as raw values or as a function of baseline [89]. It should be noted that the skin penetration depth of LSCI is about 300 µm, whereas it is deeper (about 1-1.5 mm) with laser Doppler techniques [53, 114].

There is little data about the linearity between the LSCI signal and actual skin blood flow in human skin, whereas LDI has been shown to provide a valid measure of skin blood flow [115, 116]. Recent work based on computer simulations and laboratory measurements has shown that LDI and LSCI similarly provide a perfusion index proportional to the concentration and mean velocity of red blood cells [117]. In vivo, Stewart et al have shown a very good correlation between the two techniques in burn scar perfusion assessment [118].

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Such correlation between LSCI and LDI is maintained over a wide range of human skin perfusion when data are expressed as raw arbitrary perfusion units [119] (Figure 7). Subtracting biological zero (BZ) from raw arbitrary perfusion units did not affect the correlation between LSCI and LDI but shifted the regression line towards the origin [119].

A potential problem of LSCI is its sensitivity to movement artifacts. Mahé et al recently showed that movement-induced artifacts may be overcome by subtracting the signal backscattered from an opaque adhesive surface adjacent to the region of interest (ROI) [120]. This simple method could be useful in many investigations of skin microvascular function when strict immobility cannot be ensured.

Analyzing LSCI is challenging, partly because of the large amount of data (i.e. an acquisition rate of 18 Hz provides more than 40 000 images for a single 40-min LTH measurement). Rousseau et al recently demonstrated that increasing the size of the ROI improves the reproducibility of PORH assessed with LSCI (18 Hz), whatever the "time of interest" (TOI) [121]. The authors suggest that at this frequency, ROIs should be larger than 10 mm² and TOIs longer than 1 s.

In conclusion, LSCI seems to be a remarkable tool to assess skin blood flux, especially when coupled with PORH and LTH. However, data acquisition requires caution, particularly regarding movement artifacts.

Methodological issues

Recording conditions

Blood circulation in the skin plays a key role in the body's thermoregulation through complex interactions between systemic and local mechanisms. Therefore, besides the issue of local thermal challenges (discussed above), environmental temperature influences skin blood flow. As a consequence, the room temperature should be controlled when studying skin

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microcirculation, especially on the fingers. A three degree Celsius increase in room temperature (i.e. from 24°C to 27°C) significantly increased resting CVC, but also the PORH peak and the LTH peak and plateau on the finger pad, whereas cooling to 21°C tended to decrease resting CVC and the PORH peak but did not affect LTH [54]. The influence of room temperature is less obvious for forearm measurements [54].

In healthy subjects, local non-nociceptive external pressure to the skin induces vasodilation (often referred to as "pressure-induced vasodilation", or PIV) to protect the tissue from pressure-induced ischemic damage [122]. It is of interest that PIV has been successfully used as a reactivity test to show the inability of the skin of diabetic patients to adapt to localized pressure [123, 124] and suimilarly in older subjects [125]. Although PIV has been observed over a wide range of pressures [126], it is unlikely to occur as a result of the weight of the LDF probe alone. Nonetheless, LDI and LCSI are immune to artifacts of this nature.

The influence of mental stress and fear on the LDF signal has also been studied, with conflicting conclusions. Mild mental stress has been shown to drastically decrease baseline skin blood flow (from 32 to 42%) whereas it had little influence (8% increase) on mean arterial pressure [127]. A similar tendency has been observed by using a Stroop color test [54]. In the same way, fear-induced stress evoked marked skin vasoconstriction in the finger [128]. On the forearm however, mental stress does not influence skin blood flow during normothermia [54, 129] or reactivity tests such as PORH and LTH [54], or slightly increases skin blood flux [127]. Although these results suggest regional differences in the effects of mental stress, these discrepancies between studies may also reflect differences in methodology.

In conclusion, room temperature (and possibly stress) influence laser Doppler measurements, especially when studying digital skin blood flux. Experiments should therefore be performed in a temperature-controlled room and recording should start after the

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participant's acclimatization. A vacuum cushion may be used to maintain the hand and forearm as still as possible and thus reduce movement artifacts.

Characteristics of the population

Although aging does not affect resting cutaneous blood flow [130], human skin vascular response to thermal challenges is impaired in elderly subjects compared to younger adults in non-glabrous skin (a subject expertly reviewed by Holowatz and Kenney [131]). No difference was shown however after local heating on the finger pad [132].

Gender is another concern when studying microvascular function. Hormone level variations across the physiological menstrual cycle or due to the oral contraceptive pill (OCP) regimen affect endothelium-dependent vasodilation of conductance arteries in different ways, depending on the OCP formulation [133-135]. The effect of the phase of the menstrual cycle or of OCPs on microvascular function has been explored with conflicting results. Resting cutaneous blood flux and conductance are affected by gender, females having lower values than males [130]. In the same way, local heating induces a lower increase in females than in males [130]. The menstrual cycle did not influence microvascular reactivity assessed by the iontophoresis of ACh and SNP combined with laser Doppler [136]. However, a recent controlled study has shown a small increase in the initial LTH peak after the administration of 17-β-estradiol, progesterone and a combination in young women in whom the sex hormones were suppressed with a gonadotropin-releasing hormone antagonist, whereas there was no effect on the LTH plateau or PORH [137]. Finally, healthy females showed greater vasoconstriction due to local cooling than males, the response being more pronounced during the luteal phase than the follicular phase [138]. The influence of female hormone levels across menstrual cycle or OCP on microvascular reactivity deserves further exploration, but it could introduce a confounding factor in studies [139].

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Age, gender, phase of the menstrual cycle and contraception should be taken into account to limit bias in controlled studies, by appropriate matching or randomization. Finally, vasoactive drugs and cigarette smoking also affect microvascular function [140, 141] and should therefore be avoided where possible.

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Skin sites and data expression

As previously mentioned, skin site influences the study of microvascular reactivity. The spatial variability of single-point LDF results has been described for almost three decades [142]. Braverman explained the variability of the signal by the anatomy of the underlying vasculature. Indeed, a high skin blood flux corresponds to underlying ascending arterioles whereas lower flux indicates venular predominance [53]. As skin arterioles are separated by an average of 1.7 mm on the forearm [53], flux may vary consistently according to the position of the LDF probe. This is the cause of the poor inter-day reproducibility of single-point LDF discussed above, which is a limitation of the technique.

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On the finger pad however (and on non-glabrous skin in general), the skin contains a high proportion of arteriovenous anastomoses, making baseline flux highly variable over time when assessed with single-point LDF. There is also a higher vessel density and thus baseline flux is more elevated than on the forearm. This higher density and easier probe positioning decreases spatial variability and therefore improves reproducibility of flux recorded with single-point LDF on the finger pad compared to the forearm [54]. This is untrue when data are expressed as a function of baseline, probably because of the influence of recording conditions on basal digital skin blood flux.

One major limitation of laser techniques is that they do not provide absolute perfusion values (i.e. cutaneous blood flow in mL/min relative to the volume or weight of tissue) [52]. Measurements are often expressed as arbitrary PU and referred to as flux. Some groups have

proposed to take into account blood pressure variations when expressing laser Doppler data [52]. They correct for the short and long term variations in blood pressure which would result in variations in cutaneous blood flow. However, this approach may be hampered by regional blood flow autoregulation. Blood flow autoregulation is the adjustment of vascular resistances in order to maintain constant flow over a wide range of pressures. This phenomenon is very efficient in the "protected" cerebral, coronary and renal circulatory systems, while it is much inferior in squeletal muscle and intestinal circulation, and absent in pulmonary circulation [143]. However, there is little information concerning the relationship between systemic blood pressure and skin perfusion pressure. Using large cutaneous island flaps in anesthetized dogs, it was shown that a decrease in cutaneous blood pressure was linearly correlated with a decrease in cutaneous blood flow, with no evidence of any plateau at a given flow value in this model [144], suggesting a lack of consistent autoregulation [145]. Therefore, it would be wise to correct for cutaneous blood flux by mean arterial pressure or if possible using peripheral blood pressure. When blood pressure is taken into account, expressing data as conductance is more appropriate than when it is expressed as resistance [146].

However, this does not permit the comparison of absolute flux or conductance values across studies in which different probes and/or brands of device and/or sites of measurement are used. An illustration of this issue is the comparison between LSCI and LDI. Although both signals (expressed as perfusion units) are very well correlated (R>0.85) [118, 119], there is a proportional bias between the two techniques whether data are expressed as raw PUs or as a percentage increase from baseline, suggesting that one should not assimilate PUs provided by the two systems [119].

The consequence of the latter limitation is that baseline flux or baseline CVC are of little interest when considered individually. Instead, microvessels are challenged with the various tests described in this review. Data is then expressed as raw flux or CVC, as a

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function of baseline (i.e. peak/plateau minus baseline, percentage increase/decrease from baseline, area under the curve) or as a percentage of maximal flux or CVC. According to the technique (single-point LDF, LDI or LSCI) and the test, the reproducibility of the measurements is drastically influenced by the way of expressing data, as detailed above and summarized in Table 1.

Recent work has shown that normalizing data to maximum flux provides similar responses to thermal stimuli (skin-surface cooling and whole body heat stress) whether assessed with single-point LDF, integrated LDF or LDI [147]. Scaling data to maximal vasodilation after local heating to 42-44°C is acceptable in mechanistically driven, carefully controlled studies, when skin blood flux is assessed with LDF or LSCI [1, 89]. However, such data expression may not be appropriate when studying reactivity in patients, in whom maximal vasodilation may be altered [1]. Full-field techniques such as LDI or LSCI may be of particular interest in such situations.

Biological zero

For laser Doppler measurements skin blood flux does not reach the value of zero when perfusion is absent due to brownian motion of macromolecules (reached after 3–5 min of cuff occlusion) [148]. Part of this signal may also be attributed to remaining red blood cells in venules. Whether data analysis should take into account this residual flux (referred to as "biological zero", BZ) remains controversial. Indeed, BZ (recorded with LDF) has been shown to be additive to the flow signal [148]. The authors therefore suggested measuring BZ under every experimental condition and subtracting it from the flux signal [148]. This is technically a wise precaution, but in practice is only possible when considering PORH (during which BZ is obtained *de facto*). In other conditions, occluding large vessels for 3 to 5 min would induce tremendous changes in microvascular reactivity, and bias the response. A

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solution would be to occlude arterial flow after other challenges, but this is not advisable as temperature or drugs (i.e. conditions of high blood flux) increase BZ recorded with LDF [148] and LDI [149]. In such circumstances, as the absolute difference is small, BZ subtraction has little influence when quantifying absolute hyperemic perfusion. Subtracting the biological zero did not improve one-week PORH reproducibility [54]. Furthermore, it may introduce bias when data are expressed as a percentage increase from baseline flux [149].

To our knowledge, little data are available concerning BZ assessed with LSCI. A recent study has shown higher BZ with LSCI than with LDI, thus again raising the issue of its influence on data analysis [119]. Subtracting BZ did not alter its correlation with LDI but shifted the regression line towards the origin. However, BZ subtraction introduced some variability in baseline, thus worsening the correlation when data was expressed as a percentage increase from baseline.

In conclusion, correction for BZ could be considered when studying PORH with laser Doppler or laser speckle. In the latter case, LSCI data should be expressed as raw perfusion units, but not as a function of baseline. Overall, correction for BZ makes data analysis more complicated without improving reproducibility.

Limits and perspectives

Among the different techniques reviewed, each has advantages and drawbacks. Microscopy-derived techniques are semi-quantitative, implemented in small devices that can be used at the bedside; they are mostly used to assess morphology rather than the function of the microvasculature. On the other hand, the advantage of laser Doppler and laser speckle techniques is that they can be coupled with various reactivity tests to challenge microvessels. However, these tests do not specifically assess distinct pathways but provide an overall assessment of microvascular function. Indeed, recent studies have shown that the

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mechanisms underlying of common reactivity tests (i.e. Ach iontophoresis, PORH and LTH) are complex and involve several different pathways [150]. Besides deeper an exploration into their mechanisms, these tests should be standardized if they are to be used as surrogate markers of microvascular function.

Another approach which has not been explored in this review concerns signal processing. Indeed, cutaneous blood flow has been studied through several processing tools such as the Fourier transform and the wavelet transform [52]. Other methods such as multifractality and sample entropy have recently been applied to LDF signals [151].

Conclusion

In conclusion, different techniques have been developed in the past thirty years to assess microvascular function. Although optical microscopy-derived techniques (such as nailfold videocapillaroscopy) have found clinical applications they mainly provide morphological information about the microvessels. Laser Doppler techniques coupled to reactivity tests are widespread in the field of microvascular function research. Post-occlusive reactive hyperemia (PORH) and local thermal hyperemia (LTH) have been shown to be reliable tests, although their underlying mechanisms are not fully understood yet. Despite its wide use as a specific test of endothelial function, acetylcholine iontophoresis has many limitations. In a general way, all these tests suffer from a lack of standardization and show highly variable reproducibility according to the skin site, recording conditions and the way of expressing data. Recent techniques such as laser speckle contrast imaging are promising tools, although further work is needed to determine the strength of the technique.

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References

1. Minson CT (2010). Thermal provocation to evaluate microvascular reactivity in human skin. *J Appl Physiol* 109:1239-46.

2. Sax FL, Cannon RO, 3rd, Hanson C, Epstein SE (1987). Impaired forearm vasodilator reserve in patients with microvascular angina. Evidence of a generalized disorder of vascular function? *N Engl J Med* 317:1366-70.

3. Chang CH, Tsai RK, Wu WC, Kuo SL, Yu HS (1997). Use of dynamic capillaroscopy for studying cutaneous microcirculation in patients with diabetes mellitus. *Microvasc Res* 53:121-7.

4. Antonios TF, Singer DR, Markandu ND, Mortimer PS, MacGregor GA (1999). Structural skin capillary rarefaction in essential hypertension. *Hypertension* 33:998-1001.

5. Levy BI, Schiffrin EL, Mourad JJ, Agostini D, Vicaut E, Safar ME, Struijker-Boudier HA (2008). Impaired tissue perfusion: a pathology common to hypertension, obesity, and diabetes mellitus. *Circulation* 118:968-76.

6. Feihl F, Liaudet L, Waeber B, Levy BI (2006). Hypertension: a disease of the microcirculation? *Hypertension* 48:1012-7.

7. Yamamoto-Suganuma R, Aso Y (2009). Relationship between post-occlusive forearm skin reactive hyperaemia and vascular disease in patients with Type 2 diabetes--a novel index for detecting micro- and macrovascular dysfunction using laser Doppler flowmetry. *Diabet Med* 26:83-8.

8. Kruger A, Stewart J, Sahityani R, O'Riordan E, Thompson C, Adler S, Garrick R, Vallance P, Goligorsky MS (2006). Laser Doppler flowmetry detection of endothelial dysfunction in end-stage renal disease patients: correlation with cardiovascular risk. *Kidney Int* 70:157-64.

9. Engelberger RP, Pittet YK, Henry H, Delodder F, Hayoz D, Chiolero RL, Waeber B, Liaudet L, Berger MM, Feihl F (2011). Acute endotoxemia inhibits microvascular nitric oxide-dependent vasodilation in humans. *Shock* 35:28-34.

10. Holowatz LA, Thompson-Torgerson CS, Kenney WL (2008). The human cutaneous circulation as a model of generalized microvascular function. *J Appl Physiol* 105:370-2.

11. Herrick AL (2000). Vascular function in systemic sclerosis. *Curr Opin Rheumatol* 12:527-33.

12. Wigley FM (2009). Vascular disease in scleroderma. *Clin Rev Allergy Immunol* 36:150-75.

13. Carpentier PH (1999). New techniques for clinical assessment of the peripheral microcirculation. *Drugs* 59 Spec No:17-22.

14. Cutolo M, Sulli A, Secchi ME, Paolino S, Pizzorni C (2006). Nailfold capillaroscopy is useful for the diagnosis and follow-up of autoimmune rheumatic diseases. A future tool for the analysis of microvascular heart involvement? *Rheumatology (Oxford)* 45 Suppl 4:iv43-6.

15. Groner W, Winkelman JW, Harris AG, Ince C, Bouma GJ, Messmer K, Nadeau RG (1999). Orthogonal polarization spectral imaging: a new method for study of the microcirculation. *Nat Med* 5:1209-12.

16. Goedhart PT, Khalilzada M, Bezemer R, Merza J, Ince C (2007). Sidestream Dark Field (SDF) imaging: a novel stroboscopic LED ring-based imaging modality for clinical assessment of the microcirculation. *Opt Express* 15:15101-14.

17. Stern MD (1975). In vivo evaluation of microcirculation by coherent light scattering. *Nature* 254:56-8.

18. De Backer D, Ospina-Tascon G, Salgado D, Favory R, Creteur J, Vincent JL (2010). Monitoring the microcirculation in the critically ill patient: current methods and future approaches. *Intensive Care Med* 36:1813-25.

19. Michoud E, Poensin D, Carpentier PH (1994). Digitized nailfold capillaroscopy. *Vasa* 23:35-42.

20. De Angelis R, Grassi W, Cutolo M (2009). A growing need for capillaroscopy in rheumatology. *Arthritis Rheum* 61:405-10.

21. Cutolo M, Pizzorni C, Tuccio M, Burroni A, Craviotto C, Basso M, Seriolo B, Sulli A (2004). Nailfold videocapillaroscopic patterns and serum autoantibodies in systemic sclerosis. *Rheumatology (Oxford)* 43:719-26.

22. Maricq HR, LeRoy EC (1973). Patterns of finger capillary abnormalities in connective tissue disease by "wide-field" microscopy. *Arthritis Rheum* 16:619-28.

23. Hudson M, Taillefer S, Steele R, Dunne J, Johnson SR, Jones N, Mathieu JP, Baron M (2007). Improving the sensitivity of the American College of Rheumatology classification criteria for systemic sclerosis. *Clin Exp Rheumatol* 25:754-7.

24. Ingegnoli F, Boracchi P, Gualtierotti R, Lubatti C, Meani L, Zahalkova L, Zeni S, Fantini F (2008). Prognostic model based on nailfold capillaroscopy for identifying Raynaud's phenomenon patients at high risk for the development of a scleroderma spectrum disorder: PRINCE (prognostic index for nailfold capillaroscopic examination). *Arthritis Rheum* 58:2174-82.

25. Ingegnoli F, Zeni S, Meani L, Soldi A, Lurati A, Fantini F (2005). Evaluation of nailfold videocapillaroscopic abnormalities in patients with systemic lupus erythematosus. *J Clin Rheumatol* 11:295-8.

26. Zaric D, Worm AM, Stahl D, Clemmensen OJ (1981). Capillary microscopy of the nailfold in psoriatic and rheumatoid arthritis. *Scand J Rheumatol* 10:249-52.

27. Shore AC (2000). Capillaroscopy and the measurement of capillary pressure. *Br J Clin Pharmacol* 50:501-13.

28. Antonios TF, Rattray FE, Singer DR, Markandu ND, Mortimer PS, MacGregor GA (1999). Maximization of skin capillaries during intravital video-microscopy in essential hypertension: comparison between venous congestion, reactive hyperaemia and core heat load tests. *Clin Sci (Lond)* 97:523-8.

29. Debbabi H, Uzan L, Mourad JJ, Safar M, Levy BI, Tibirica E (2006). Increased skin capillary density in treated essential hypertensive patients. *Am J Hypertens* 19:477-83.

30. Debbabi H, Bonnin P, Levy BI (2010). Effects of blood pressure control with perindopril/indapamide on the microcirculation in hypertensive patients. *Am J Hypertens* 23:1136-43.

31. Tibirica E, Rodrigues E, Cobas RA, Gomes MB (2007). Endothelial function in patients with type 1 diabetes evaluated by skin capillary recruitment. *Microvasc Res* 73:107-12.

32. Kelly RI, Pearse R, Bull RH, Leveque JL, de Rigal J, Mortimer PS (1995). The effects of aging on the cutaneous microvasculature. *J Am Acad Dermatol* 33:749-56.

33. Bollinger A, Frey J, Jager K, Furrer J, Seglias J, Siegenthaler W (1982). Patterns of diffusion through skin capillaries in patients with long-term diabetes. *N Engl J Med* 307:1305-10.

34. Hettema ME, Zhang D, Stienstra Y, Oomen PN, Smit AJ, Kallenberg CG, Bootsma H (2008). Decreased capillary permeability and capillary density in patients with systemic sclerosis using large-window sodium fluorescein videodensitometry of the ankle. *Rheumatology (Oxford)* 47:1409-12.

35. Bull RH, Bates DO, Mortimer PS (1992). Intravital video-capillaroscopy for the study of the microcirculation in psoriasis. *Br J Dermatol* 126:436-45.

36. Lamby P, Prantl L, Gais S, Walter M, Bachthaler M, Nerlich M, Feuerbach S, Jung EM (2008). Evaluation of the vascular integrity of free flaps based on microcirculation imaging techniques. *Clin Hemorheol Microcirc* 39:253-63.

37. Eren S, Rubben A, Krein R, Larkin G, Hettich R (1995). Assessment of microcirculation of an axial skin flap using indocyanine green fluorescence angiography. *Plast Reconstr Surg* 96:1636-49.

38. Kaiser M, Yafi A, Cinat M, Choi B, Durkin AJ (2011). Noninvasive assessment of burn wound severity using optical technology: a review of current and future modalities. *Burns* 37:377-86.

39. Pennings FA, Ince C, Bouma GJ (2006). Continuous real-time visualization of the human cerebral microcirculation during arteriovenous malformation surgery using orthogonal polarization spectral imaging. *Neurosurgery* 59:167-71.

40. Perez-Barcena J, Goedhart P, Ibanez J, Brell M, Garcia R, Llinas P, Jimenez C, Ince C (2011). Direct observation of human microcirculation during decompressive craniectomy after stroke. *Crit Care Med* 39:1126-9.

41. Schmitz V, Schaser KD, Olschewski P, Neuhaus P, Puhl G (2008). In vivo visualization of early microcirculatory changes following ischemia/reperfusion injury in human kidney transplantation. *Eur Surg Res* 40:19-25.

42. Puhl G, Schaser KD, Vollmar B, Menger MD, Settmacher U (2003). Noninvasive in vivo analysis of the human hepatic microcirculation using orthogonal polorization spectral imaging. *Transplantation* 75:756-61.

43. De Backer D, Hollenberg S, Boerma C, Goedhart P, Buchele G, Ospina-Tascon G, Dobbe I, Ince C (2007). How to evaluate the microcirculation: report of a round table conference. *Crit Care* 11:R101.

44. De Backer D, Creteur J, Preiser JC, Dubois MJ, Vincent JL (2002). Microvascular blood flow is altered in patients with sepsis. *Am J Respir Crit Care Med* 166:98-104.

45. Donadello K, Favory R, Salgado-Ribeiro D, Vincent JL, Gottin L, Scolletta S, Creteur J, De Backer D, Taccone FS (2011). Sublingual and muscular microcirculatory alterations after cardiac arrest: A pilot study. *Resuscitation* 82:690-5.

46. Virgini-Magalhaes CE, Porto CL, Fernandes FF, Dorigo DM, Bottino DA, Bouskela E (2006). Use of microcirculatory parameters to evaluate chronic venous insufficiency. *J Vasc Surg* 43:1037-44.

47. Milner SM, Bhat S, Gulati S, Gherardini G, Smith CE, Bick RJ (2005). Observations on the microcirculation of the human burn wound using orthogonal polarization spectral imaging. *Burns* 31:316-9.

48. Goertz O, Ring A, Kohlinger A, Daigeler A, Andree C, Steinau HU, Langer S Orthogonal polarization spectral imaging: a tool for assessing burn depths? *Ann Plast Surg* 64:217-21.

49. Lindert J, Werner J, Redlin M, Kuppe H, Habazettl H, Pries AR (2002). OPS imaging of human microcirculation: a short technical report. *J Vasc Res* 39:368-72.

50. Treu CM, Lupi O, Bottino DA, Bouskela E (2011). Sidestream dark field imaging: the evolution of real-time visualization of cutaneous microcirculation and its potential application in dermatology. *Arch Dermatol Res* 303:69-78.

51. Zijlstra WG, Buursma A, Meeuwsen-van der Roest WP (1991). Absorption spectra of human fetal and adult oxyhemoglobin, de-oxyhemoglobin, carboxyhemoglobin, and methemoglobin. *Clin Chem* 37:1633-8.

52. Cracowski JL, Minson CT, Salvat-Melis M, Halliwill JR (2006). Methodological issues in the assessment of skin microvascular endothelial function in humans. *Trends Pharmacol Sci* 27:503-8.

53. Braverman IM (2000). The cutaneous microcirculation. *J Investig Dermatol Symp Proc* 5:3-9.

54. Roustit M, Blaise S, Millet C, Cracowski JL (2010). Reproducibility and methodological issues of skin post-occlusive and thermal hyperemia assessed by single-point laser Doppler flowmetry. *Microvasc Res* 79:102-108.

55. Ahn H, Johansson K, Lundgren O, Nilsson GE (1987). In vivo evaluation of signal processors for laser Doppler tissue flowmeters. *Med Biol Eng Comput* 25:207-11.

56. Clough GF (2005). Microdialysis of large molecules. *AAPS J* 7:E686-92.

57. Minson CT, Berry LT, Joyner MJ (2001). Nitric oxide and neurally mediated regulation of skin blood flow during local heating. *J Appl Physiol* 91:1619-26.

58. Wong BJ, Wilkins BW, Holowatz LA, Minson CT (2003). Nitric oxide synthase inhibition does not alter the reactive hyperemic response in the cutaneous circulation. *J Appl Physiol* 95:504-10.

59. Kalia YN, Naik A, Garrison J, Guy RH (2004). Iontophoretic drug delivery. *Adv Drug Deliv Rev* 56:619-58.

60. Turner J, Belch JJ, Khan F (2008). Current concepts in assessment of microvascular endothelial function using laser Doppler imaging and iontophoresis. *Trends Cardiovasc Med* 18:109-16.

61. Murray AK, Moore TL, King TA, Herrick AL (2008). Vasodilator iontophoresis a possible new therapy for digital ischaemia in systemic sclerosis? *Rheumatology (Oxford)* 47:76-9.

62. Murray AK, Herrick AL, Gorodkin RE, Moore TL, King TA (2005). Possible therapeutic use of vasodilator iontophoresis. *Microvasc Res* 69:89-94.

63. Noon JP, Walker BR, Hand MF, Webb DJ (1998). Studies with iontophoretic administration of drugs to human dermal vessels in vivo: cholinergic vasodilatation is mediated by dilator prostanoids rather than nitric oxide. *Br J Clin Pharmacol* 45:545-50.

64. Holowatz LA, Thompson CS, Minson CT, Kenney WL (2005). Mechanisms of acetylcholine-mediated vasodilatation in young and aged human skin. *J Physiol* 563:965-73.

65. Durand S, Tartas M, Bouye P, Koitka A, Saumet JL, Abraham P (2004). Prostaglandins participate in the late phase of the vascular response to acetylcholine iontophoresis in humans. *J Physiol* 561:811-9.

66. Berghoff M, Kathpal M, Kilo S, Hilz MJ, Freeman R (2002). Vascular and neural mechanisms of ACh-mediated vasodilation in the forearm cutaneous microcirculation. *J Appl Physiol* 92:780-8.

67. Dalle-Ave A, Kubli S, Golay S, Delachaux A, Liaudet L, Waeber B, Feihl F (2004). Acetylcholine-induced vasodilation and reactive hyperemia are not affected by acute cyclo-oxygenase inhibition in human skin. *Microcirculation* 11:327-36.

68. Berliner MN (1997). Skin microcirculation during tapwater iontophoresis in humans: cathode stimulates more than anode. *Microvasc Res* 54:74-80.

69. Durand S, Fromy B, Bouye P, Saumet JL, Abraham P (2002). Current-induced vasodilation during water iontophoresis (5 min, 0.10 mA) is delayed from current onset and involves aspirin sensitive mechanisms. *J Vasc Res* 39:59-71.

70. Durand S, Fromy B, Bouye P, Saumet JL, Abraham P (2002). Vasodilatation in response to repeated anodal current application in the human skin relies on aspirin-sensitive mechanisms. *J Physiol* 540:261-9.

71. Durand S, Fromy B, Koitka A, Tartas M, Saumet JL, Abraham P (2002). Oral single high-dose aspirin results in a long-lived inhibition of anodal current-induced vasodilatation. *Br J Pharmacol* 137:384-90.

72. Tartas M, Bouye P, Koitka A, Durand S, Gallois Y, Saumet JL, Abraham P (2005). Early vasodilator response to anodal current application in human is not impaired by cyclooxygenase-2 blockade. *Am J Physiol Heart Circ Physiol* 288:H1668-73.

73. Ferrell WR, Ramsay JE, Brooks N, Lockhart JC, Dickson S, McNeece GM, Greer IA, Sattar N (2002). Elimination of electrically induced iontophoretic artefacts: implications for non-invasive assessment of peripheral microvascular function. *J Vasc Res* 39:447-55.

74. Ramsay JE, Ferrell WR, Greer IA, Sattar N (2002). Factors critical to iontophoretic assessment of vascular reactivity: implications for clinical studies of endothelial dysfunction. *J Cardiovasc Pharmacol* 39:9-17.

75. Jadhav S, Sattar N, Petrie JR, Cobbe SM, Ferrell WR (2007). Reproducibility and repeatability of peripheral microvascular assessment using iontophoresis in conjunction with laser Doppler imaging. *J Cardiovasc Pharmacol* 50:343-9.

76. Blaise S, Hellmann M, Roustit M, Isnard S, Cracowski JL (2010). Oral sildenafil increases skin hyperaemia induced by iontophoresis of sodium nitroprusside in healthy volunteers. *Br J Pharmacol* 160:1128-34.

77. Agarwal SC, Allen J, Murray A, Purcell IF (2010). Comparative reproducibility of dermal microvascular blood flow changes in response to acetylcholine iontophoresis, hyperthermia and reactive hyperaemia. *Physiol Meas* 31:1-11.

78. Sauvet F, Mahe G, Chennaoui M, Langrume C, Vasseur M, Abraham P, Leftheriotis G (2011). Acetylcholine chloride as a potential source of variability in the study of cutaneous vascular function in man. *Microvasc Res* 82:190-7.

79. Roustit M, Blaise S, Cracowski JL (2009). Sodium nitroprusside iontophoresis on the finger pad does not consistently increase skin blood flow in healthy controls and patients with systemic sclerosis. *Microvasc Res* 77:260-4.

80. Tibirica E, Matheus AS, Nunes B, Sperandei S, Gomes MB (2011). Repeatability of the evaluation of systemic microvascular endothelial function using laser doppler perfusion monitoring: clinical and statistical implications. *Clinics (Sao Paulo)* 66:599-605.

81. Droog EJ, Henricson J, Nilsson GE, Sjoberg F (2004). A protocol for iontophoresis of acetylcholine and sodium nitroprusside that minimises nonspecific vasodilatory effects. *Microvasc Res* 67:197-202.

82. Lorenzo S, Minson CT (2007). Human cutaneous reactive hyperaemia: role of BKCa channels and sensory nerves. *J Physiol* 585:295-303.

83. Larkin SW, Williams TJ (1993). Evidence for sensory nerve involvement in cutaneous reactive hyperemia in humans. *Circ Res* 73:147-54.

84. Binggeli C, Spieker LE, Corti R, Sudano I, Stojanovic V, Hayoz D, Luscher TF, Noll G (2003). Statins enhance postischemic hyperemia in the skin circulation of hypercholesterolemic patients: a monitoring test of endothelial dysfunction for clinical practice? *J Am Coll Cardiol* 42:71-7.

85. Medow MS, Taneja I, Stewart JM (2007). Cyclooxygenase and nitric oxide synthase dependence of cutaneous reactive hyperemia in humans. *Am J Physiol Heart Circ Physiol* 293:H425-32.

86. Nohria A, Gerhard-Herman M, Creager MA, Hurley S, Mitra D, Ganz P (2006). Role of nitric oxide in the regulation of digital pulse volume amplitude in humans. *J Appl Physiol* 101:545-8.

87. Charkoudian N (2003). Skin blood flow in adult human thermoregulation: how it works, when it does not, and why. *Mayo Clin Proc* 78:603-12.

88. Yvonne-Tee GB, Rasool AH, Halim AS, Rahman AR (2005). Reproducibility of different laser Doppler fluximetry parameters of postocclusive reactive hyperemia in human forearm skin. *J Pharmacol Toxicol Methods* 52:286-92.

89. Roustit M, Millet C, Blaise S, Dufournet B, Cracowski JL (2010). Excellent reproducibility of laser speckle contrast imaging to assess skin microvascular reactivity. *Microvasc Res* 80:505-11.

90. Keymel S, Sichwardt J, Balzer J, Stegemann E, Rassaf T, Kleinbongard P, Kelm M, Heiss C, Lauer T (2010). Characterization of the non-invasive assessment of the cutaneous microcirculation by laser Doppler perfusion scanner. *Microcirculation* 17:358-66.

91. Yvonne-Tee GB, Rasool AH, Halim AS, Wong AR, Rahman AR (2008). Method optimization on the use of postocclusive hyperemia model to assess microvascular function. *Clin Hemorheol Microcirc* 38:119-33.

92. Corretti MC, Anderson TJ, Benjamin EJ, Celermajer D, Charbonneau F, Creager MA, Deanfield J, Drexler H, Gerhard-Herman M, Herrington D, Vallance P, Vita J, Vogel R (2002). Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: a report of the International Brachial Artery Reactivity Task Force. *J Am Coll Cardiol* 39:257-65.

93. Schmelz M, Luz O, Averbeck B, Bickel A (1997). Plasma extravasation and neuropeptide release in human skin as measured by intradermal microdialysis. *Neurosci Lett* 230:117-20.

94. Wallengren J (1997). Vasoactive peptides in the skin. *J Investig Dermatol Symp Proc* 2:49-55.

95. Wong BJ, Fieger SM (2010). Transient receptor potential vanilloid type-1 (TRPV-1) channels contribute to cutaneous thermal hyperaemia in humans. *J Physiol* 588:4317-26.

96. Shastry S, Joyner MJ (2002). Geldanamycin attenuates NO-mediated dilation in human skin. *Am J Physiol Heart Circ Physiol* 282:H232-6.

97. Medow MS, Bamji N, Clarke D, Ocon AJ, Stewart JM (2011). Reactive oxygen species (ROS) from NADPH and xanthine oxidase modulate the cutaneous local heating response in healthy humans. *J Appl Physiol* 111:20-6.

98. Roustit M, Simmons GH, Carpentier P, Cracowski JL (2008). Abnormal digital neurovascular response to local heating in systemic sclerosis. *Rheumatology (Oxford)* 47:860-4.

99. Tew GA, Klonizakis M, Moss J, Ruddock AD, Saxton JM, Hodges GJ (2011). Reproducibility of cutaneous thermal hyperaemia assessed by laser Doppler flowmetry in young and older adults. *Microvasc Res* 81:177-82.

100. Johnson JM, Kellogg DL, Jr. (2010). Local thermal control of the human cutaneous circulation. *J Appl Physiol* 109:1229-38.

101. Hodges GJ, Kosiba WA, Zhao K, Johnson JM (2009). The involvement of heating rate and vasoconstrictor nerves in the cutaneous vasodilator response to skin warming. *Am J Physiol Heart Circ Physiol* 296:H51-6.

102. Maver J, Strucl M (2000). Microvascular reactivity in normotensive subjects with a familial predisposition to hypertension. *Microvasc Res* 60:241-8.

103. Cankar K, Finderle Z (2003). Gender differences in cutaneous vascular and autonomic nervous response to local cooling. *Clin Auton Res* 13:214-20.

104. Lutolf O, Chen D, Zehnder T, Mahler F (1993). Influence of local finger cooling on laser Doppler flux and nailfold capillary blood flow velocity in normal subjects and in patients with Raynaud's phenomenon. *Microvasc Res* 46:374-82.

105. Foerster J, Kuerth A, Niederstrasser E, Krautwald E, Pauli R, Paulat R, Eweleit M, Riemekasten G, Worm M (2007). A cold-response index for the assessment of Raynaud's phenomenon. *J Dermatol Sci* 45:113-20.

106. Victor RG, Leimbach WN, Jr., Seals DR, Wallin BG, Mark AL (1987). Effects of the cold pressor test on muscle sympathetic nerve activity in humans. *Hypertension* 9:429-36.

107. Johnson JM, Yen TC, Zhao K, Kosiba WA (2005). Sympathetic, sensory, and nonneuronal contributions to the cutaneous vasoconstrictor response to local cooling. *Am J Physiol Heart Circ Physiol* 288:H1573-9.

108. Roustit M, Maggi F, Isnard S, Hellmann M, Bakken B, Cracowski JL (2010). Reproducibility of a local cooling test to assess microvascular function in human skin. *Microvasc Res* 79:34-39.

109. Thompson-Torgerson CS, Holowatz LA, Flavahan NA, Kenney WL (2007). Cold-induced cutaneous vasoconstriction is mediated by Rho kinase in vivo in human skin. *Am J Physiol Heart Circ Physiol* 292:H1700-5.

110. Yamazaki F, Sone R, Zhao K, Alvarez GE, Kosiba WA, Johnson JM (2006). Rate dependency and role of nitric oxide in the vascular response to direct cooling in human skin. *J Appl Physiol* 100:42-50.

111. Roustit M, Blaise S, Millet C, Cracowski JL (2011). Impaired transient vasodilation and increased vasoconstriction to digital local cooling in primary Raynaud's phenomenon. *Am J Physiol Heart Circ Physiol* 301:H324-30.

112. Briers JD (2001). Laser Doppler, speckle and related techniques for blood perfusion mapping and imaging. *Physiol Meas* 22:R35-66.

113. Forrester KR, Tulip J, Leonard C, Stewart C, Bray RC (2004). A laser speckle imaging technique for measuring tissue perfusion. *IEEE Trans Biomed Eng* 51:2074-84.

114. O'Doherty J, McNamara P, Clancy NT, Enfield JG, Leahy MJ (2009). Comparison of instruments for investigation of microcirculatory blood flow and red blood cell concentration. *J Biomed Opt* 14:034025.

115. Forrester K, Doschak M, Bray R (1997). In vivo comparison of scanning technique and wavelength in laser Doppler perfusion imaging: measurement in knee ligaments of adult rabbits. *Med Biol Eng Comput* 35:581-6.

116. Kernick DP, Shore AC (2000). Characteristics of laser Doppler perfusion imaging in vitro and in vivo. *Physiol Meas* 21:333-40.

117. Thompson OB, Andrews MK (2010). Tissue perfusion measurements: multiple-exposure laser speckle analysis generates laser Doppler-like spectra. *J Biomed Opt* 15:027015.

118. Stewart CJ, Frank R, Forrester KR, Tulip J, Lindsay R, Bray RC (2005). A comparison of two laser-based methods for determination of burn scar perfusion: laser Doppler versus laser speckle imaging. *Burns* 31:744-52.

119. Millet C, Roustit M, Blaise S, Cracowski JL (2011). Comparison between laser speckle contrast imaging and laser Doppler imaging to assess skin blood flow in humans. *Microvasc Res* 82:147-51.

120. Mahe G, Rousseau P, Durand S, Bricq S, Leftheriotis G, Abraham P (2011). Laser speckle contrast imaging accurately measures blood flow over moving skin surfaces. *Microvasc Res* 81:183-8.

121. Rousseau P, Mahe G, Haj-Yassin F, Durand S, Humeau A, Leftheriotis G, Abraham P (2011). Increasing the "region of interest" and "time of interest", both reduce the variability of blood flow measurements using laser speckle contrast imaging. *Microvasc Res* 82:88-91.

122. Fromy B, Abraham P, Saumet JL (1998). Non-nociceptive capsaicin-sensitive nerve terminal stimulation allows for an original vasodilatory reflex in the human skin. *Brain Res* 811:166-8.

123. Fromy B, Abraham P, Bouvet C, Bouhanick B, Fressinaud P, Saumet JL (2002). Early decrease of skin blood flow in response to locally applied pressure in diabetic subjects. *Diabetes* 51:1214-7.

124. Koitka A, Abraham P, Bouhanick B, Sigaudo-Roussel D, Demiot C, Saumet JL (2004). Impaired pressure-induced vasodilation at the foot in young adults with type 1 diabetes. *Diabetes* 53:721-5.

125. Fromy B, Sigaudo-Roussel D, Gaubert-Dahan ML, Rousseau P, Abraham P, Benzoni D, Berrut G, Saumet JL (2010). Aging-associated sensory neuropathy alters pressure-induced vasodilation in humans. *J Invest Dermatol* 130:849-55.

126. Abraham P, Fromy B, Merzeau S, Jardel A, Saumet JL (2001). Dynamics of local pressure-induced cutaneous vasodilation in the human hand. *Microvasc Res* 61:122-9.

127. Silverman DG, Jotkowitz AB, Gutter V, Braverman IM, O'Connor TZ (1996). Regional vs systemic responses to mental stress: a potential mechanism for non-demand-related ischemia. *Microvasc Res* 51:396-9.

128. Hayashi N, Someya N, Maruyama T, Hirooka Y, Endo MY, Fukuba Y (2009). Vascular responses to fear-induced stress in humans. *Physiol Behav* 98:441-6.

129. Klein JC, Crandall CG, Brothers RM, Carter JR (2010). Combined heat and mental stress alters neurovascular control in humans. *J Appl Physiol* 109:1880-6.

130. Hodges GJ, Sharp L, Clements RE, Goldspink DF, George KP, Cable NT (2010). Influence of age, sex, and aerobic capacity on forearm and skin blood flow and vascular conductance. *Eur J Appl Physiol* 109:1009-15.

131. Holowatz LA, Kenney WL (2010). Peripheral mechanisms of thermoregulatory control of skin blood flow in aged humans. *J Appl Physiol* 109:1538-44.

132. Evans E, Rendell M, Bartek J, Connor S, Bamisedun O, Dovgan D, Giitter M (1993). Thermally-induced cutaneous vasodilatation in aging. *J Gerontol* 48:M53-7.

133. Meendering JR, Torgrimson BN, Miller NP, Kaplan PF, Minson CT (2010). A combined oral contraceptive containing 30 mcg ethinyl estradiol and 3.0 mg drospirenone does not impair endothelium-dependent vasodilation. *Contraception* 82:366-72.

134. Torgrimson BN, Meendering JR, Kaplan PF, Minson CT (2007). Endothelial function across an oral contraceptive cycle in women using levonorgestrel and ethinyl estradiol. *Am J Physiol Heart Circ Physiol* 292:H2874-80.

135. Torgrimson BN, Meendering JR, Kaplan PF, Minson CT (2011). Depot-medroxyprogesterone acetate and endothelial function before and after acute oral, vaginal, and transdermal estradiol treatment. *Hypertension* 57:819-24.

136. Ketel IJ, Stehouwer CD, Serne EH, Poel DM, Groot L, Kager C, Hompes PG, Homburg R, Twisk JW, Smulders YM, Lambalk CB (2009). Microvascular function has no menstrual-cycle-dependent variation in healthy ovulatory women. *Microcirculation* 16:714-24.

137. Brunt VE, Miner JA, Meendering JR, Kaplan PF, Minson CT (2011). 17beta-estradiol and progesterone independently augment cutaneous thermal hyperemia but not reactive hyperemia. *Microcirculation* 18:347-55.

138. Cankar K, Finderle Z, Strucl M (2000). Gender differences in cutaneous laser doppler flow response to local direct and contralateral cooling. *J Vasc Res* 37:183-8.

139. Cracowski JL (2011). Female hormones and skin microvascular function. *Microcirculation* 18:356-7.

140. Dalla Vecchia L, Palombo C, Ciardetti M, Porta A, Milani O, Kozakova M, Lucini D, Pagani M (2004). Contrasting effects of acute and chronic cigarette smoking on skin microcirculation in young healthy subjects. *J Hypertens* 22:129-35.

141. Lehr HA (2000). Microcirculatory dysfunction induced by cigarette smoking. *Microcirculation* 7:367-84.

142. Tenland T, Salerud EG, Nilsson GE, Oberg PA (1983). Spatial and temporal variations in human skin blood flow. *Int J Microcirc Clin Exp* 2:81-90.

143. Tuma RF, Duran WF, Ley K. Handbook of Physiology: Microcirculation. *2nd Edition.* Elsevier Amsterdam, 2008.

144. Fischer JC, Parker PM, Shaw WW (1985). Laser Doppler flowmeter measurements of skin perfusion changes associated with arterial and venous compromise in the cutaneous island flap. *Microsurgery* 6:238-43.

145. Heistad DD, Abboud FM (1974). Factors that influence blood flow in skeletal muscle and skin. *Anesthesiology* 41:139-56.

146. O'Leary DS (1991). Regional vascular resistance vs. conductance: which index for baroreflex responses? *Am J Physiol* 260:H632-7.

147. Brothers RM, Wingo JE, Hubing KA, Crandall CG (2010). Methodological assessment of skin and limb blood flows in the human forearm during thermal and baroreceptor provocations. *J Appl Physiol* 109:895-900.

148. Kernick DP, Tooke JE, Shore AC (1999). The biological zero signal in laser Doppler fluximetry - origins and practical implications. *Pflugers Arch* 437:624-31.

149. Mayrovitz HN, Leedham JA (2001). Laser-Doppler imaging of forearm skin: perfusion features and dependence of the biological zero on heat-induced hyperemia. *Microvasc Res* 62:74-8.

150. Brunt VE, Minson CT (2011). Cutaneous thermal hyperemia: more than skin deep. *J Appl Physiol* 111:5-7.

151. Humeau A, Chapeau-Blondeau F, Rousseau D, Rousseau P, Trzepizur W, Abraham P (2008). Multifractality, sample entropy, and wavelet analyses for age-related changes in the peripheral cardiovascular system: preliminary results. *Med Phys* 35:717-23.

Table 1. Reproducibility of post-occlusive reactive hyperemia (PORH) and local thermal hyperemia (LTH) on the forearm of healthy subjects.

			LDF		LDI	LSCI
			Single-point* [54, 89]	Integrating [77, 99]	[89]	[89, 121]
PORH	Peak	CVC / PU	45 / 30	NA	NA	8 / 3.1[#]
		CVC_{PK}-CVC_{BL}	19.4[¶]-48 / 33	NA	NA	11
		$\%CVC_{BL}$	22.7[¶]-38 / 32	NA	NA	15
		AUC	89 / 36	NA	NA	NA
		$\%CVC_{max}$	41 / 39	NA	NA	35
LTH	Peak	CVC	57 / 40	19	39	15
		$\%CVC_{BL}$	87 / 51	32	52	21
		$\%CVC_{max}$	19 / 25	11	42	9
	Plateau	CVC	40 / 42	19	17	15
		$\%CVC_{BL}$	92 / 58	30 / 38.5[†]	34	24

Data are expressed as within subject coefficient of variation (in %) of cutaneous vascular conductance (CVC), peak CVC minus baseline CVC (CVC_{PK}-CVC_{BL}), percentage change from baseline CVC ($\%CVC_{BL}$), area under the curve of flux (in PU.s), or percentage of maximal CVC ($\%CVC_{max}$). * without normalizing baseline skin temperature / after normalizing baseline skin temperature to 33°C. [†]From ref [77]. [#]Peak was expressed as perfusion units (PU), from ref [121]; [¶]From ref [77]; NA: not available

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Figure legends

Figure 1. Representative images of nailfold videocapillaroscopy (NVC) with a magnification x 100**.** A, Normal pattern showing homogenous distribution of capillary loops. B, Pattern observed in a patient with systemic sclerosis, showing disorganized enlarged/giant capillaries.

Figure 2. A, Cathodal iontophoresis of vasoadilator drug and control while recording skin blood flux with laser Doppler imaging (LDI); 1, active electrode containing the drugs; 2, passive electrode; 3, current generators connected to the electrodes; vacuum cushion to reduce movement artifacts; 5, head of the imager. B, skin blood flux recorded during iontophoresis (20 min, 20 µA) of sodium nitroprusside (bottom) and saline (top) after local anesthesia to avoid axon reflex vasodilation. C, intensity allows easier positioning of the regions of interest.

Figure 3. Example of current-induced vasodilation observed during cathodal iontophoresis (15 min, 20 or 100 µA) of saline and deionized water. The black bar represents the length of iontophoresis. Skin blood flux was assessed with laser Doppler imaging (frame rate: 3 images/min). PU: perfusion units.

Figure 4. Example of post-occlusive reactive hyperemia (PORH) recorded on the forearm with laser Doppler flowmetry (LDF). Hyperemia may be either expressed as peak raw value (PK), as a function of baseline: peak minus baseline (PK-BL), percentage increase from baseline (PK%BL) or area under curve (AUC); or as the percentage of vasodilation maximal vasodilation (reached by heating locally to 4244°C. The kinetics of the response is sometimes reported as the time to peak (TP) hyperemia (time from cuff release to peak hyperemia, in seconds). BL: baseline; BZ: biological zero.

Figure 5. Example of local thermal hyperemia (LTH) recorded on the forearm with laser speckle contrast imaging (LSCI). Flux is averaged over 3 min for baseline and plateau, and over 1 min for peak (light bars). PU: perfusion units.

Figure 6. Typical tracing of skin blood flux assessed with laser Doppler flowmetry during a 30-min local cooling at 15 °C on the forearm. An inconstant cold-induced vasodilation is observed within the first 10 min. Data are expressed as perfusion units (PU). Reproduced with permission from ref [108].

Figure 7. Measurement of skin blood flux on different skin sites of the forearm (numbered 1 to 5): unheated, heated to 36°C, to 39°C, to 42°C and to 44°C, respectively, using laser speckle contrast imaging (A) and laser Doppler imaging (B).

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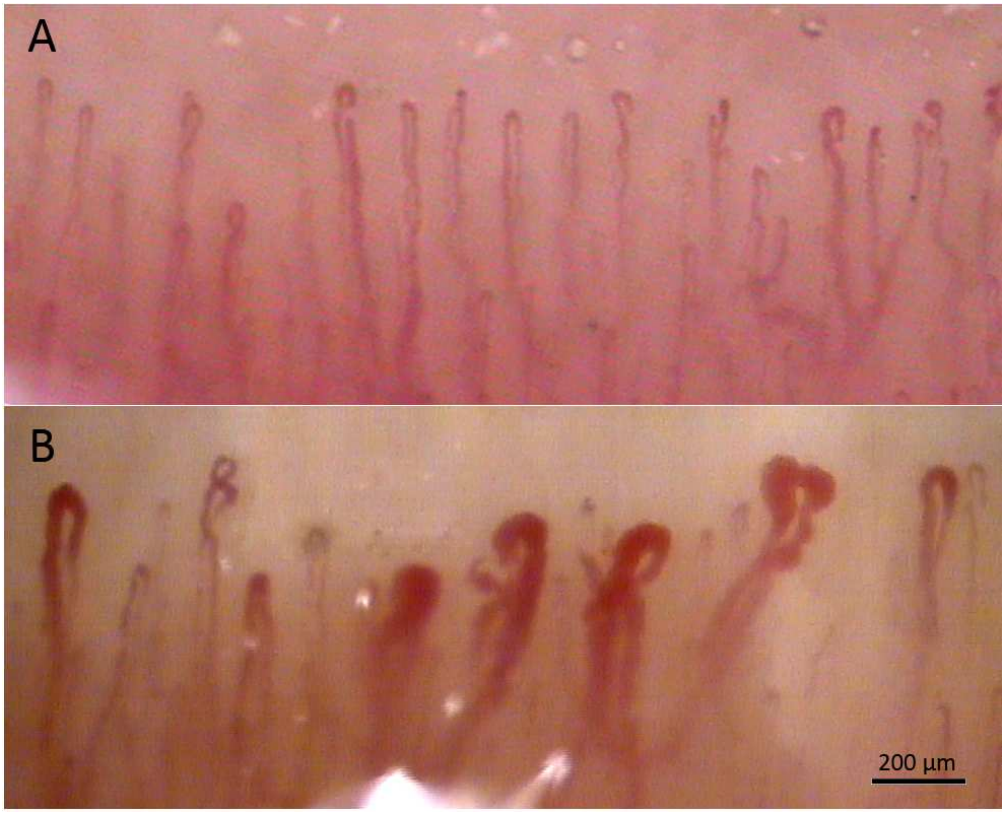

Representative images of nailfold videocapillaroscopy (NVC) with a magnification x 100. A, Normal pattern showing homogenous distribution of capillary loops. B, Pattern observed in a patient with systemic sclerosis, showing disorganized enlarged/giant capillaries.
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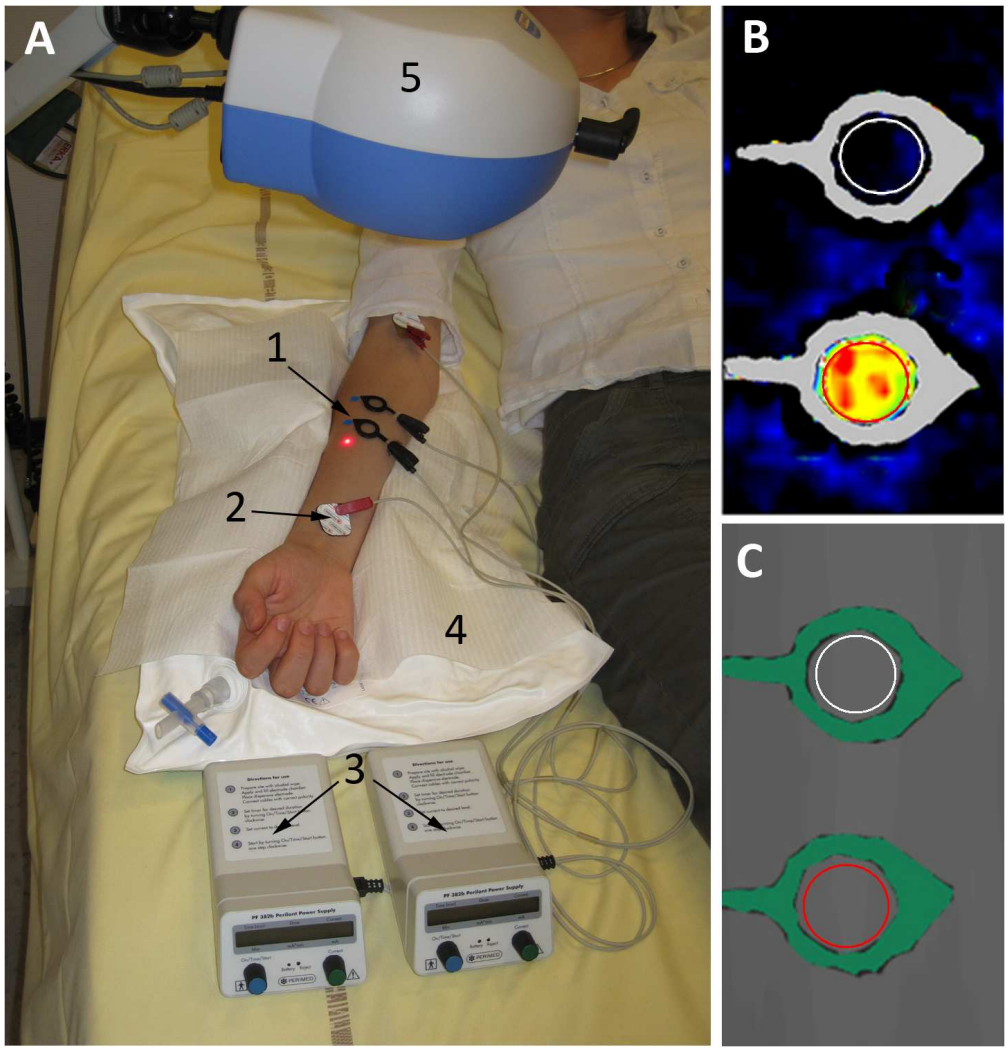

A, Cathodal iontophoresis of vasoadilator drug and control while recording skin blood flux with laser Doppler imaging (LDI); 1, active electrode containing the drugs; 2, passive electrode; 3, current generators connected to the electrodes; vacuum cushion to reduce movement artifacts; 5, head of the imager. B, skin blood flux recorded during iontophoresis (20 min, 20 µA) of sodium nitroprusside (bottom) and saline (top) after local anesthesia to avoid axon reflex vasodilation. C, intensity allows easier positioning of the regions of interest.
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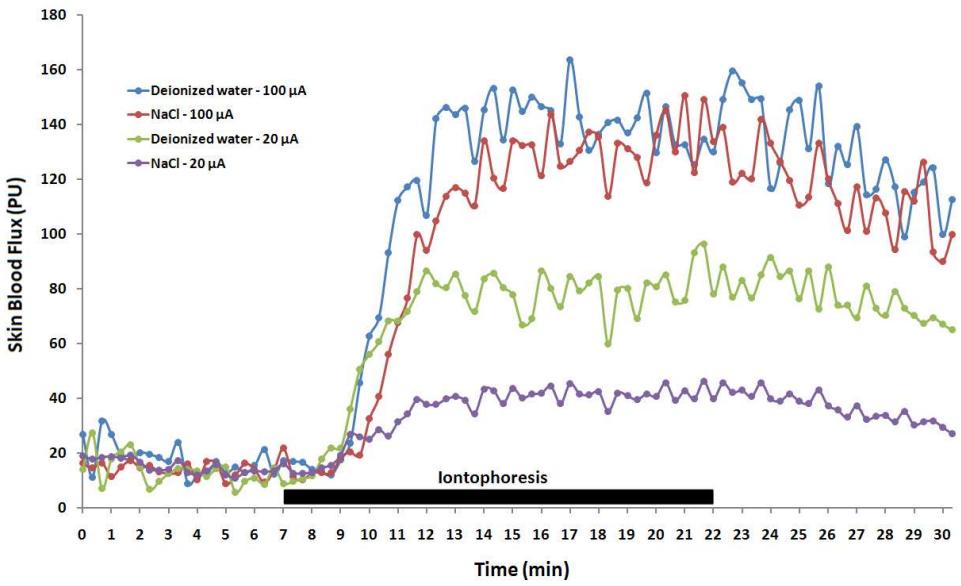

Example of current-induced vasodilation observed during cathodal iontophoresis (15 min, 20 or 100 µA) of saline and deionized water. The black bar represents the length of iontophoresis. Skin blood flux was assessed with laser Doppler imaging (frame rate: 3 images/min). PU: perfusion units.
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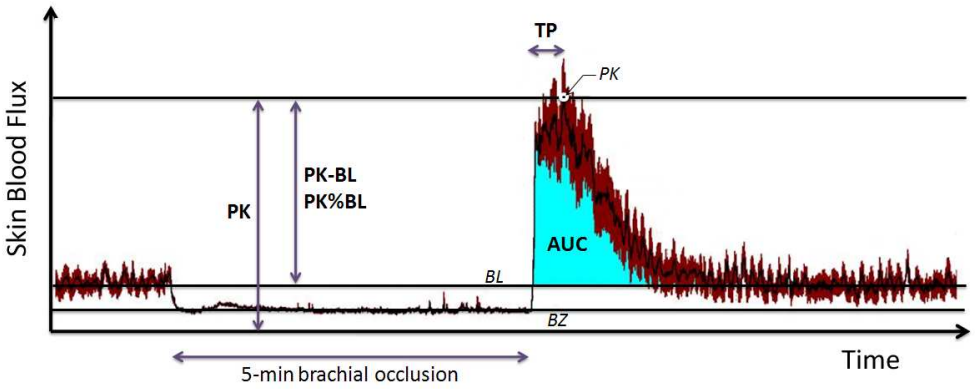

Example of post-occlusive reactive hyperemia (PORH) recorded on the forearm with laser Doppler flowmetry (LDF). Hyperemia may be either expressed as peak raw value (PK), as a function of baseline: peak minus baseline (PK-BL), percentage increase from baseline (PK%BL) or area under curve (AUC); or as the percentage of vasodilation maximal vasodilation (reached by heating locally to 4244°C. The kinetics of the response is sometimes reported as the time to peak (TP) hyperemia (time from cuff release to peak hyperemia, in seconds). BL: baseline; BZ: biological zero.
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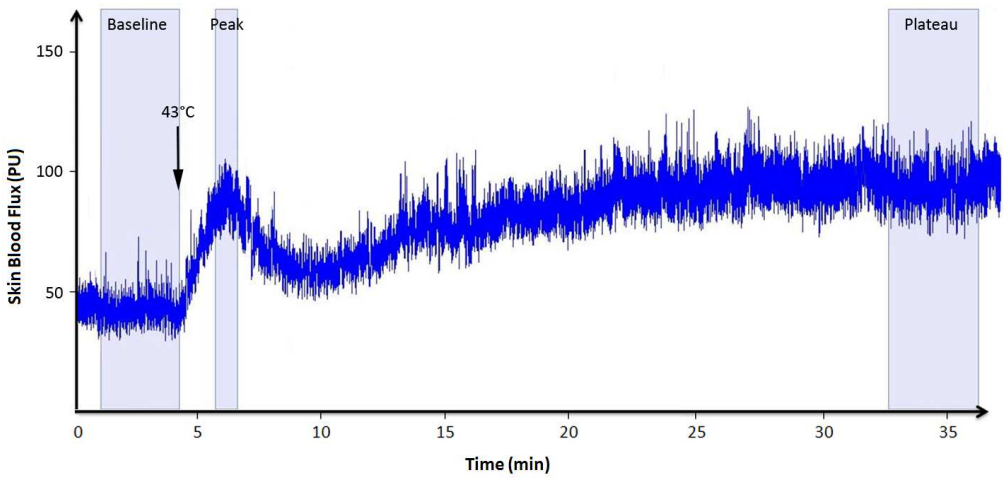

Example of local thermal hyperemia (LTH) recorded on the forearm with laser speckle contrast imaging (LSCI). Flux is averaged over 3 min for baseline and plateau, and over 1 min for peak (light bars). PU: perfusion units.
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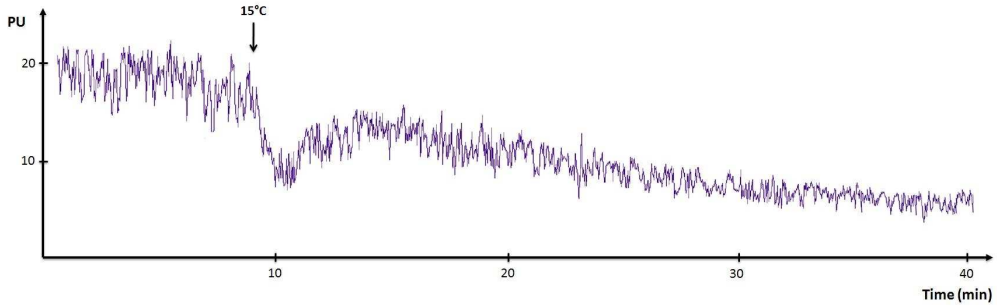

Typical tracing of skin blood flux assessed with laser Doppler flowmetry during a 30-min local cooling at 15 °C on the forearm. An inconstant cold-induced vasodilation is observed within the first 10 min. Data are expressed as perfusion units (PU). Reproduced with permission from ref [108].
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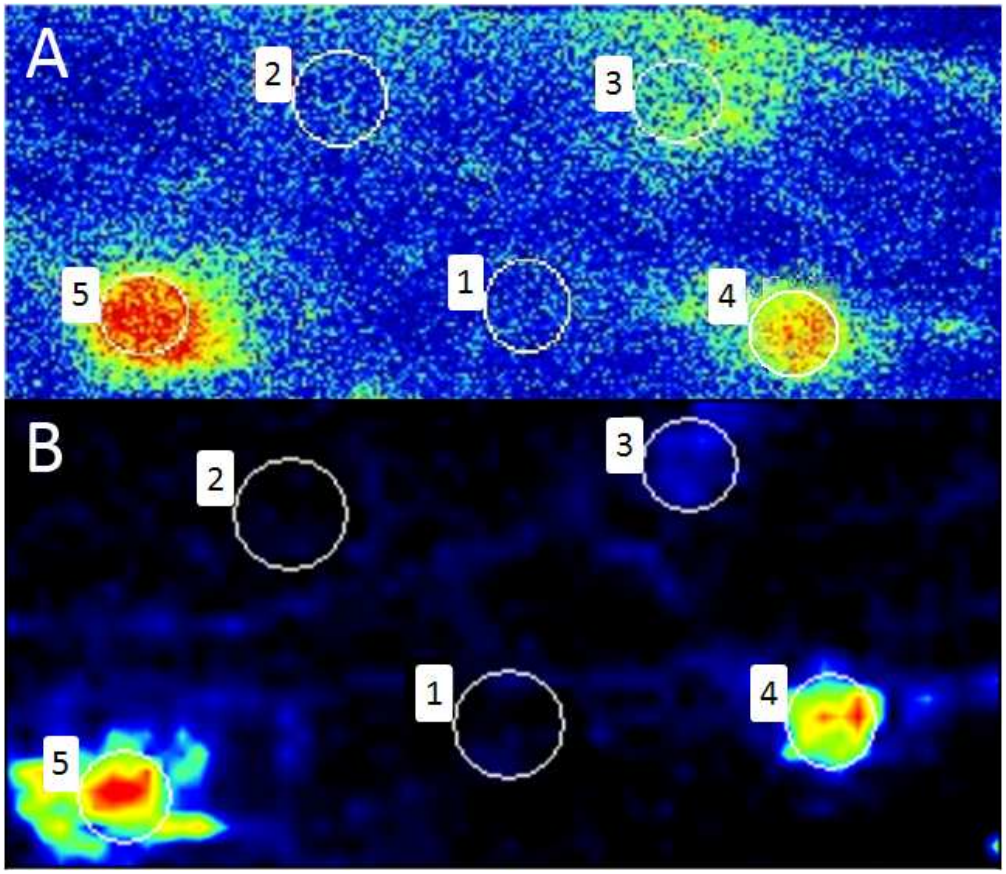

Measurement of skin blood flux on different skin sites of the forearm (numbered 1 to 5): unheated, heated to 36°C, to 39°C, to 42°C and to 44°C, respectively, using laser speckle contrast imaging (A) and laser Doppler imaging (B).
170x147mm (96 x 96 DPI)