Study of skin microvascular function in Raynaud's phenomenon : a pathophysiological and pharmacological approach

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Etude de la fonction microvasculaire cutanée dans le syndrome de Raynaud : approches physiopathologique et pharmacologique

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Résumé

Etude de la fonction microvasculaire cutanée dans le syndrome de Raynaud : approches physiopathologique et pharmacologique

La microcirculation cutanée a été proposée comme modèle d'étude de la dysfonction microvasculaire globale dans les maladies cardiovasculaires. Par ailleurs, elle est spécifiquement atteinte dans le syndrome de Raynaud, qui est une ischémie paroxystique des extrémités déclenchée notamment par le froid. L'exploration de la fonction microvasculaire cutanée suscite donc un réel intérêt, mais les méthodes d'étude souffrent d'une hétérogénéité importante, et leur variabilité intra-individuelle est mal connue. La première partie de ce travail fait la synthèse des différentes méthodes d'étude la fonction microvasculaire cutanée, et rapporte les résultats de deux études consacrées à leur reproductibilité. Nous avons dans une seconde partie étudié grâce à ces tests la réactivité microvasculaire cutanée dans le syndrome de Raynaud, et mis en évidence des anomalies chez ces patients, notamment du contrôle neuro-vasculaire. La dernière partie de cette thèse est consacrée à l'étude d'approches pharmacologiques ciblées sur les anomalies de la microcirculation cutanée identifiées chez les patients. Nous avons évalué l'effet du sildenafil, un inhibiteur de la phosphodiesterase-5, sur le flux sanguin digital et montré son effet vasodilatateur lors d'un refroidissement local dans le syndrome de Raynaud. Enfin, nous avons étudiés chez l'animal et chez l'homme l'iontophorèse de vasodilatateurs, une approche innovante d'administration locale de médicaments pour augmenter le flux sanguin cutané.

Mots-clés : microcirculation – Raynaud – sclérodermie systémique – refroidissement local – flux sanguin cutané – sildenafil – sitaxentan

Summary

Study of skin microvascular function in Raynaud's phenomenon: a pathophysiological and pharmacological approach

The cutaneous microcirculation has been proposed as a model to study generalized microvascular function in various diseases. Moreover, it is specifically affected in Raynaud's phenomenon, characterized by transient ischemia in the digits in response to cold. Despite recent advances in methods exploring the cutaneous microcirculation, they still suffer from a lack of standardization. In the first part of this dissertation, we have reviewed the different techniques used to assess skin microvascular reactivity, and studied the reproducibility of reactivity tests. We then used these tests to assess cutaneous microvascular reactivity in Raynaud's phenomenon, and showed abnormal neurovascular control of the microvasculature in these patients. The third part of this dissertation is dedicated to pharmacological studies targeting the cutaneous microcirculation in Raynaud's phenomenon. We tested the effect of sildenafil, a phosphodiesterase-5 inhibitor, on digital skin blood flow while cooling locally, and showed increase in cutaneous vascular conductance in patients with Raynaud's phenomenon. Finally, we assessed in animals and in humans the effect of locally administered vasodilating drugs on the cutaneous microcirculation, by using iontophoresis. This innovative approach may be an interesting treatment in Raynaud's phenomenon.

Key words: microcirculation – Raynaud – systemic sclerosis – local cooling – skin blood flow – sildenafil - sitaxentan

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Résumé substantiel

La microcirculation désigne l'ensemble des vaisseaux sanguins de petit calibre que sont les artérioles, les capillaires et les veinules. Elle joue un rôle fondamental dans les apports d'oxygène et de nutriments aux cellules des différents tissus, et est donc indispensable au bon fonctionnement des organes [1]. Les artérioles jouent par ailleurs un rôle central dans la régulation des résistances périphériques, afin de protéger les capillaires en cas d'élévation trop importante de la pression artérielle [2].

L'exploration de la microcirculation a mis en évidence une dysfonction microvasculaire périphérique chez des patients atteints de maladies cardiovasculaire, suggérant la possible implication d'une dysfonction microvasculaire généralisée à l'origine de ces pathologies [3-5].

Outre la perfusion des différentes structures de la peau, la microcirculation cutanée joue un rôle clé dans la régulation thermique humaine. La réactivité microvasculaire cutanée est régulée de façon fine par des mécanismes centraux ainsi que des facteurs locaux sensibles aux variations de température [6]. Grâce aux avancées récentes dans le domaine de l'exploration de la microcirculation, l'évaluation du flux sanguin cutané lors d'un refroidissement ou d'un chauffage local a été proposée comme test de réactivité microvasculaire.

La microcirculation cutanée pourrait donc être le marqueur d'une dysfonction microvasculaire généralisée, facilement accessible, donc facile à évaluer, et non invasif [7]. A ce jour, la peau a été utilisée comme modèle pour explorer la fonction microvasculaire de nombreuses maladies [1, 4, 8-12].

Par ailleurs, certaines maladies affectent spécifiquement la microcirculation cutanée. C'est le cas du syndrome de Raynaud, caractérisé par des ischémies paroxystiques des extrémités en réponse au froid ou à des émotions. Le syndrome de Raynaud peut être primaire (idiopathique) ou secondaire à une autre pathologie telle que la sclérodermie systémique [13]. Bien qu'à l'origine d'une gêne parfois importante, le syndrome de Raynaud primaire est bénin. En revanche, le Raynaud secondaire à la sclérodermie peut conduire à des ischémies prolongées associées à une morbidité importante. Sur le plan physiopathologique, cette différence peut s'expliquer par le caractère essentiellement fonctionnel de l'anomalie de la microcirculation dans le Raynaud primaire, alors qu'elle s'associe fréquemment à une anomalie de structure dans le Raynaud secondaire [13, 14]. La physiopathologie du syndrome de Raynaud est multifactorielle et complexe, aboutissant à un déséquilibre entre vasoconstriction et vasodilatation, en faveur d'une vasoconstriction accrue [14, 15].

L'exploration de la microcirculation cutanée suscite donc un intérêt à la fois comme marqueur de dysfonction microvasculaire globale dans de nombreuses pathologies cardiovasculaires, mais aussi afin de mieux caractériser la dysfonction localisée que l'on retrouve dans le syndrome de Raynaud, et d'envisager de nouvelles perspectives thérapeutiques.

Les méthodes les plus utilisées depuis le début des années 1980 sont les techniques dérivées de la microscopie, notamment la vidéocapillaroscopie, ainsi que le laser Doppler [16]. L'évaluation de l'oxygénation tissulaire est également couramment utilisée.

Le signal obtenu avec le laser Doppler est corrélé au flux microvasculaire, et ce de façon linéaire. Néanmoins, il ne renseigne pas sur le débit, ce qui justifie son utilisation en association à des tests de réactivité. Parmi ces tests, l'iontophorèse d'acétylcholine et de nitroprussiate de sodium ont été proposés pour évaluer la vasodilatation endothélium-

dépendante et indépendante, respectivement. Néanmoins, ces tests ont de nombreux inconvénients.

Parmi les autres tests de réactivité microvasculaire, l'hyperhémie post-occlusive (HPO), l'hyperthermie thermique (HT) ou le refroidissement local sont potentiellement intéressants. Néanmoins, ces tests ne sont pas standardisés et souffrent d'une hétérogénéité importante quant aux modalités pratiques de leur réalisation, mais aussi de l'expression des résultats. Par ailleurs, leur variabilité intra-individuelle dans le temps ou sur différents sites cutanés est mal connue. La première partie de cette thèse fait la synthèse des avantages et des inconvénients des différents tests couplés à la mesure du flux sanguin cutané par laser Doppler. Elle rapporte également les résultats de deux études consacrées à la reproductibilité de l'HPO et de l'HT, au niveau du doigt et de l'avant-bras, en utilisant différentes techniques telle que la fluxmétrie laser Doppler, l'imagerie laser Doppler, ou l'imagerie laser speckle [17, 18].

Nous avons ensuite comparé la réactivité microvasculaire cutanée de patients atteints de Raynaud primaire ou secondaire, en enregistrant le flux sanguin cutané lors d'un chauffage local, avec et sans anesthésie locale. Cette étude a permis de mettre en évidence une anomalie du contrôle neurovasculaire des vaisseaux de la peau chez les patients sclérodermiques [19]. En revanche, nous n'avons retrouvé aucune anomalie chez les patients ayant un syndrome de Raynaud primaire.

Le froid étant le principal facteur déclenchant un vasospasme chez les patients, nous avons mis au point une sonde de fluxmétrie laser Doppler permettant d'enregistrer le flux sanguin tout en refroidissant localement [20]. Nous avons ensuite testé la reproductibilité d'un test de refroidissement local chez des volontaires sains, qui s'est avérée bonne pour un refroidissement de 30 minutes, mais pas de 5 minutes [20].

Cette sonde nous a permis de comparer la réactivité microvasculaire cutanée au froid local entre des patients ayant un syndrome de Raynaud primaire et des volontaires sains appariés, grâce au test de 30 minutes. Les résultats obtenus suggèrent une vasoconstriction en réponse au froid exagérée chez les patients [21]. Une anesthésie locale permet en revanche de corriger la phase précoce de cette vasoconstriction excessive chez les patients mais pas chez les volontaires sains, ce qui suggère une implication des nerfs sensitifs [21]. La vasoconstriction tardive au froid local étant également accrue chez les patients, d'autres mécanismes sont potentiellement impliqués. Les travaux récents ayant abouti à une meilleure compréhension des voies impliquées dans la réponse microvasculaire au froid permettent d'émettre plusieurs hypothèses.

En effet, le refroidissement local entraîne une vasoconstriction faisant intervenir la noradrénaline, grâce à la translocation de récepteurs α_{2c}-adrénergiques depuis le cytosol (appareil de Golgi) jusqu'à la membrane cellulaire, sous l'influence du système RhoA/Rho kinase (ROCK) [22]. L'autre mécanisme impliqué dans la réponse est l'inhibition de la voie du monoxyde d'azote (NO), puissant vasodilatateur, de par une diminution de l'activité des NO synthases et par un autre mécanisme en aval, encore méconnu [23]. La génération de radicaux libres par la mitochondrie des cellules musculaires lisses pourrait être le premier signal à l'origine de la vasoconstriction au froid local [24], en activant le système ROCK et en inhibant la voie du NO.

Depuis le début des années 1990, l'implication de l'endothéline dans la physiopathologie du syndrome de Raynaud a fait l'objet de plusieurs études, suggérant une augmentation de la libération d'endothéline chez les patients par rapport à des sujets contrôles [13, 25].

L'étude de la microcirculation cutanée dans le syndrome de Raynaud permet de mieux caractériser la dysfonction et donc de proposer de nouvelles cibles thérapeutiques, dont certaines sont explorées dans la troisième partie de cette thèse. Ainsi, les travaux que nous avons réalisés chez les patients atteints d'un phénomène de Raynaud primaire suggèrent une vasoconstriction cutanée exagérée au froid local [21], faisant intervenir un mécanisme neurovasculaire précoce, et un mécanisme retardé, probablement l'activation du système ROCK ou l'inhibition du NO. Nous avons donc émis l'hypothèse qu'un traitement augmentant l'activité de la voie du NO pourrait s'avérer efficace pour restaurer le flux sanguin cutané digital des patients ayant un Raynaud primaire exposés à un refroidissement local.

L'effet vasodilatateur du NO est lié à la génération de guanosine-5-monophosphate cyclique (GMPc), qui induit une relaxation des fibres musculaires lisses. Le GMPc est ensuite métabolisé par la phosphodiesterase de type 5 (PDE5). Le sildenafil est un inhibiteur de la PDE5 qui inhibe donc la dégradation du GMPc, et potentialise ainsi la vasodilatation. Nous avons testé l'effet du sildenafil sur la réactivité microvasculaire digitale au froid chez des patients atteints de Raynaud primaire. Les résultats montrent qu'une dose unique de 100 mg augmente significativement le flux, alors que seule une tendance est observée avec la dose de 50 mg. Les phases précoce et tardive de la réponse au froid sont augmentées, suggérant un effet vasodilatateur non spécifique du sildénafil 100 mg. Bien que le refroidissement local ne soit pas un critère de substitution dans le syndrome de Raynaud, cet effet vasodilatateur sur la microcirculation lors d'un refroidissement local suggère un potentiel intérêt d'un traitement « à la demande » avant exposition au froid. Cette hypothèse devra être confirmée par essai clinique contrôlé, randomisé en double-aveugle, avec des critères de jugement cliniques. Il est d'ailleurs intéressant de noter que de nombreux essais cliniques sont actuellement en cours pour évaluer le potentiel intérêt des inhibiteurs de la PDE5 dans le syndrome de Raynaud.

D'autres essais évaluent l'intérêt d'inhibiteurs du système ROCK ou encore d'antagonistes sélectifs des récepteurs adrénergiques α_{2c}.

Enfin, bien que des traitements soit actuellement disponibles, notamment dans le Raynaud secondaire, leur utilisation par voie systémique est limitée par des effets indésirables dose-dépendants parfois graves. La dysfonction microvasculaire du syndrome de Raynaud étant préférentiellement située au niveau des doigts, l'administration locale de vasodilatateurs pourrait permettre d'atteindre des concentrations thérapeutiques en s'affranchissant des effets indésirables de la voie systémique. L'iontophorèse est une technique non invasive d'administration transcutanée des médicaments, qui repose sur le transfert de molécules ionisées sous l'influence d'un courant de faible intensité [26].

Lors d'études précliniques chez le rat nous avons testé l'effet de l'iontophorèse de vasodilatateurs sur le flux sanguin cutané. A l'état de base, l'iontophorèse de bosentan et de sitaxentan (antagonistes des récepteurs de l'endothéline) n'a pas augmenté le flux sanguin cutané. En revanche, en administrant de l'endothéline par voie intra-artérielle, nous avons observé un effet du sitaxentan, suggérant un passage de la molécule par voie iontophorétique. L'étape suivante fut de tester l'iontophorèse de sitaxentan chez l'homme. Cette étude a été interrompue par un retrait du marché de la molécule et l'arrêt de tous les essais cliniques en cours, à cause de cas de toxicité hépatique graves [27].

En revanche, l'iontophorèse de dérivés de la prostacycline (PGI_2), le tréprostinil et l'iloprost, a entrainé une vasodilatation importante et prolongée chez l'animal [28]. Ces résultats ont été confirmés chez des volontaires sains pour le tréprostinil (Blaise *et al*, manuscrit soumis). Enfin l'évaluation de ce traitement va se poursuivre, en étudiant notamment la réponse au niveau digital, chez des sujets sains ainsi que des patients atteints de syndrome de Raynaud secondaire à la sclérodermie systémique.

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Abbreviations

AAC: area above the curve

Ach: acetylcholine

ADMA: asymmetric dimethylarginine

AUC: area under the curve

BKCa: large conductance calcium-activated potassium channels

BL: baseline

BZ: biological zero

cGMP: cyclic guanosine-5-monophosphate

CGRP: calcitonin gene-related peptide

CMV: cytomegalovirus

COX: cyclooxygenase

CV: within subject coefficient of variation

CVC: cutaneous vascular conductance

EDHF: endothelium-derived hyperpolarizing factor

ECE: endothelin-converting enzyme

eNOS: endothelial nitric oxide synthase

ERA: endothelin receptor antagonist

ET-1: endothelin-1

FMD: flow-mediated dilation

HSP90: heat shock protein 90

ICC: intra-class coefficient of correlation

LDF: laser Doppler flowmetry

LDI: laser Doppler imaging

LDPI: laser Doppler perfusion imaging

LDPM: laser Doppler perfusion monitoring

LSCI: laser speckle contrast imaging

LTH: local thermal hyperemia

NO: nitric oxide

NOS: nitric oxide synthase

NVC: nailfold videocapillaroscopy

OCP: oral contraceptive pill

OPS: orthogonal polarization spectral

PDE5: phosphodiesterase 5

PGI_2: prostacyclin

PIV: pressure-induced vasodilation

PORH: post-occlusive reactive hyperemia

PU: perfusion units

RBC: red blood cell

ROCK: RhoA-Rho kinase

ROI: region of interest

ROS: reactive oxygen species

RP: Raynaud's phenomenon

SDF: sidestream dark field

SNP: sodium nitroprusside

SSc: systemic sclerosis

TIMP: tissue inhibitor of metalloproteinases

TOI: time of interest

TRP: transient receptor potential protein

TRPV: transient receptor potential vanilloid channels

Introduction

The microcirculation refers to the smallest segments of the vascular system, i.e. arterioles, capillaries and venules. It plays a role of primary importance in gas and nutrient exchange. Adequate perfusion through the microcirculation is therefore essential for the integrity of tissue and organ function [1]. Arterioles are also the principal site of control of vascular resistance through myogenic tone, which leads to acute vasoconstriction in response to increased transmural pressure [2]. This property allows the capillaries to be protected from potentially damaging elevated pressure.

The role of generalized microvascular dysfunction in the pathophysiology, or as a consequence of, cardiovascular disease has 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 [3]. Microvascular dysfunction has also been reported in patients with hypertension as well as in experimental models of hypertension [4]. Beside functional changes, remodeling of the microvasculature and rarefaction may occur at an early stage in the development of hypertension [4]. The resulting increase in peripheral resistance amplifies the phenomenon and further exacerbates hypertension. A similar vicious circle has been suggested in the pathophysiology of diabetes [1]. Indeed, insulin enhances microvascular recruitment by skeletal muscle, and the resulting increase in blood flow contributes to the subsequent glucose uptake [5]. Therefore, impaired microvascular function and/or capillary rarefaction may reduce glucose uptake and aggravate insulin resistance.

Changes in generalized microvascular function has also been proposed as a mechanism underlying organ dysfunction and multiple organ failure in patients with sepsis [29]. Although the exact cause of microvascular dysfunction in sepsis has not been completely elucidated, it could be impaired by the mediators of the inflammatory response [29]. The subsequent mechanisms may include reduced functional capillary density [30] and impaired nitric oxide-dependent vasodilation [11]. Microvascular impairment was positively correlated with a poorer outcome [30].

In conclusion, as impaired tissue perfusion related to microvascular dysfunction is common among cardiovascular risk factors such as hypertension, diabetes, dyslipidemia and obesity [1], generalized microvasculature dysfunction has been suggested to be a hallmark of cardiovascular disease. In critical care medicine, similarly, microcirculatory failure has now been considered as a clinical concept, particularly in patients with sepsis [31].

Skin microcirculation as a model of generalized microvascular dysfunction

The cutaneous circulation is organized as two horizontal plexuses in the dermis: the upper network from which capillary loops arise is located in the papillary dermis; it represents the most important part of skin microcirculation [32]. It is connected with a lower network, located at the dermal-subcutaneous interface, through ascending arterioles and descending venules (Figure 1) [32].

The cutaneous microcirculation presents anatomical differences according to its location. Arteriovenous anastomoses have been identified in the digits, nose and ears. Finally, although the organization of the microvasculature did not differ between the arms, the legs, the chest and the face, the density of capillary loops and ascending arterioles is heterogeneous [32].

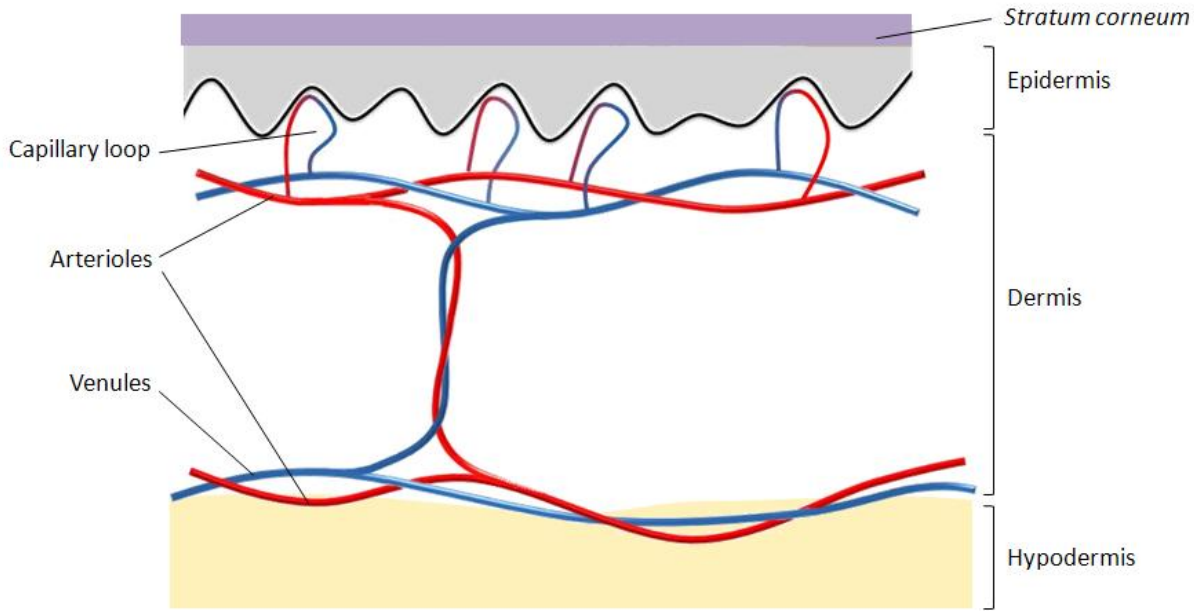

Figure 1. *Schematic representation of microvascular organization in human skin (adapted from ref. [33])*

Besides its nutritive role, the cutaneous microcirculation plays a central role in thermoregulation in humans. Indeed, resting skin blood flow in thermoneutral conditions is approximately 250 µL/min (which dissipates 80-90 kcal/h) but it can be increased to 6-8 L/min during severe hyperthermia, which represents 60% of cardiac output [6]. It is interesting to note that skin blood flow changes to thermal stimuli are regulated both by sympathetic vasoconstrictor and vasodilator systems, and also by local mechanisms [6]. The evaluation of skin blood flow in response to local thermal challenges has been proposed as a test of microvascular reactivity [34].

Indeed, recent technological advances have provided simple and non-invasive methods to estimate skin blood flow. As the skin is readily accessible, it could provide an appropriate site to assess peripheral microvascular reactivity. The human cutaneous circulation could therefore be used as a surrogate marker of systemic microvascular function in various diseases [7]. However, this raises the issue of how representative the microcirculation in the skin is of 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 [1, 4, 8]. The study of skin microvascular reactivity has also been shown to be correlated to retinal microvasculature in diabetic patients [9]. In the same way, skin microvascular function has been shown to be an independent marker of cardiovascular disease in patients with type 2 diabetes [10] or end-stage kidney disease [12]. Skin microcirculation has also been used as a model of microvascular function in experimental shock [11].

Microvascular dysfunction in Raynaud's phenomenon

Besides being studied as a model of the systemic microcirculation, the cutaneous microvasculature may be specifically affected in several pathological conditions such as burns, flaps, wounds or Raynaud's phenomenon.

Raynaud's phenomenon (RP) is characterized by transient ischemia in the digits in response to cold or to emotions. It manifests clinically as palor (ischemia), cyanosis (due to deoxygenation) and rubor (reperfusion) often accompanied by pain. Raynaud's phenomenon in the absence of other disease is called primary RP, whereas secondary RP is associated with autoimmune, inflammatory, hematopoietic or connective tissue diseases such as systemic sclerosis (SSc) [13]. The estimated prevalence of RP is approximately 5% with significant geographic variations, and is more frequent in women [35].

While primary RP has a benign course, secondary RP can lead to irreversible ischemia associated with significant morbidity (e.g. gangrene). From a pathophysiological point of view, this discrepancy may be explained by structural vascular abnormalities of the vessels (including microvessels and digital arteries) in secondary RP [13, 14], whereas the vascular defect in primary RP is mostly functional [14]. Indeed, although minor structural changes in the microvascular bed have been reported in primary RP, they are not as frequent or

pronounced as those found in patients with scleroderma [36]. The consequence of this difference is that digital blood flow in SSc patients is affected even in thermoneutral conditions, and that any superimposed vasospasm may lead to irreversible tissue damage [14].

The pathophysiology of primary RP is multifactorial and complex, and results in microvascular vasospasms [14, 15]. The underlying mechanisms responsible for the imbalance between vasodilation and vasoconstriction have not yet been fully understood, but it probably involves both neural and non-neural mechanisms that closely interact.

As RP is triggered by exposure to cold or stress, central neural mechanisms, particularly the sympathetic nervous system, have long been thought to be the main cause of digital vasospasm. However, local factors have since been shown to participate in RP, including modulation of the expression of postjunctional α_2-adrenoceptors in response to cooling. Non-adrenergic mechanisms such as the vasodilator role of calcitonin gene-related peptide (CGRP) have raised interest in the 1990s.

Besides neural mechanisms, endothelium dysfunction is likely to be involved in the pathophysiology of vascular disease in SSc [37]. Available evidence also suggests impaired endothelial function in primary RP, through increased vasoconstriction via the endothelin-1 (ET-1) pathway, and decreased endothelium-dependent vasodilation [14]. The depressed or limited dilating action of nitric oxide (NO) has finally been suggested both in secondary and primary RP, although data are conflicting in the latter case [13, 14].

The cutaneous microcirculation as a pharmacological target

Considering the specific impairment of skin microvascular function in RP, it could represent an interesting target for treatments. As endothelial dysfunction has been suggested to be the key to microvascular impairment in RP [14], the NO pathway has raised interest in

the past few years. Indeed, the exogenous delivery of NO donors has been suggested as a treatment of primary [38] and secondary RP [39].

On the other hand, increased plasma levels of ET-1 have been associated with primary RP and with SSc [13] and increased ET-1 receptors have been found in the skin of patients with SSc [40]. Moreover, bosentan, a non specific endothelin receptor antagonist (ERA) is indicated in the prevention of digital ulcers in SSc patients at risk [41, 42].

However, systemic administration of vasodilating drugs is limited by dose-dependent adverse reactions (e.g. hepatotoxicity with bosentan). Therefore, local drug delivery may be a way of getting around the toxicity of systemic treatments. Iontophoresis is a simple, non-invasive transdermal drug delivery method using a low-intensity electric current [26]. Moreover, it provides faster administration and better control of the delivered dose than usual passive transdermal administration.

The organization of the different studies included in this dissertation is represented Figure 2. In the first part of this work we will describe the different non-invasive tools used to assess microvascular function in the human skin. We will focus on recent advances in methods and discuss the issue of data expression. The second part will be dedicated to the study of the cutaneous microcirculation in primary and secondary RP, in order to better understand the mechanisms underlying microvascular dysfunction in RP. The third part of this work will describe pharmacological approaches that target the skin microcirculation. Particularly, we will study therapies aiming at increasing microvascular blood flow during exposure to cold. Finally, we will report the results of laboratory and clinical pharmacology studies assessing innovating drug delivery systems such as iontophoresis.

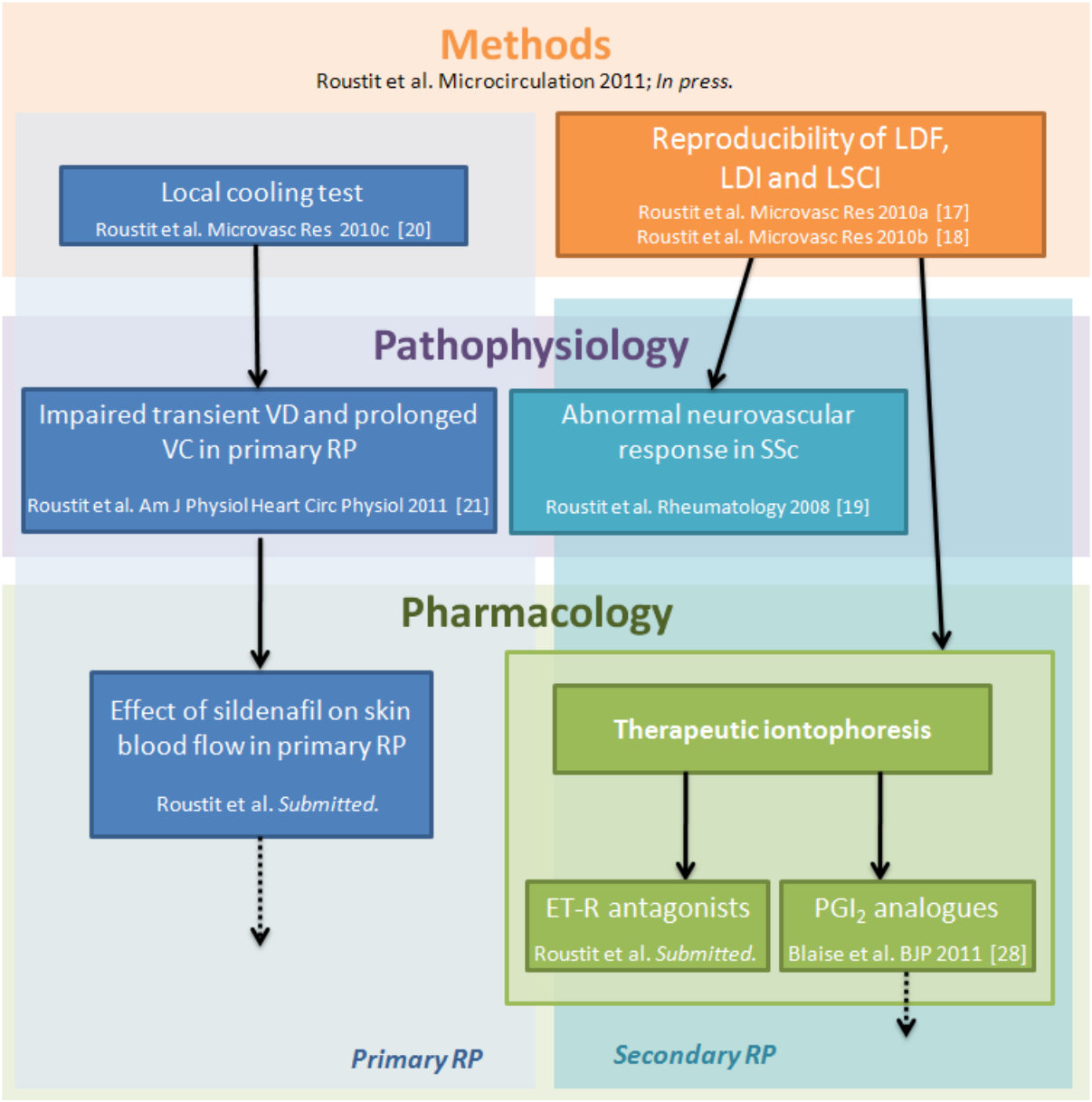

Figure 2. *Organization of the studies in the dissertation. LDF: laser Doppler flowmetry; LDI: laser Doppler imaging; LSCI: laser speckle contrast imaging; ET-R: endothelin-1 receptor; PGI_2: prostacyclin; RP: Raynaud's phenomenon; SSc: systemic sclerosis; VC: vasoconstriction; VD: vasodilation.*

Part I. Methods for the non-invasive assessment of microvascular function

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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Microcirculation, i*n press*

For more than two decades, methods for the noninvasive exploration of cutaneous microcirculation have raised considerable interest, whether to explore skin microcirculation as a model of generalized microvascular impairment or to identify specific abnormalities, e.g. in secondary Raynaud's phenomenon. Optical microscopy and laser Doppler techniques have been mainly used [16], 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 [43]. More sophisticated techniques have recently been developed, including orthogonal polarization spectral (OPS) imaging [44] and more recently sidestream dark field (SDF) imaging [45]. 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 [46]. 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 chapter we will review the different techniques used to study the microvasculature, focusing on the tests used to assess microvascular function. Evaluation of tissue oxygenation will not be treated in the present dissertation (for detail please refer to the expert review by De Backer [47]). Different methodological issues such as data expression will be discussed. A particular attention will be given to the reproducibility of laser Doppler measurement as it constituted the basis of our work on methods [17, 18, 20].

I. Optical microscopy-derived techniques

1. 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 [48]. Recently available digital systems have made the technique more reliable and user-friendly [49].

The skin site most studied using videocapillaroscopy is the periungueal region. Indeed, nailfold capillaries are parallel to the skin's surface 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 [16]. The normal NVC pattern is characterized by a homogeneous distribution of parallel capillary loops from 6 to 15 µm in diameter [16] (Figure 3A).

Abnormal patterns are observed in diseases affecting digital skin microvasculature (e.g. systemic sclerosis, Figure 3B), showing morphological abnormalities of the capillaries (enlarged loops, giant capillaries, ramifications, capillary disorganization), micro-hemorrhages and lower density (capillary loss) [49]. Capillary abnormalities in systemic sclerosis have been classified into early, active or late patterns by Cutolo et al [50]. Since the first description of abnormal finger capillary patterns in connective tissue diseases using capillaroscopy [51], the technique has played an increasing role in the early diagnosis of scleroderma spectrum disorder [49], and significantly improves the sensitivity of the American College of Rheumatology criteria in the diagnosis of patients with limited systemic sclerosis [52]. 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 [53].

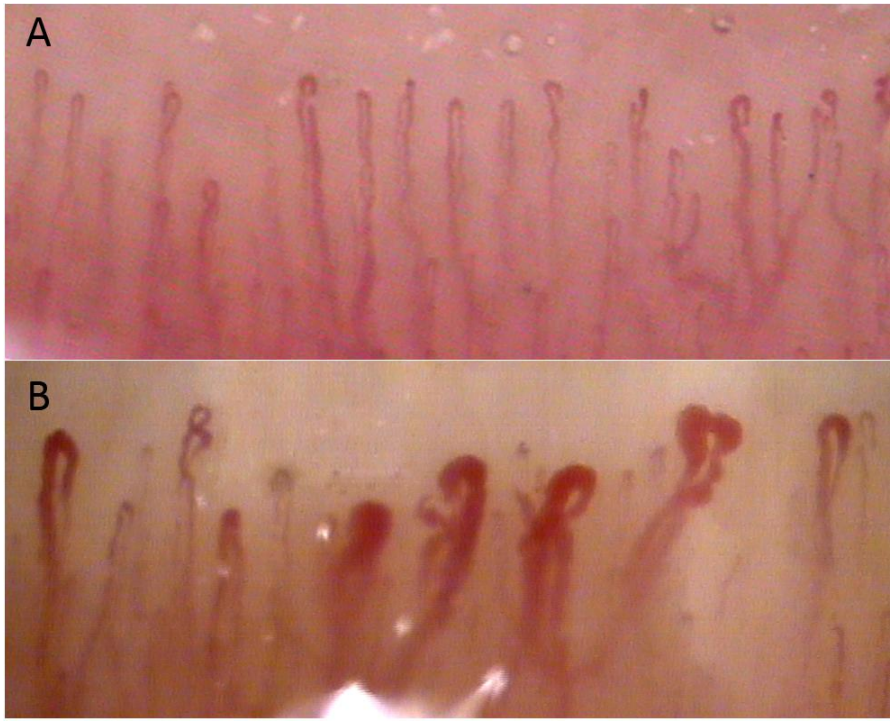

Figure 3. *Representative images of nailfold videocapillaroscopy (NVC) with a magnification x 100. A: normal pattern showing homogenous distribution of the capillary loops. B: Pattern observed in a patient with SSc, showing disorganized enlarged/giant capillaries.*

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 [54] or rheumatoid arthritis [55], although no specific patterns have been identified.

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 functional 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

[56]. When performed on the dorsum of the finger, venous congestion showed better results than brachial PORH [57]. Using such methods, both baseline and maximal capillary recruitment were significantly lower in patients with essential hypertension than in normotensive controls [8]. We note that some authors have described a reversion of both functional and structural capillary rarefaction in patients under effective antihypertensive treatment [58, 59]. Similar studies have shown impaired capillary recruitment (i.e. an absolute difference or percentage change between functional and maximal densities) in patients with type 1 diabetes compared to controls, although the baseline density was higher in these patients [60]. 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 [9].

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 [61] and in various diseases including diabetes [62], systemic sclerosis [63], psoriasis [64], or to evaluate the vascular integrity of skin flaps [65, 66]. 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.

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.

2. Orthogonal polarization spectral and sidestream dark field imaging

In OPS imaging the tissue is illuminated with linearly polarized green light and the remitted illumination is provided by depolarized photons scattered by the deeper layers of the tissue, imitating transillumination of the superficial layer [44]. SDF imaging is a closely related technique, but illumination is provided by concentrically placed light emitting diodes surrounding a central light guide [45]. The green light is scattered by the deeper layers of the tissue while it is absorbed by hemoglobin, providing an image in which red blood cells (RBCs) appear as dark moving globules against a white/grayish background [45].

Orthogonal polarization spectral imaging is a relatively inexpensive technique and has the advantage of being portable [67]. 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 [67].

OPS and SDF have been used during surgery to assess the microcirculation of several organs including the brain [68, 69], the kidney [70] or the liver [71]. The most studied site however is the sublingual region, where the density of perfused capillaries can be non-invasively assessed [47]. 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 [72]. The main applications of OPS 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 [30]. 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 [73]. 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 [74]. Other

applications of skin OPS imaging include the assessment of microcirculation in burn wounds [75, 76]. Nonetheless, OPS use in burn wound severity is still predominantly used for research [67].

Application of pressure with OPS or SDF probes during examination modifies the flow velocity in vessels under investigation [77] 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 [78].

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 [47].

II. Laser Doppler

1. 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 [46]. 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 [79].

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 frequency (often 32 Hz) and is accurate at detecting and quantifying relative changes in skin blood flow in response to a given stimulus [80]. However, the regional heterogeneity of skin perfusion [33] leads to spatial variability, which contributes to the relatively poor reproducibility of the technique [17] (this reference is available at the end of this chapter).

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 [80]. 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. For each image the matrix of the points provides an index of cutaneous vascular flow heterogeneity in addition to the flux values. Therefore, LDI decreases spatial variability but 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.

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 [81]. However, it does not provide an exact measure of flow (i.e. $mL.min^{-1}$) as can be obtained by extrapolation 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)] [80].

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 [82]. Nonetheless, it can also be used to deliver drugs to a small area of tissue, avoiding confounding systemic effects [80]. Although minimally invasive, microdialysis offers the advantage of a controlled drug infusion rate and the absence of current-induced vasodilation. 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 [83, 84].

2. 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 4). 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 (this will be discussed in detail in the third part of this dissertation) [26]. Combined with laser Doppler, acetylcholine (Ach) and sodium nitroprusside (SNP) iontophoresis have been widely used to assess microvascular endothelium-dependent and independent vasodilation, respectively [80, 85]. It is of note that vasodilator iontophoresis has been proposed as a new therapy in diseases affecting skin microcirculation of the digits, such as SSc [86, 87]. We will discuss this point in the third part or this dissertation.

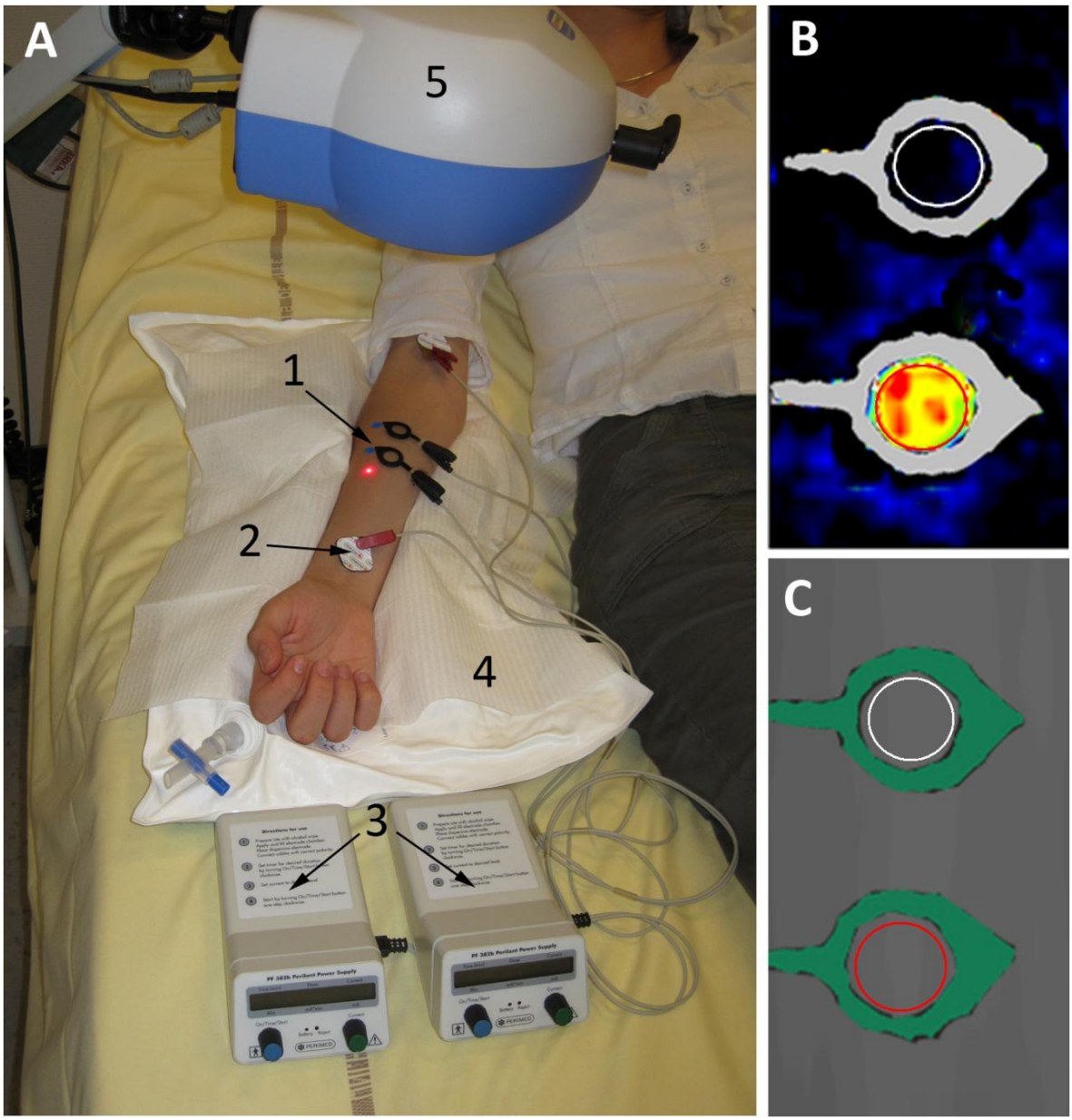

Figure 4. *A, Cathodal iontophoresis of vasoadilator drug and control while recording skin blood flux with laser Doppler imaging (LDI); 1, active electrode containing the drugs; 2, passive electrode; 3, current generators connected to the electrodes; vacuum cushion to reduce movement artifacts; 5, head 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.*

The mechanisms by which Ach iontophoresis induces vasodilation of the microvessels remain unclear [80, 85]. A Cyclooxygenase (COX)-dependent pathway seems to be

predominant [88-90], although data are conflicting [91, 92]. On the other hand, nitric oxide (NO) does not extensively contribute to the response [88, 89]. Interactions between prostaglandin and NO pathways could explain the discrepancies between the results of these different studies [85]. Besides the endothelium-dependent vasodilation, iontophoresis of Ach induces C-fiber (axon reflex)-mediated vasodilation [91]. The variable effect of COX inhibition and local anesthesia [91, 92] on Ach-induced vasodilation may be attributed to these different components of the response to Ach iontoporesis.

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 [93, 94]. The amplitude of current-induced vasodilation depends on the delivered electrical charge (i.e. the product of current intensity by duration of application) [94] (Figure 5) and the current delivery pattern. For a similar charge, repeated applications induce more non specific effects than continuous iontophoresis [95]. Durand et al showed that current-induced vasodilation was abolished by local anesthesia and largely reduced after desensitization of C-nociceptive fibers by capsaicin [94], suggesting a role of neural axon reflex. Moreover, prostaglandins are likely to be essential for this axon reflex-related vasodilatation [96], mainly through the COX-1 pathway [97]. Nonetheless, the exact underlying mechanisms of the interference of current with vasodilation remain unclear.

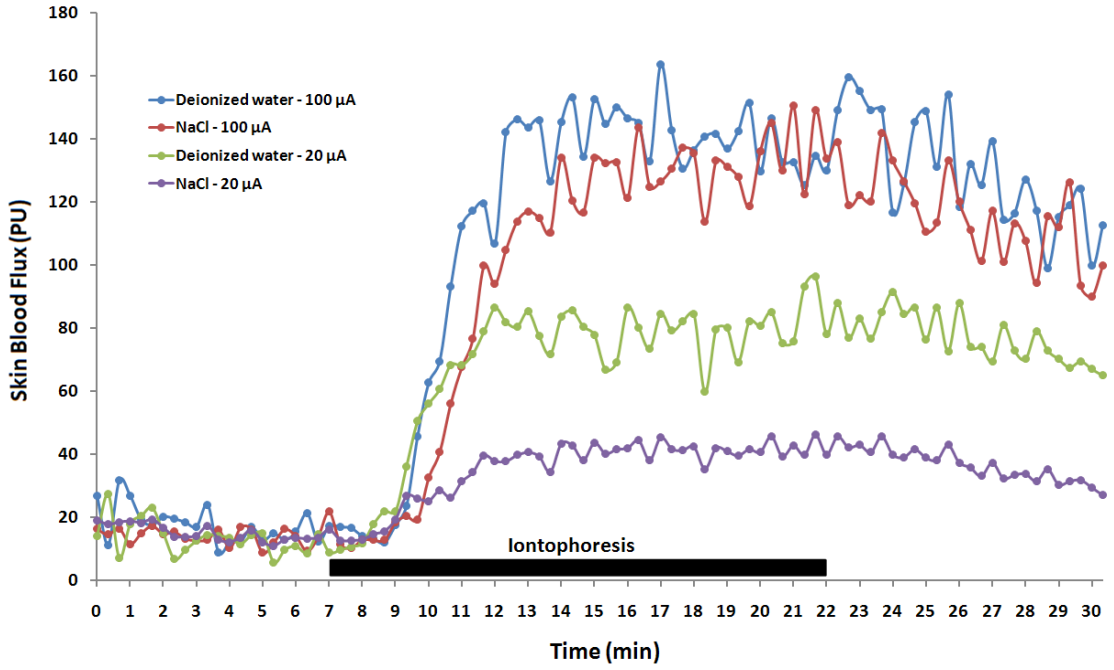

Figure 5. *Example of current-induced vasodilation observed during cathodal iontophoresis (15 min, 20 or 100 µA) of saline and deionized water. The black bar represents the length of iontophoresis. Skin blood flux was assessed with laser Doppler imaging (frame rate: 3 images/min). PU: perfusion units.*

Different vehicles have been used to dilute drugs (e.g. tap water, deionized water and saline) with various galvanic responses [85]. In the excellent paper by Ferrell *et al* [98], the authors have shown that distilled water alone induces a more pronounced current-induced vasodilation than saline [98]. 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 [98]. 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 of the integral of voltage over time (between 60 and 70 V.min) 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 [99]. Skin resistance is variable between individuals and between different skin areas, depending on the density of sweat ducts or hair follicles [85]. Ramsay *et al* showed a significant linear inverse correlation between skin resistance and the response to Ach or SNP iontophoresis [99]. Monitoring voltage across the iontophoretic circuit may be 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 [85].

The reproducibility of Ach and SNP iontophoresis is good when assessed with LDI, especially when the perfusion is corrected by the resistance time integral [100]. 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) of 0.72 [101]. When using LDF, the reproducibility of Ach iontophoresis was poorer (ranging from 25% to 35% depending on the way of expressing data) [102]. 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 [103]. 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 [104]. 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 [86].

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 [34]. Moreover, other limitations must be acknowledged, including non-specific effects and poor reproducibility when LDF is used [105]. 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 5). Pre-treatment with a local anesthetic is a way to limit axon reflex-induced vasodilation [101]. Limiting current density (<0.01 mA/cm²) and charge density (<7.8 mC/cm²) also decreases current-induced vasodilation [106]. Finally, skin resistance may be reported and can be readily approximated by connecting a voltmeter in parallel [100]. Perfusion data may then be normalized to skin resistance, or resistance can be standardized by adjusting the distance between the electrodes.

3. 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. It is also called post-ischemic or reactive hyperemia [80]. Many mediators contribute to PORH. Sensory nerves are partially involved through an axon reflex response [107, 108]. Local mediators include large conductance calcium-activated potassium (BKCa) channels that seem to play a major role [107], suggesting that endothelium-derived hyperpolarizing factor (EDHF) is involved; while results are conflicting concerning the implication of prostaglandins [92, 109, 110]. The inhibition of NO synthesis does not alter PORH on the forearm [84], but recent work suggests that COX inhibition unmasks the NO dependence of reactive hyperemia in human cutaneous

circulation [110]. On the finger pad however, the response seems to be partly NO-dependent [111]. 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 6). 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 [6]. Time to peak perfusion after cuff release is another parameter quantified when performing PORH, but its physiological significance as a marker of skin microvascular reactivity remains to be established.

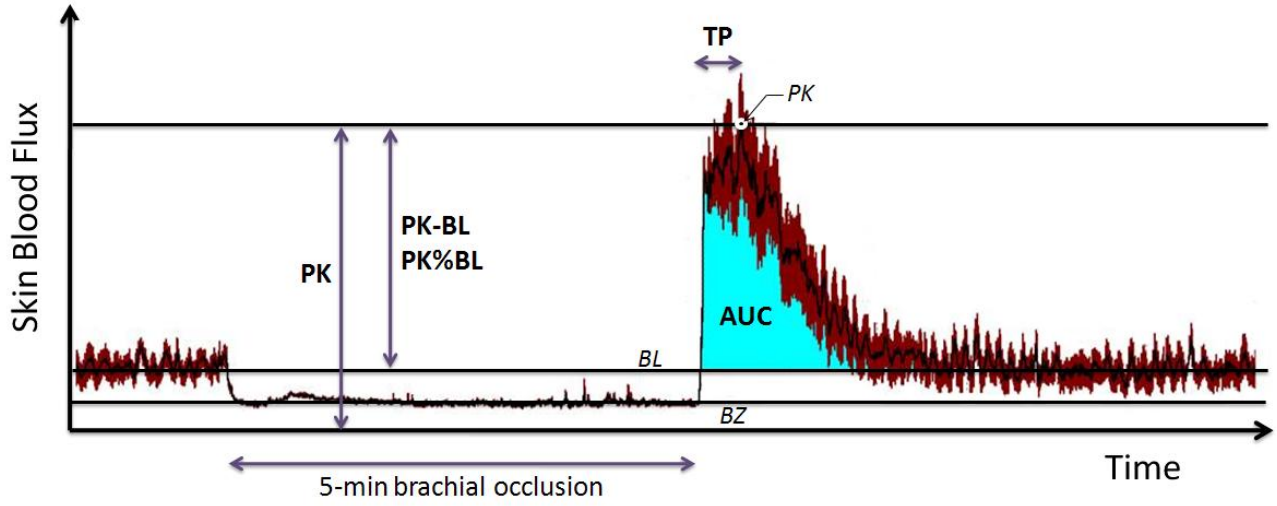

Figure 6. *Example of post-occlusive reactive hyperemia (PORH) recorded on the forearm with laser Doppler flowmetry (LDF). Hyperemia may be either expressed as peak raw value (PK), as a function of baseline: peak minus baseline (PK-BL), percentage increase from baseline (PK%BL) or area under the curve (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*

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%) [17]. However, reproducibility is poor (CV are 45% or higher) when peak perfusion is expressed as a function of baseline [17, 105]. 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 [112]. However, reproducibility was only fair to good (CV around 20%) when the position of the probe was recorded with less precision [102] and decidedly poor when the skin sites were randomly chosen (CV were 40% or higher) [17] (Table 1).

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) [18] (Table 1) (this reference is available at the end of this chapter).

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 [18]. It is likely that the variation in capillary density between different skin sites is the major source of variability when using single-point LDF and the use of full-field techniques such as LDI could lessen this variability.

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

	Single-point* LDF [17, 18]	LSCI [18, 113]
CVC / PU	45 / 30	8 / 3.1[#]
CVC_{PK}-CVC_{BL}	19.4[¶]-48 / 33	11
$\%CVC_{BL}$	22.7[¶]-38 / 32	15
AUC	89 / 36	NA
$\%CVC_{max}$	41 / 39	35

Data are expressed as within subject coefficient of variation (in %) of peak 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. [#]Peak was expressed as perfusion units (PU), from ref [113]; [¶] From ref [102]; NA: not available

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 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%) [114]. 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 [18] (see below).

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 [84, 114, 115]. 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 [116]. 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 [114].

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 pathway (potentially interacting with NO) 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 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.

4. Local thermal hyperemia

Among thermal challenges, local heating induces an increase in skin blood flux often referred to as local thermal hyperemia (LTH). It provides an integrated index of neurovascular and nitric oxide-dependent cutaneous blood flow regulation [80]. In healthy subjects, LTH is

characterized by an initial peak within the first 5 min, a subsequent nadir followed by a sustained plateau (Figure 7). The initial peak mainly depends on sensory nerves as it is significantly attenuated by local anesthesia [83]. Although to date, there has been no positive evidence to support this claim, it has been suggested that calcitonin gene-related peptide (CGRP) [117], possibly co-released with substance P, is responsible for this initial peak [118]. 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 [119]. The late plateau phase however is insensitive to local anesthesia and is mostly NO-dependent [83]. 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 [120]. As NO synthase inhibition does not completely abolish the response, other contributors are thought to be involved, including norepinephrine and neuropeptide Y [34]. Recently, reactive oxygen species (ROS) have been shown to play a role in plateau hyperemia by limiting the availability of NO [121].

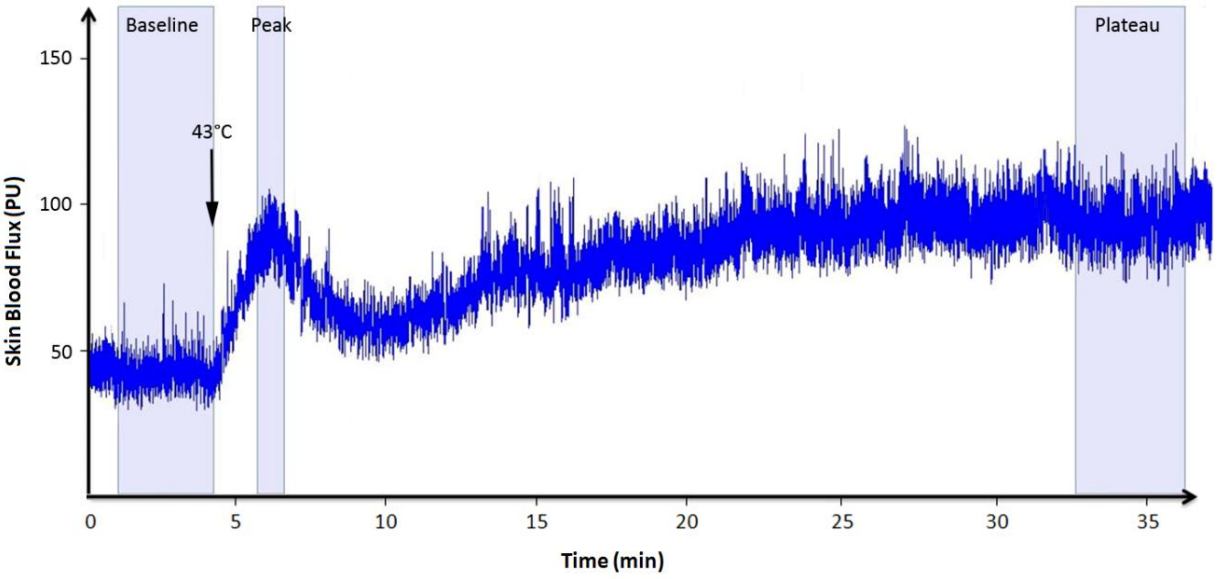

Figure 7. *Example of local thermal hyperemia (LTH) recorded on the forearm with laser speckle contrast imaging (LSCI). Flux is averaged over 3 min for baseline and plateau, and over 1 min for peak (light bars). PU: perfusion units.*

The two independent phases of LTH imply a dichotomized analysis of the recording. Figure 7 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. 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 [19]. 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 [12].

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%) [17]. 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 [18]. 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 [102]; 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 [102]. 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) [122].

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) [18]. However, LDI was not as accurate to assess the LTH peak on the forearm, probably because of its slow kinetics (CV for peak was 39% when expressed as raw CVC) over wide areas. The good inter-site reproducibility of peak CVC simultaneously assessed at two sites on the same forearm strengthens this hypothesis [18]. 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 2).

Table 2. Reproducibility of local thermal hyperemia (LTH) on the forearm of healthy subjects.

		LDF		LDI	LSCI
		Single-point* [17, 18]	Integrating [102, 122]	[18]	[18, 113]
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 peak an plateau cutaneous vascular conductance (CVC), percentage change from baseline CVC ($\%CVC_{BL}$), or percentage of maximal CVC ($\%CVC_{max}$). * without normalizing baseline skin temperature / after normalizing baseline skin temperature to 33°C. [†]From ref [102].

Although many heating protocols have been proposed, local warming to 42-43°C is usually sufficient to induce maximal vasodilation [123]. 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 [34] 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 [123], it starts at around the 45^{th}-50^{th} min [124], 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 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 $\%CVC_{max}$.

5. 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 [125], flexible cold packs [126] or use of a stream of carbon dioxide [127]. Due to its relative ease of use, immersion in cold water has been extensively used, including in patients with RP [128]. However, this technique induces a systemic sympathetic activation [129], 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 [130], without inducing any effect on ipsilateral and contralateral controls [20] (this reference is available in the second part of this dissertation), 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 [123] (Figure 8). 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 [131] and inhibition of the NO system [123]. Sensory nerves could play a role in the transient vasodilation, which is less well understood [123]. Such transient vasodilation is more obvious when the cooling is rapid [132], making the rate of cooling an important parameter to consider when studying microvascular reactivity to local cooling.

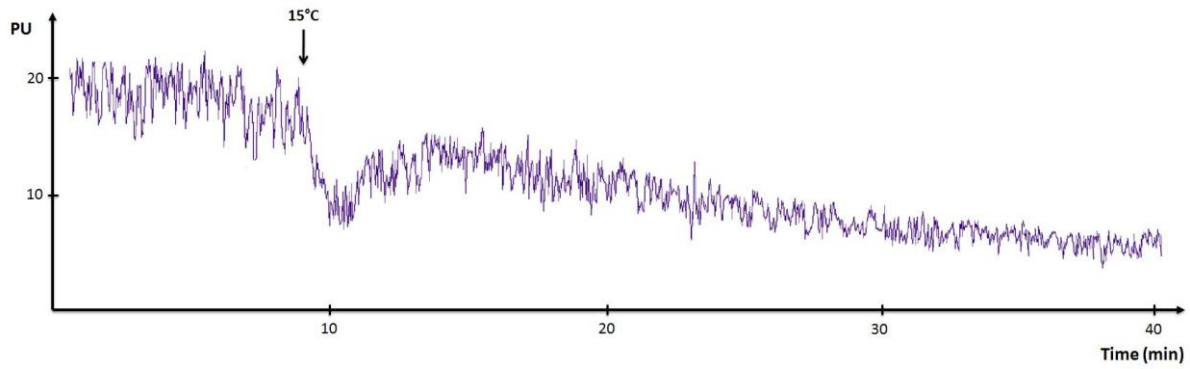

Figure 8. *Typical tracing of skin blood flux assessed with laser Doppler flowmetry during a 30-min local cooling at 15 °C on the forearm. An inconstant cold-induced vasodilation is observed within the first 10 min. Data are expressed as perfusion units (PU). Reproduced with permission from ref [20].*

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%) [20]. 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 [21], which will be detailed in the second part of this work.

III. 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 [133, 134]. 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 [18]. 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 [33, 135].

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 [136, 137]. Recent work has shown that LDI and LSCI provide the same information using computer simulations and laboratory measurements, LSCI having the advantage of generating full images at video rates [138]. In vivo, Stewart et al have shown a very good correlation between the two techniques in burn scar perfusion assessment [139]. Such correlation between LSCI and LDI is maintained over a wide range of perfusion in human skin when data are expressed as raw arbitrary perfusion units [140] (Figure 9). 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, suggesting a proportional bias [140].

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) [141]. 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) [113]. The authors suggest that at this frequency, ROIs should be larger than 10 mm^2 and TOIs longer than 1 s.

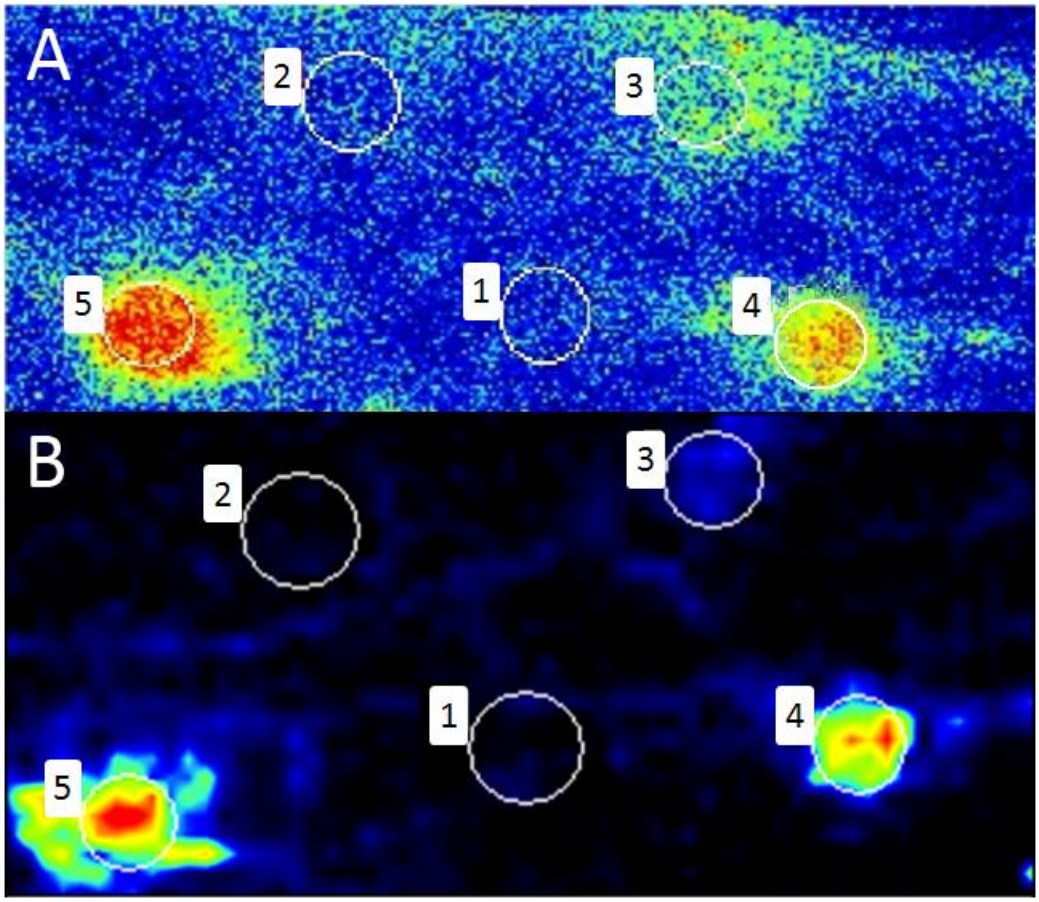

Figure 9. *Measurement of skin blood flux on different skin sites of the forearm (numbered 1 to 5): unheated, heated to 36°C, to 39°C, to 42°C and to 44°C, respectively, using laser speckle contrast imaging (A) and laser Doppler imaging (B).*

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.

IV. **Methodological issues**

1. 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 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 [17]. The influence of room temperature is less obvious for forearm measurements [17].

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 [142]. 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 [143, 144] and similarly in older subjects [145]. Although PIV has been observed over a wide range of pressures [146], 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 [147]. A similar tendency has been observed by using a Stroop color test [17]. In the same way, fear-induced stress evoked marked skin vasoconstriction in the finger [148]. On the forearm however, mental stress does not influence skin blood flow during normothermia [17, 149] or reactivity tests such as PORH and LTH [17], or slightly increases skin blood flux [147]. 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 participant's acclimatization. A vacuum cushion may be used to maintain the hand and forearm as still as possible and thus reduce movement artifacts.

2. Characteristics of the population

Although aging does not affect resting cutaneous blood flow [150], 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 [151]). No difference was shown however after local heating on the finger pad [152].

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 [153-155]. 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 [150]. In the same way, local heating induces a lower increase in females than in males [150]. The menstrual cycle did not influence microvascular reactivity assessed by the iontophoresis of ACh and SNP combined with laser Doppler [156]. 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 [157]. 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 [158]. The influence of female hormone levels across menstrual cycle or OCP on microvascular reactivity deserves further exploration, but it could introduce a confounding bias in studies [159].

Age, gender, phase of the menstrual cycle and contraception should be taken into account to limit confusing bias in controlled studies, by appropriate matching or randomization. Finally, vasoactive drugs and cigarette smoking also affect microvascular function [160, 161] and should therefore be taken into account.

3. 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 [162]. 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 [33]. As skin arterioles are separated by an average of 1.7 mm on the forearm [33], 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.

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 [17]. 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) [80]. 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 [80]. 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. Indeed, there is little information concerning the relationship between systemic blood pressure and skin perfusion pressure. 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 [163]. 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 [164], suggesting a lack of consistent autoregulation [165]. 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 [166].

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 ($R>0.85$) [139, 140], there is a major 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 [140].

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 the first part of this work. Data is then expressed as raw flux or CVC, as a 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 Tables 1 and 2.

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 [167]. 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 [18, 34]. However, such data expression may not be appropriate when studying reactivity in patients, in whom maximal vasodilation may be altered [34]. Full-field techniques such as LDI or LSCI may be of particular interest in such situations.

4. 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) [168]. 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 [168]. The authors therefore suggested measuring BZ under every experimental condition and subtracting it from the flux signal [168]. 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 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 [168] and LDI [169]. 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 [17]. Furthermore, it may introduce bias when data are expressed as a percentage increase from baseline flux [169].

To our knowledge, little data are available concerning BZ assessed with LSCI. In a recent study we have shown higher BZ with LSCI than with LDI, thus again raising the issue of its influence on data analysis [140]. 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 [140].

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.

V. Limits and perspectives

Among the different techniques reviewed, each has advantages and drawbacks. Microscopy-derived techniques are semi-quantitative, implemented using small devices that can be installed 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 mechanisms underlying of common reactivity tests (i.e. Ach iontophoresis, PORH and LTH) are complex and involve several different pathways [170]. Besides deeper 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 discussed in this dissertation concerns signal processing. Indeed, cutaneous blood flow has been studied through several processing tools such as the Fourier transform and the wavelet transform [80]. Other methods such as multifractality and sample entropy have recently been applied to LDF signals [171, 172].

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 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.

Study 1. Reproducibility and methodological issues of skin post-occlusive and thermal hyperemia assessed by single-point laser Doppler flowmetry

The primary objective of this study was to determine the 1-week reproducibility of skin microvascular reactivity assessed with single-point LDF on the forearm and the finger pad. We also determined the spatial variability of simultaneous measurements, as well as the influence of mental stress and room temperature on post-occlusive and thermal hyperemia.

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Regular Article

Reproducibility and methodological issues of skin post-occlusive and thermal hyperemia assessed by single-point laser Doppler flowmetry

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ABSTRACT

Objective: The primary objective of this study was to evaluate 1-week reproducibility of post-occlusive reactive hyperemia (PORH) and local thermal hyperemia (LTH) assessed by single-point laser-Doppler flowmetry (LDF) on different skin sites. We also evaluated spatial reproducibility of both tests on the forearm. Finally, we assessed the influence of mental stress and room temperature variations on PORH and LTH.

Methods: We performed PORH and LTH assessing skin blood flow on the forearm and on the finger pad with LDF. We repeated the sequence 1 week later. We also performed PORH and LTH during mental stress (Stroop test) and at room temperatures of 21 °C and 27 °C. Data were expressed as cutaneous vascular conductance (CVC), as a function of baseline and as a function of 44 °C vasodilation (%CVC_{44}). Reproducibility was expressed as within subject coefficients of variation (CV) and intra-class correlation coefficients (ICC).

Results: Fourteen Caucasian healthy volunteers were recruited. Median age was 25 (2.7) and 50% were female. Median body mass index was 21.2 (5). PORH was reproducible on the finger, whether expressed as raw CVC (CV = 25%; ICC = 0.56) or as %CVC_{44} (CV = 24%; ICC = 0.60). However, PORH showed poor reproducibility on the forearm. In the same way, LTH was reproducible on the finger pad when expressed as CVC (CV = 17%; ICC = 0.81) but not on the forearm. Spatial reproducibility was poor on the forearm. Elevated room temperature (27 °C) affected PORH and LTH on the finger pad ($p<0.05$) but not on the forearm.

Conclusion: Single-point LDF is a reproducible technique to assess PORH and LTH on the finger pad when data are expressed as raw CVC or %CVC_{44}. On the forearm, however, it shows great inter-day variability, probably due to spatial variability of capillary density. These results highlight the need for alternative techniques on the forearm.

Introduction

Microvascular function in the skin can be routinely studied in humans using laser Doppler flowmetry (LDF), which provides an index of skin blood flow, coupled with various reactivity tests. Among them, post-occlusive reactive hyperemia (PORH) and local thermal hyperemia (LTH) are commonly used as functional markers of microvascular function (Cracowski et al., 2006). However, the mediators involved differ between these tests. Many mediators are involved in PORH. Sensory nerves are partially involved through an axon reflex response (Larkin and Williams, 1993; Lorenzo and Minson, 2007). Local mediators are also involved: endothelium-derived hyperpolarizing factors (EDHF) seems to play a major role (Lorenzo and Minson, 2007), while results are conflicting about the implication of prostaglandins. Recent work suggests that cyclo-oxygenases inhibition unmasks nitric oxide (NO) dependence of reactive hyperemia in human cutaneous circulation (Medow et al., 2007). On the other hand, the initial peak of LTH is nearly exclusively mediated by a CGRP-dependent axon reflex whereas the late plateau is primarily mediated by NO (Minson et al., 2001).

These techniques have been used as clinical surrogate markers in various diseases such as primary Raynaud's phenomenon and systemic sclerosis (Murray et al., 2006; Roustit et al., 2008a; Wigley et al., 1990) or to evaluate the effect of drugs on microcirculation (Binggeli et al., 2003; Cankar and Strucl, 2007). However, the lack of standardization of data expression limits the use of these tests in routine practice. Moreover, methodological issues compromise the reproducibility of such techniques, emphasizing the need to study the conditions in which LDF measurements are performed. Indeed, the variability of LDF recording in different physiological and environmental conditions is unknown, and is currently a limitation to its use in a clinical setting, where such conditions may not be fully controlled. Another major source of variation is the site of measurement (Cracowski et al., 2006). Indeed, different responses are observed between the forearm and the finger (Braverman, 2000), and even between different sites on the same forearm.

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Finally, the way of expressing data remains an issue; several authors use raw values whereas others scale them over baseline or 44 °C flux.

The primary objective of the present study was to determine the 1-week reproducibility of skin microvascular reactivity assessed using single-point LDF, both on the forearm and on the finger pad. Moreover, we aimed to compare different ways of expressing data in order to optimize reproducibility of the measurements. We also evaluated the spatial reproducibility of simultaneous recordings on different areas of the forearm. In order to obtain reproducibility data in the conditions of a routine use, locations of the laser Doppler probes were not marked, and variations due to menstrual cycle phases in women were taken into account in the variability. Finally, we assessed the influence of mental stress (Stroop test) and of different room temperatures on PORH and LTH assessed with single-point LDF.

Patients and methods

Study population

Healthy volunteers aged 18 years or older were recruited through local newspaper advertisements. Non-inclusion criteria included any significant medical history and tobacco smoking. Grenoble Institutional Review Board approval was obtained on November 26, 2007 and each subject gave written informed consent before participation.

Study design

Upon arrival at the laboratory, participants were placed in a temperature-controlled room (24 ± 1 °C). All participants were fasted for at least 6 h. After a 30-min acclimatization period, post-occlusive reactive hyperemia (PORH) was performed followed by local thermal hyperemia (LTH) (day 0). This sequence was repeated 1 week later (day 7), at the same hour. The Stroop test was performed on day 0, whereas room temperature was randomly set at 21 °C or 27 °C between both visits (Fig. 1). The participants were supine for the duration of the whole experiment. For each sequence (i.e. day 0, day 7, Stroop, 21 °C, 27 °C), blood pressure was recorded manually on the contralateral arm at baseline, during PORH peak, LTH peak and plateau.

Laser Doppler measurements

Two skin sites were randomly chosen on the ventral side of the upper left forearm (5 cm apart, avoiding visible veins) and the third one was on the finger pad (index finger). A vacuum cushion was used to decrease artifacts associated with arm movements. Cutaneous blood flow was measured using single-point laser Doppler flowmetry (Periflux System 5000, Perimed, Järfälla, Sweden). PORH and LTH were performed with LDF probes with integrated heaters (Probe 457, Perimed, Järfälla, Sweden). After a 5 min baseline recording, blood flow was occluded for 5 min by inflating a cuff placed on the left arm to 50 mm Hg above the systolic blood pressure. The cuff was then released and PORH was recorded as previously described (Roustit et al., 2008a). After return to baseline, LTH was induced at all sites by heating the skin at 42 °C (1 °C s^{-1}) during 30 min (heating plateau). Finally, this sequence was followed by a 5 min 44 °C heating period to achieve maximal blood flow. Probes were removed after each sequence and a 30-min rest allowed skin blood flow to decrease to baseline (Fig. 1).

Variability of LDF measurements according to room temperature and mental stress

PORH and LTH were performed on all participants at different room temperatures on two separate days (21 ± 1 °C and 27 ± 1 °C, the order of these sequences was randomly chosen) and compared with 24 °C ± 1 °C measurements. Volunteers also underwent a mental stress test, the Stroop color test (Stroop, 1935). The Stroop task measures the ability to selectively attend to the color of a word while filtering out its meaning. The Stroop is attractive for use as a laboratory stressor because of the absence of a significant learning effect and its consistent influence on autonomic reactivity (Boutcher and Boutcher, 2006). The Stroop test increases splanchnic nerve activity (Freyschuss et al., 1988), heart rate and catecholamine response (Boutcher and Boutcher, 2006). The Stroop test was performed 5 times: at baseline, during PORH, during the initial thermal peak, the 42 °C plateau and the 44 °C vasodilation. Heart rate was continuously recorded during each test. Laser Doppler measurements were performed as described above.

Data analysis

Data were digitized, stored on a computer, and analyzed off-line with signal processing software (PeriSoft 2.5.5; Perimed, Järfälla, Sweden). Data were expressed as cutaneous vascular conductance (CVC), which is the flux in mV divided by the mean arterial pressure in mm Hg. Indeed, expressing data as CVC is a more physiological approach, as it takes into account differences and variations in blood pressure (O'Leary, 1991).

As there is no consensus about PORH data expression, the amplitude of the response was determined by raw peak CVC, raw peak CVC minus baseline CVC (PK-BL), the percentage increase between peak and baseline (PK%BL) and the area under curve (AUC) (Fig. 2A). We also expressed peak hyperemia as a percentage of CVC after heating at 44 °C (%CVC_{44}), i.e. 44 °C thermal plateau CVC, as previously described (Lorenzo and Minson, 2007). The kinetics of the response was determined by the time to peak (time from cuff release to peak hyperemia, in seconds).

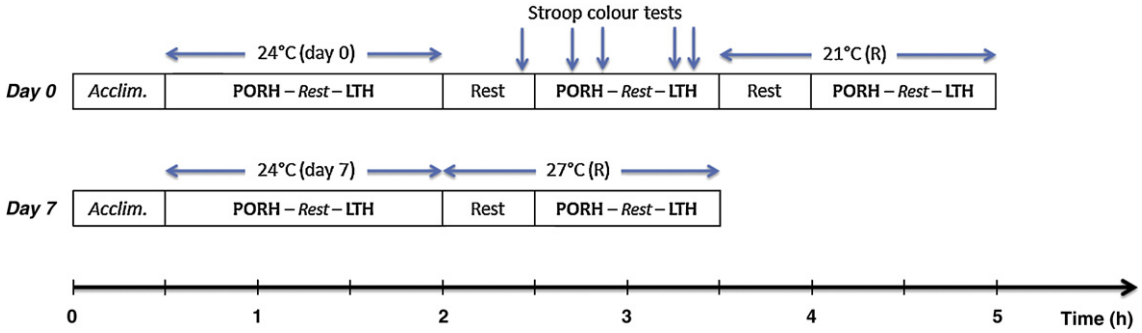

Fig. 1. The study design included two visits, 7 days apart. One week reproducibility of post-occlusive reactive hyperemia (PORH) and local thermal hyperemia (LTH) was assessed between two measurements recorded at the same hour at 24 °C. The Stroop color test was performed on day 0 at 24 °C. Afterward, room temperature was randomly set at 21 °C or 27 °C between day 0 and day 7. Acclim, acclimatization; R, randomized sequences.

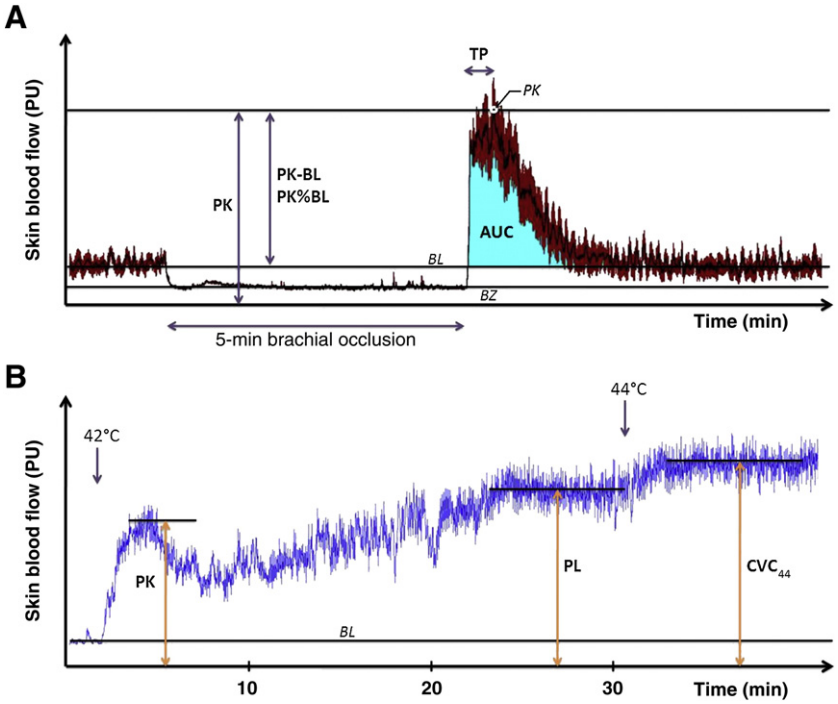

Fig. 2. Examples of post-occlusive reactive hyperemia (PORH) and local thermal hyperemia (LTH) performed on the forearm. (A) PORH data are expressed as peak raw value (PK), peak minus baseline (PK-BL), the percentage increase between peak and baseline (PK%BL), the area under curve (AUC) and the percentage of vasodilation to 44 °C local heating (B, CVC_{44}). The kinetics of the PORH response was determined by the time to peak (TP) hyperemia (time from cuff release to peak hyperemia, in seconds). BL, baseline; BZ, biological zero. (B) LTH data are expressed as peak raw value (PK), plateau raw value (PL), as a percentage of baseline (BL) or as a percentage of 44 °C vasodilation (CVC_{44}).

The amplitude of LTH was determined by initial raw peak CVC and 42 °C plateau CVC (Fig. 2B). Skin blood flux values were averaged over 1 min for the initial peak, and over 3 min for the 42 °C plateau and the CVC_{44}. We also expressed LTH peak and plateau as percentages of baseline. Finally, peak was scaled to 44 °C vasodilatation, as previously described (Roustit et al., 2008b).

To assess the spatial reproducibility of simultaneous recordings on the forearm, data from the two sequences at 24 °C (day 0 and day 7) were pooled.

Statistical analysis

Quantitative data are expressed as the median and interquartile in parenthesis. Quantitative data were analyzed with the Friedman test and with the Wilcoxon test for paired analyses, with each subject serving as his/her own control. We considered p values <0.05 as significant, corrected by Bonferroni's method for multiple comparisons. Reproducibility was expressed as within subject coefficients of variation (CV) (Bland, 2000; Donald et al., 2008; Roustit et al., 2010) CV <35% were deemed acceptable (Harris et al., 2007). Repeatability of measurements was expressed as intra-class correlation coefficients (ICC) (Bland, 2000). ICC values of <0.40, 0.40 to 0.75 and >0.75 represent poor, fair to good and excellent agreements, respectively (Landis and Koch, 1977).

Results

Study population

Fourteen Caucasian healthy volunteers were recruited in the study. Median age was 25 (2.7) and 50% were female, all taking oral contraceptives. Median body mass index was 21.2 (5). Resting systolic/diastolic blood pressure was 124 (12)/69 (19) mm Hg. All subjects had normal lipids profile and normal blood cell count.

Post-occlusive reactive hyperemia

One-week reproducibility of PORH amplitude was good on the finger pad when data were expressed as peak CVC or as $\%CVC_{44}$ (Table 1). The kinetics of the response (time to peak) was also reproducible (CV = 26%; ICC = 0.93) on the finger pad. However, reproducibility of PORH was poor on the forearm, whatever the way of expressing data.

Table 1
Reproducibility of post-occlusive reactive hyperemia (PORH) on the finger pad and the forearm between day 0 (D0) and day 7 (D7).

		D0	D7	CV	ICC
Peak CVC	Finger pad	28.47 (14.7)	37.63 (16.6)	**25**	**0.56**
	Forearm	3.66 (3.1)	5.83 (2.5)	45	0.26
PK-BL	Finger pad	18.18 (10.5)	19.49 (13.8)	45	0.08
	Forearm	3.12 (2.5)	4.6 (2.3)	48	0.29
PK%BL	Finger pad	282.76 (403.6)	242.60 (330.3)	63	0.52
	Forearm	413.5 (297)	509.2 (291.6)	38	0.33
AUC	Finger pad	13,149.3 (14,772)	8932.1 (8669)	48	0.23
	Forearm	5806.7 (6903)	1631.8 (1436)	89	0.13
$\%CVC_{44}$	Finger pad	128.3 (54)	138.6 (62)	**24**	**0.60**
	Forearm	64 (58)	61.5 (57)	41	0.62

Data are expressed as raw values, as a function of 44 °C vasodilation or as a function of baseline.
PORH amplitude at D0 and D7 is expressed as median (interquartile) peak cutaneous vascular conductance (Peak CVC, in mV/mm Hg), peak CVC minus baseline CVC (PK-BL), peak as a percentage of baseline (PK%BL), area under the curve (AUC, in PU s) and percentage of 44 °C vasodilation ($\%CVC_{44}$). Variability between D0 and D7 is expressed as within subject coefficients of variation (CV, in %). Repeatability is expressed as intra-class coefficients of correlation (ICC). CV<35% were deemed acceptable (Harris et al., 2007). ICC values of <0.40, 0.40 to 0.75 and >0.75 represent poor, fair to good and excellent agreements, respectively (Landis and Koch, 1977). Results appearing in bold fulfill both conditions.

Table 2
Reproducibility of local thermal hyperemia (LTH) on the finger pad and the forearm between day 0 (D0) and day 7 (D7).

		D0	D7	CV	ICC
Peak (CVC)	Finger pad	34.04 (22.29)	38.57 (22.01)	**17**	**0.81**
	Forearm	6.88 (5.4)	6.94 (2.63)	57	0.72
Plateau (CVC)	Finger pad	25.71 (23.81)	26.94 (21.86)	**25**	**0.78**
	Forearm	11.04 (9.8)	9.17 (9.11)	40	0.71
Peak (%CVC_{44})	Finger pad	117.62 (29.4)	124.30 (55.9)	24	0.28
	Forearm	69.99 (15.3)	76.28 (31.9)	**19**	**0.55**
Peak (%BL)	Finger pad	276.56 (535.8)	301.67 (392.4)	**34**	**0.84**
	Forearm	714.70 (488.9)	592.32 (683.3)	87	0.07
Plateau (%BL)	Finger pad	215.78 (177.4)	154.02 (304.9)	50	0.74
	Forearm	946.98 (1709.2)	644.04 (1211.4)	92	0.04

Data are expressed as raw values, as a function of 44 °C vasodilation or as a function of baseline.
Thermal peak and plateau data at D0 and D7 are expressed as median (interquartile) cutaneous vascular conductance (CVC, in mV/mm Hg), as a percentage of 44 °C vasodilation (%CVC_{44}) and as a percentage of baseline (%BL). Variability between D0 and D7 is expressed as within subject coefficients of variation (CV, in %). Repeatability is expressed as intra-class coefficients of correlation (ICC). CV<35% were deemed acceptable (Harris et al., 2007). ICC values of <0.40, 0.40 to 0.75 and >0.75 represent poor, fair to good and excellent agreements, respectively (Landis and Koch, 1977). Results appearing in bold fulfill both conditions.

Local thermal hyperemia

Reproducibility of LTH was good on the finger pad, for peak and plateau, when expressed as raw CVC (Table 2). Other ways of expressing data, however, were poorly reproducible for both the finger pad and the forearm. Overall reproducibility of LTH on the forearm was poor.

Spatial variability of LDF measurements on the forearm

Spatial reproducibility results on the forearm are summarized in Table 3. We observed poor reproducibility of PORH expressed either as raw CVC, as a function of baseline or scaled to CVC_{44}. Spatial reproducibility of LTH was good only when expressed as %CVC_{44} for both the initial peak and the late plateau.

Table 3
Spatial variability of simultaneously recorded post-occlusive reactive hyperemia (PORH) and thermal hyperemia (LTH) on two sites of the same forearm.

		Site 1	Site 2	CV	ICC
PORH	Peak CVC	5.25 (3.17)	5.78 (4.34)	47	0.12
	PK-BL	4.35 (2.7)	4.85 (3.96)	55	0.54
	PK%BL	467.27 (285.81)	533.07 (325.62)	45	0.55
	%CVC_{44}	62.85 (49.88)	56.41 (35.82)	86	0.27
	AUC	1465.65 (384.9)	1731.3 (777.33)	62	0.34
LTH	Peak CVC	6.94 (3.36)	6.89 (7.2)	53	0.34
	Plateau CVC	10.33 (9.7)	11.13 (9.87)	46	0.57
	Peak %CVC_{44}	71.61 (31.14)	67.43 (28.44)	**25**	**0.67**
	Peak %BL	671.48 (706.16)	618.84 (655.08)	69	0.29
	Plateau %BL	814.11 (1247.19)	892.56 (1271.53)	58	0.57

Data are expressed as raw values, as a function of 44 °C vasodilation or as a function of baseline.
PORH amplitude is expressed as median (interquartile) peak cutaneous vascular conductance (Peak CVC, in mV/mm Hg), peak CVC minus baseline CVC (PK-BL), peak as a percentage of baseline (PK%BL), area under the curve (AUC, in PU s) and percentage of 44 °C vasodilation (%CVC_{44}). Thermal peak and plateau data are expressed as median (interquartile) cutaneous vascular conductance (CVC, in mV/mm Hg), as a percentage of 44 °C vasodilation (%CVC_{44}) and as a percentage of baseline (%BL). Variability between D0 and D7 is expressed as within subject coefficients of variation (CV, in %). Repeatability is expressed as intra-class coefficients of correlation (ICC). CV<35% were deemed acceptable (Harris et al., 2007). ICC values of <0.40, 0.40 to 0.75 and >0.75 represent poor, fair to good and excellent agreements, respectively (Landis and Koch, 1977). Results appearing in bold fulfill both conditions.

Variability of LDF measurements according to room temperature or mental stress

Physiological and environmental variations of PORH and LTH were assessed in all volunteers. Elevated room temperature (27 °C) significantly influenced PORH on the finger pad (Table 4), increasing both baseline and peak CVCs. However, the effect was greater on baseline flux, resulting in significantly decreased PK%BL ($p<0.01$). Lowered room temperature (21 °C) tended to decrease PORH baseline and peak CVCs, but we did not show a significant difference compared to the 24 °C recordings. Similar results were observed for LTH, with significantly increased baseline, peak, plateau and CVC_{44} at 27 °C on the finger pad. Room temperature had no significant effect on PORH and LTH performed on the forearm.

Finally, PORH and LTH were not influenced by the Stroop test, although mental stress had a significant effect on heart rate for all sequences, with no obvious habituation effect. The heart rate was 65 (6) and 79 (16) beats/min at baseline, 65 (5) and 82 (14) during PORH, 64 (5) and 82 (13) at thermal peak, 64 (6) and 83 (12) at plateau, 67 (8) and 81 (10) at CVC_{44} ($p<0.01$ for all sequences).

Discussion

In the present study, we show that PORH and LTH assessed with single-point LDF are reproducible on the finger pad but not on the forearm. We further suggest that the lack of reproducibility on the forearm is related to spatial variability. Finally, we showed no influence of mental stress on PORH and LTH whereas we observed a significant influence of room temperature when performing PORH and LTH on the finger pad, but not when on the forearm.

Laser Doppler flowmetry, when coupled with reactivity tests, has been widely used to study skin microvascular function. One of the most commonly used of these tests is PORH. However, there is a lack of standardization of several methodological issues such as the length of artery occlusion or the position of the cuff (Cracowski et al., 2006). Moreover, data expression still remains controversial and looking at the literature clearly shows there are many ways of expressing PORH amplitude. Indeed, some authors have used peak raw values expressed as millivolts (Gomes et al., 2008; Zhao et al., 2004) or as CVC (Roustit et al., 2008a; Yvonne-Tee et al., 2005). PORH amplitude may also be expressed as %CVC_{44} (Lorenzo and Minson, 2007) or as a function of baseline flux, i.e. PK-BL (Roustit et al., 2008a; Yvonne-Tee et al., 2005), PK%BL (Binggeli et al., 2003; Yvonne-Tee et al., 2005), as AUC (Gomes et al., 2008; Wong et al., 2003) or a ratio of AUC before and after cuff inflation (Yamamoto-Suganuma and Aso, 2009). Finally, some groups have expressed PORH amplitude as a function of LDF signal during occlusion, also called biological zero (Morales et al., 2005). In the present study, we have assessed the reproducibility and repeatability of PORH using most of these ways of expressing data. We did not express PORH as a function of the biological zero, however, as subtracting the biological zero from our results worsened PORH reproducibility (data not shown for clarity).

Most of the published data assessing skin blood flow in humans focus on the forearm, which is more accessible and more representative of general skin microvascular function than the finger pad. Moreover, basal skin blood flow is supposed to be more variable on the finger pad, probably due to arteriovenous communications (Braverman, 2000). However, our results show poor reproducibility on the forearm whatever the way of expressing data. As we did not use markers to identify recording sites on the skin, this discrepancy may be explained by the spatial variation due to a wide disparity in skin capillary density (Cracowski et al., 2006; Johnson et al., 1984). The poor spatial reproducibility of simultaneously performed PORH on the same forearm strengthens this hypothesis. Conversely, PORH amplitude on the finger pad was reproducible when expressed as peak raw CVC or as %CVC_{44}. This may be due to easier positioning of

Table 4
Physiological variations of post-occlusive reactive hyperemia (PORH) and thermal hyperemia (LTH) assessed with LDF measurements according to room temperature or mental stress.

			24 °C (control)	Stroop	21 °C	27 °C	p
PORH	Baseline CVC	Finger pad	7.01 (18.92)	4.54 (5.3)	3.86 (3.7)	23.88* (13.59)	<0.01
		Forearm	0.94 (0.1)	1.02 (0.7)	0.86 (0.4)	1.27 (0.9)	NS
	Peak CVC	Finger pad	28.47 (14.7)	34.23 (15.05)	20.12 (10.6)	42.62* (16.3)	<0.01
		Forearm	3.66 (3.1)	6.41 (2.77)	4.25 (3.5)	8.42 (3.8)	NS
	PK-BL	Finger pad	18.18 (10.5)	26.74 (10.5)	15.78 (6.1)	21.42 (13.2)	NS
		Forearm	3.12 (2.5)	5.06 (2.3)	3.47 (3.1)	4.97 (2.7)	NS
	PK%BL	Finger pad	282.76 (403.6)	566.15 (409.5)	499.70 (672.8)	107.39 (55.6)	<0.01
		Forearm	413.5 (297)	502.94 (328.4)	453.33 (334)	393.77 (305.6)	NS
	AUC	Finger pad	13,149.35 (14,772.7)	18,818.8 (39,889)	9308.8 (8887)	13,493.1 (5179.9)	NS
		Forearm	5806.7 (6903)	1739.45 (927.5)	1202.60 (969.1)	1247.90 (1891.6)	NS
	$\%CVC_{44}$	Finger pad	128.27 (53.9)	112.8 (66.3)	96.6 (77.3)	116.2 (23.5)	NS
		Forearm	64 (58.2)	50.5 (21.9)	43.67 (31)	59.1 (33.9)	NS
LTH	Baseline CVC	Finger pad	11.46 (11.98)	7.41 (14.55)	3.08* (4.73)	27.45* (17.7)	<0.01
		Forearm	0.83 (1.02)	0.94 (0.28)	0.69 (0.52)	1.14 (0.76)	NS
	Peak CVC	Finger pad	34.04 (22.29)	35.88 (18.9)	32.58 (26.51)	52.97* (17.9)	<0.05
		Forearm	6.22 (6.39)	9.28 (5.55)	6.9 (5.31)	9.66 (2.83)	NS
	Plateau CVC	Finger pad	25.71 (23.81)	26.38 (20.36)	24.48 (17.65)	38.58* (23.06)	<0.05
		Forearm	10.89 (13.24)	12.27 (9.86)	10.04 (7.03)	11.98 (4.47)	NS
	CVC_{44}	Finger pad	28.48 (21.65)	32.43 (15)	29.83 (15.49)	43.47* (23.64)	<0.05
		Forearm	12.25 (15.32)	13.80 (10.12)	11.48 (5.87)	13.48 (6.23)	NS

PORH amplitude is expressed as median (interquartile) peak cutaneous vascular conductance (Peak CVC, in mV/mm Hg), peak CVC minus baseline CVC (PK-BL), peak as a percentage of baseline (PK%BL) and area under the curve (AUC, in PU s). LTH data are expressed as median (interquartile) cutaneous vascular conductance (CVC, in mV/mm Hg). *p*-values in the last column refer to comparison between all measurements (Friedman test). Values <0.05 were considered as significant and then paired comparisons were calculated (Wilcoxon rank test).

* $p<0.05$ vs. 24 °C control. NS, not significant.

the probe on the finger pad. Skin blood flow presents certain specificities on the finger pad, i.e. baseline CVC is more elevated and depends highly on temperature, which was confirmed by the present work. As a consequence, the poor 1-week reproducibility observed when expressing data as a function of baseline may be explained by the variations in baseline flux. Thus, we shall recommend expressing PORH amplitude as raw CVC or $\%CVC_{44}$ rather than as a function of baseline on the finger pad.

Thermal hyperemia is another reactivity test which, coupled to LDF, allows assessment of axon reflex-dependent (initial peak) and NO-dependent (late plateau) skin vasodilation (Minson et al., 2001). On the finger pad, the reproducibility for both peak and plateau was correct when data were expressed as raw CVC values, but worsened when expressed as percentages of baseline. This further supports the idea that most of the finger pad variability relies on baseline flux. On the forearm, however, we showed poor 1-week reproducibility of all measurements except the initial peak expressed as $\%CVC_{44}$. This index could be a useful tool to study the initial axon reflex-dependent vasodilation. We have previously used these methods to investigate skin neurovascular function in patients with systemic sclerosis (Roustit et al., 2008b). Room temperature and mental stress had no effect on LTH when performed on the forearm. However, as previously discussed for PORH, we observed great variability when comparing simultaneously recorded LTH on the same forearm, suggesting preponderant spatial variability in the poor one-week reproducibility.

We used within subject coefficients of variation (CV) to express the reproducibility of our data, as previously described (Bland, 2000; Donald et al., 2008; Harris et al., 2007). In order to assess the reliability of the measurement, we also calculated intra-class correlation coefficients (ICC) (Bland, 2000). We considered that CV<35% were deemed acceptable, as previously described (Harris et al., 2007). However, to our knowledge, there is no well-established threshold below which within-subject CV are good. In the present study, some of our results show CVs between 25 and 35%, which may be considered as borderline. On the other hand, ICC>0.75 represent excellent agreements (Landis and Koch, 1977). Although these parameters have been used in other studies with comparable sample sizes (Svalestad et al., 2010; Yvonne-Tee et al., 2005), we shall note that our study may be underpowered when considering low ICCs. However, for expected ICCs of 0.75, we obtain a distance from correlation to limit of the 95% confidence interval ω of 0.238 with $n=14$, which remains within the range of fair to good agreements (Landis and Koch, 1977). Using the same parameters, another group showed comparable CV (33%) but lower ICC (0.31) when heating locally on the cheek of ten healthy volunteers (Svalestad et al., 2010). Agarwal et al. found comparable CVs for PORH ($n=10$) but lower CVs for LTH on the forearm (ranging from 9 to 38%, depending on data expression). However, the heating protocol was different: baseline temperature was normalized to 34 °C, heating rate was slow (0.1 °C s^{-1}, ours was 1 °C s^{-1}) and the authors used lidocaine/prilocaine pre-treatment to avoid axon reflex (Agarwal et al., 2010). On the contrary, we assessed the reproducibility of the initial axon reflex-dependant peak. Another group has assessed reproducibility of PORH on the forearm ($n=18$) and showed dramatically lower CVs and better ICCs than we did in the present study (Yvonne-Tee et al., 2005). Nonetheless, the recording conditions were very different. First, they marked the locations of the laser probes to study exactly the same sites from one day to another to get around spatial variability. Indeed, as we discussed above, variation in capillary density between different skin sites is probably the major source of variability using LDF (Braverman, 2000). However, identifying skin sites on which to perform measurements precisely in the same area over time is not easy in routine use. We thus deliberately placed the probes on random sites on the forearm. Moreover, we did not test spatial variability on the finger pad, as positioning is less variable than on the forearm due to the anatomy of the digits. Therefore, the better reproducibility we observed on the finger pad may be due to lower heterogeneity in capillary density.

The present study reveals that further work is needed to optimize reproducibility of PORH and LTH assessment, especially on the forearm. In order to do so, some authors have standardized baseline skin temperature at 33 °C (Minson et al., 2001) while recording with LDF. In the present study, we used a temperature controlled room, but we need to further test whether baseline skin temperature normalization improves reproducibility. Integrating LDF probes may also reduce spatial variability by processing an integrated signal taken as the average flow value from seven different scattering volumes simultaneously. We did not use such probes in the present study

because they are too large to be used on the finger pad. On the forearm, however, they could be appropriate. Another way of getting around spatial variability would be to assess skin blood flow over wider skin areas, which is possible with techniques such as laser Doppler imaging (LDI). However, the kinetics of PORH makes the use of LDI difficult. Recently developed instruments based on laser speckle contrast imaging (LSCI) may be able to overcome this problem. To our knowledge, however, there are no data about the reproducibility of such techniques.

Besides the issue of the heterogeneity of capillary density, we recruited both young males and females, which may increase variability. A limitation of our study is that we included young women in different menstrual phases. Moreover, the study design (measurements at day 0 and day 7) implied different hormonal states for the measurements. Again, this was deliberately done in order to meet real life conditions when comparing different subgroups of subjects or following a patient over time. Yet, menstrual phase does not dramatically influence PORH amplitude (Yvonne-Tee et al., 2008). However, recent work has shown that oral contraceptives influence endothelial function assessed with flow-mediated dilation of the brachial artery (Torgrimson et al., 2007). The population of our study does not allow for subgroup analysis. In order to test whether female hormones influence PORH response, a larger specific study is therefore required.

Part of the present work shows the significant influence of elevated (27 °C) room temperature on skin blood flow for both tests. Of interest, baseline and PORH peak CVC were both affected, baseline flux being more dramatically increased by temperature. Lowered room temperature (21 °C) tended to decrease skin blood flow but to a lesser extent. The difference between 21 °C and 24 °C did not reach significance, probably because of a lack of power. Of interest, room temperature did not affect PORH on the finger pad when expressed as $\%CVC_{44}$, which was shown to be reproducible. Baseline LTH, however, was significantly modified by both lowered and elevated room temperatures on the finger pad, whereas the peak and the plateau were only affected by elevated temperature, suggesting a potentiating effect of systemic heating on the local heating response at the finger pad. Such findings confirm that the 44 °C thermal plateau is actually not the maximal CVC on the finger pad, as previously described (Roustit et al., 2008b). Overall, these results emphasize the need for controlling room temperature while performing PORH and LTH on the finger pad.

The Stroop test did not influence LDF measurements significantly. However, baseline flux tended to be lower while the Stroop test was being performed, which may be related to increased basal sympathetic activity during the test. This is consistent with increased heart rate during all sequences. However, such a tendency was not obvious for PORH or LTH peak and plateau.

When coupled to LDF, PORH and LTH are both easy to perform and non-invasive tests able to discriminate healthy subjects from patients. For example, they have been the object of particular interest to assess microvascular dysfunction in several diseases like diabetes (Caselli et al., 2006; Gomes et al., 2008), hypercholesterolemia (Binggeli et al., 2003), peripheral arterial obstructive disease (Morales et al., 2005) or pathologies affecting the digits such as primary Raynaud's phenomenon and systemic sclerosis (Murray et al., 2006; Rajagopalan et al., 2003; Roustit et al., 2008b; Salvat-Melis et al., 2006). However, the skin sites studied vary among these pathologies, and the specificities of skin blood flow at each site should be considered so as to choose the most appropriate way of expressing data.

In conclusion, single-point laser Doppler flowmetry is a reproducible technique to assess PORH and LTH on the finger pad. Data should preferentially be expressed as raw CVC for LTH and PORH or as a percentage of CVC_{44} for PORH. Room temperature must be carefully controlled. On the forearm, however, single-point LDF coupled with PORH or LTH should not be used because of great inter-day variability, probably due to the spatial variability of capillary density. This issue may be addressed by (1) normalizing baseline skin temperature or (2) assessing blood flux in larger skin areas to attenuate the influence of capillary density variation between two different sites. Integrated LDF probes, laser Doppler imaging or the recently developed laser speckle contrast imaging could therefore be useful tools. However, further studies are needed to assess the reproducibility of these techniques.

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Study 2. Excellent reproducibility of laser speckle contrast imaging to assess skin microvascular reactivity.

In the continuation of the previous study, the objective of this work was to determine the 1-week reproducibility of skin microvascular reactivity assessed with single-point LDF homogenizing baseline skin temperature. We also determined the reproducibility of full-field techniques, i.e. LDI and LSCI.

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Regular Article

Excellent reproducibility of laser speckle contrast imaging to assess skin microvascular reactivity

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ABSTRACT

Objective: We compared the inter-day reproducibility of post-occlusive reactive hyperemia (PORH) assessed by single-point laser Doppler flowmetry (LDF) and laser speckle contrast analysis (LSCI), and the reproducibility of local thermal hyperemia (LTH) assessed by LDF, laser Doppler imaging (LDI) and LSCI. We also tested whether skin blood flow assessment by LDF and by LSCI are correlated.
Methods: Skin blood flow was evaluated during PORH and LTH using LDF, LDI (for LTH only) and LSCI on the forearms of healthy volunteers, at a 7 day interval. Data are expressed as cutaneous vascular conductance (CVC), as a function of baseline and scaled to the thermal plateau. Reproducibility is expressed as within subject coefficients of variation (CV, in %) and intra-class correlation coefficients (ICC).
Results: Twenty-eight healthy participants were enrolled in this study. The reproducibility of the PORH peak CVC was better when assessed with LSCI compared to LDF (CV = 8%; ICC = 0.76 and CV = 30%; ICC = 0.54, respectively). Inter-day reproducibility of the LTH plateau was better when assessed with LSCI or LDI than LDF (CV = 15%, ICC = 0.66; CV = 17%, ICC = 0.51 and CV = 42%, ICC = 0.28 respectively). Finally, we observed significant correlation between simultaneous LDF and LSCI measurements of the PORH peak CVC (R = 0.54; $p = 0.001$).
Conclusion: The recently developed LSCI technique showed very good inter-day reproducibility for assessing PORH and LTH. Moreover, we showed significant correlation between LSCI and single-point LDF for PORH. However, more data are needed to evaluate the linearity between the LSCI signal and skin blood flow.

Introduction

Laser Doppler flowmetry (LDF) and laser Doppler imaging (LDI) have been widely used to assess skin microvascular function when coupled with various reactivity tests. Among them, post-occlusive reactive hyperemia (PORH) and local thermal hyperemia (LTH) are commonly used as markers of microvascular function (Cracowski et al., 2006).

These techniques have been used as clinical surrogate markers in diseases such as primary Raynaud's phenomenon and systemic sclerosis (Murray et al., 2006; Roustit et al., 2008; Wigley et al., 1990) and to evaluate the effect of drugs on microcirculation (Binggeli et al., 2003; Cankar and Strucl, 2008). However, the lack of standardization in data expression limits the use of these tests in routine practice.

Nowadays, LDF is commonly used to assess skin blood flow. However, we recently showed that the inter-day reproducibility of single-point LDF was acceptable on the finger pad but poor on the forearm (Roustit et al., 2010). Our data suggest that this problem is related to the spatial variability of single-point LDF measurements on the forearm. Indeed, different responses are observed between the forearm and the finger (Braverman, 2000), and between different sites on the same forearm. Therefore, for routine assessment of skin blood flow, the spatial variability between different sites on the forearm raises the issue of reproducibility. Several techniques may be considered to address this issue and to improve reproducibility. As temperature plays a key role in baseline flux, homogenizing skin temperature when performing microvascular reactivity tests could improve reproducibility, especially when expressing data as a function of baseline.

Another way of getting around spatial variability could be to evaluate skin blood flow over wider areas by using LDI, which is suitable for the measurement of LTH. LDI also allows PORH to be measured over small skin areas. However, it is too slow to accurately assess the kinetics of PORH over larger areas, which limits its interest, i.e. full-field evaluation of skin blood flow.

Laser speckle contrast imaging (LSCI) is a technique based on speckle contrast analysis that provides an index of blood flow (Boas and Dunn, 2010; Briers, 2001; Draijer et al., 2009). It has been recently used to assess skin blood flow in patients with microvascular diseases,

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such as systemic sclerosis (Murray et al., 2009). Recently developed high frame rate LSCI allows continuous assessment of skin perfusion over wide areas, and could combine the advantages of both LDF and LDI. However, to our knowledge, there is no data about the reproducibility of LTH and PORH measurements using LSCI.

The primary objective of the present study was to describe and compare the inter-day reproducibility of PORH and LTH assessment on the forearm using LDF (homogenizing baseline skin temperature), LDI and LSCI. A secondary objective was to test whether skin blood flow assessment by LDF and by LSCI are correlated.

Patients and methods

Study population

Male or female healthy volunteers, aged 18 years or older were recruited through local newspaper advertisements. Non-inclusion criteria included any significant medical history and cigarette smoking. Institutional Review Board approval was obtained on March 4th 2009 (Grenoble) and each subject gave written informed consent before participation.

Protocol 1: Inter-day reproducibility of post-occlusive reactive hyperemia and local thermal hyperemia assessed by LDF

Upon arrival at the laboratory, subjects were placed in a temperature-controlled room (24 ± 1 °C). All subjects were fasted. Skin temperature was kept constant at 33 °C, as previously described (Minson et al., 2001). After a 30-min acclimatization period, post-occlusive reactive hyperemia (PORH) was performed followed by local thermal hyperemia (LTH) (day 0). This sequence was repeated 1 week later (day 7). The subjects were supine for the whole duration of the experiments. Blood pressure was recorded continuously (Nexfin monitor, Bmeye B.V., Amsterdam, The Netherlands) during skin blood flow measurements.

Two skin sites, 5 cm apart, were randomly chosen on the ventral side of the left upper forearm, more than 5 cm from the elbow and the prominence of the wrist, avoiding visible veins. A vacuum cushion was used to decrease artifacts associated with arm movements. Cutaneous blood flow was measured using laser Doppler flowmetry (Periflux System 5000, Perimed, Järfälla, Sweden). Laser wavelength was 780 nm and processing frequency bandwidth was 20 Hz to 15 kHz. PORH and LTH were performed using LDF probes with integrated heaters (Probe 457, Perimed). After a 5 min baseline recording, blood flow was occluded for 5 min by inflating a cuff placed on the left arm to 50 mmHg above the systolic blood pressure. The cuff was then released and PORH was recorded. After return to baseline, LTH was induced by heating the skin to 42 °C (1 °C s^{-1}) during 30 min. Finally, this sequence was followed by heating for 5 min at 44 °C to achieve maximal plateau blood flow ($CVC_{plateau}$).

Protocol 2: Reproducibility of local thermal hyperemia assessed by LDI

All the volunteers included in protocol 1 were enrolled in this distinct protocol that was performed on different days. Recordings of protocol 2 were carried out in the same conditions as described above. After a 30-min acclimatization period, LTH was performed on two skin sites randomly chosen on the ventral side of the forearm and equipped with SHP3 heating probes (SH02 Skin Heating Unit, Moor Instruments, Axminster, UK) filled with water at 33 °C. Skin blood flow was recorded with a laser Doppler imager (PeriScan PIM3 System, Perimed). Laser wavelength was 650–690 nm and processing frequency bandwidth was 50 Hz to 15 kHz. The laser head was placed 20 cm above the skin and the resolution was set at 1 mm step length. Frame area was 18 cm^2. After a 10 min baseline recording, skin temperature was set at 43 °C during 40 min. Scans were performed above both heating probes every minute. Blood pressure was recorded continuously as described above. This protocol was repeated 7 days later.

Protocol 3: Inter-day reproducibility of post-occlusive reactive hyperemia and local thermal hyperemia assessed by LSCI

Recordings for protocol 3 were performed in the same conditions as described above. One skin site was chosen on the forearm and equipped with a SHP3 heating probe filled with water at 33 °C. After a 30-min acclimatization period, PORH was performed followed by LTH, as described above. Skin blood flow was recorded throughout the whole experiment using a high frame rate laser speckle contrast imager (PeriCam PSI System, Perimed). Laser wavelength was 785 nm. The laser head was placed 14 cm above the skin (with a resolution of approximately 6944 pixels/cm^2). The image acquisition rate was 8 s^{-1} and frames were 81 cm^2.

Protocol 4: Comparison between post-occlusive reactive hyperemia simultaneously assessed by LSCI and by single-point LDF.

Some of the participants enrolled in protocol 3 were included in protocol 4 to test whether LDF and LSCI flux values were correlated. Besides the site equipped with the SHP3 heating probe, three other sites were equipped with single-point LDF probes on the forearm (Probe 457, Perimed) (Fig. 1), within the LSCI scanning area.

Data analysis

Data were digitized, stored on a computer, and analyzed off-line with signal processing software (PeriSoft 2.5.5 for LDF measurements, LDPIwin 3.1.2 for LDI measurements and PimSoft 1.1.1 for LSCI measurements; Perimed). Data were expressed as cutaneous vascular conductance (CVC) (O'Leary, 1991).

The amplitude of PORH assessed by single-point LDF was expressed as maximal CVC (peak), peak CVC minus baseline CVC (PK-BL), percentage increase between peak and baseline (PK%BL), area under curve (AUC) and peak as a percentage of CVC after heating at 44 °C ($\%CVC_{plateau}$), as previously described (Roustit et al., 2010). Amplitude of PORH assessed by LSCI was expressed as peak CVC, PK-BL, PK%BL and $\%CVC_{plateau}$.

The amplitude of LTH assessed by single-point LDF was expressed as raw peak and plateau CVCs, as a percentage of baseline and peak was scaled to late plateau hyperemia ($\%CVC_{plateau}$) plateau, as previously described (Roustit et al., 2010). Skin blood flux values were averaged over 1 min for the initial peak, and over 3 min for the plateau. The local heating protocol was different when using LDI and LSCI, because of the slow heating rate of SHP3 probes (4 °C min^{-1}). Indeed, we set the temperature at 43 °C, which is not painful, although in our experience, patients with systemic sclerosis experience pain with fast heating (i.e. 1 °C s^{-1}) directly to 43 °C or 44 °C. The amplitude of the response was expressed as mean CVC over 0.8 cm^2 circular regions of interest (ROIs), inside the SHP3 heating probes. Maximal CVC in the first ten minutes was set as the peak, and the plateau was averaged between the 35th and the 40th minutes after heating onset.

In protocol 4, three ROIs were chosen adjacent to the LDF probes (Fig. 1). We compared each PORH recorded using single-point LDF (skin temperature was homogenized to 33 °C) with the adjacent ROI measured simultaneously by LSCI.

To compare simultaneous recordings on the forearm, data from the two sequences (day 0 and day 7) were pooled.

Statistical analysis

Quantitative data are expressed as the median and interquartile in parenthesis. Reproducibility is expressed as within-subject coefficients of variation (CV) (Bland, 2000; Donald et al., 2008); CV < 35% were deemed

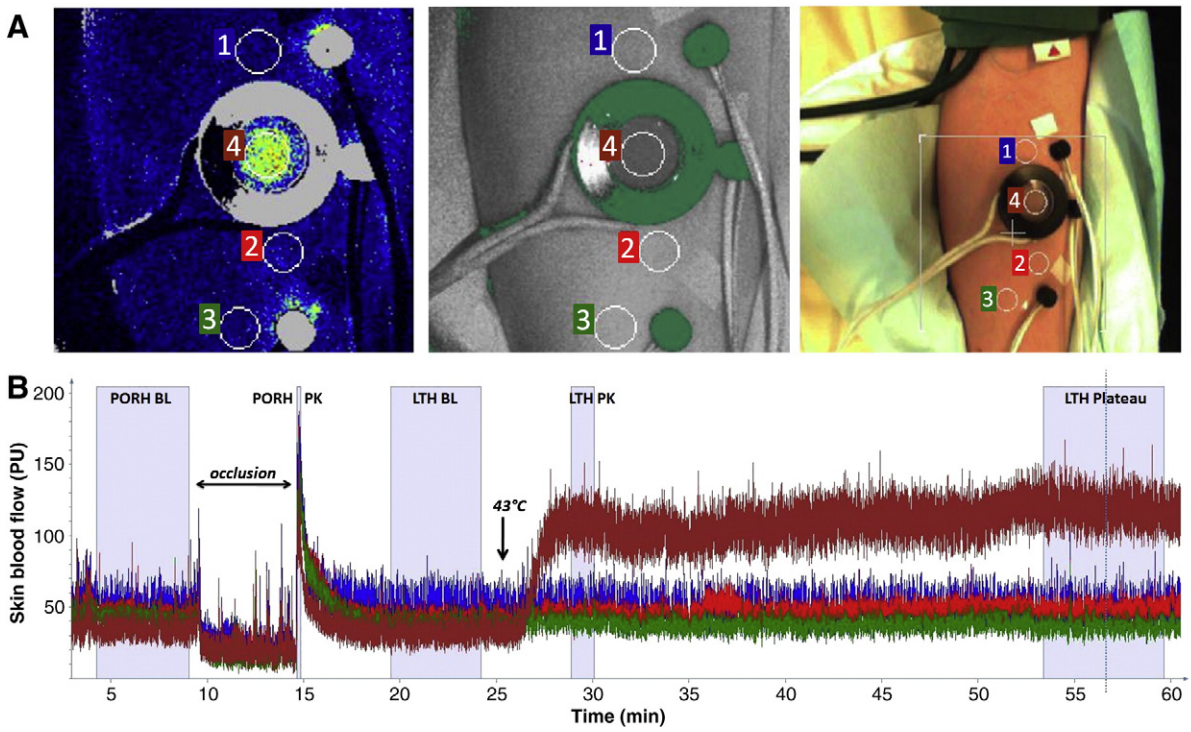

Fig. 1. (A) Laser speckle contrast imaging (LSCI) estimated perfusion (left; from low perfusion in black to high perfusion in red), intensity (middle) and picture (right) on the forearm. Regions of interests (ROIs) were adjacent to the LDF probes (numbered 1–3) and within the SHP3 heating probe (4). (B) Flux pattern recorded with LSCI for the 4 ROIs during the five periods analyzed: baseline before post-occlusive hyperemia (PORH BL), PORH peak (PORH PK), baseline before LTH (LTH BL), LTH peak (LTH PK) and LTH plateau. PU: perfusion units.

acceptable (Harris et al., 2007). Repeatability of measurements was expressed as intra-class correlation coefficients (ICC) (Bland, 2000). ICC values of <0.40, 0.40 to 0.75 and >0.75 represent poor, fair to good and excellent agreements, respectively (Landis and Koch, 1977). A Pearson correlation test was used to test the relationship between LDF and LSCI variables.

Sample size was calculated from ICCs by estimating the width of the 95% confidence interval for an expected correlation of 0.70 (between the lowest ICC value deemed acceptable, i.e. 0.4, and the highest, i.e. 1). Considering a two-sided interval, two measurements and a distance from correlation to limit ω of 0.3 (to remain within the range of fair to good agreements), the estimated number of subjects needed was 13 (nQuery Advisor®, Statistical Solutions Ltd., Cork, Ireland). Considering the risk of missing data, we enrolled 14 participants in each protocol.

Results

Protocol 1: Inter-day reproducibility of post-occlusive reactive hyperemia and local thermal hyperemia assessed by LDF

We recruited 14 healthy Caucasian volunteers in this part of the study. Their median age was 25 (4) and 78% were female. All but two females were on oral contraceptives. Their median body mass index was 21.2 (4) and resting systolic/diastolic blood pressure was 123 (14)/70 (9) mm Hg. All subjects had normal blood lipid profiles and blood cell counts.

When skin temperature was homogenized at 33 °C, the 1-week reproducibility of PORH was acceptable whether expressed as peak CVC or as a function of baseline (PK-BL and PK%BL) (Table 1). Overall reproducibility of LTH was poor except when expressed as $\%CVC_{plateau}$ (Table 1).

Protocol 2: Spatial and inter-day reproducibility of local thermal hyperemia assessed by LDI

The same subjects who participated in protocol 1, followed protocol 2, but on different days, 1 week apart.

The spatial reproducibility of the LTH peak and plateau was good when expressed as CVC, and fair to poor when expressed as a function of baseline or as $\%CVC_{plateau}$. Inter-day reproducibility of LTH plateau was good when expressed as CVC, while peak CVC and data expressed as $\%CVC_{plateau}$ or as a function of baseline showed poor reproducibility (Table 2).

Table 1
Reproducibility of post-occlusive reactive hyperemia (PORH) and local thermal hyperemia (LTH) assessed with single-point laser Doppler flowmetry (LDF) on the forearm between day 0 (D0) and day 7 (D7). Skin temperature was maintained at 33 °C during the whole measurement for PORH, or during baseline measurements for LTH.

		D0	D7	CV	ICC
PORH	Peak CVC	6.54 (5.3)	5.88 (3.4)	**30**	**0.54**
	PK-BL	5.35 (5.1)	4.88 (2.7)	**33**	**0.53**
	PK%BL	537.5 (374.2)	524.9 (240.1)	**32**	**0.54**
	AUC	2183.81 (1365.2)	1910.21 (616.1)	36	0.48
	$\%CVC_{plateau}$	54.1 (31.2)	46.8 (19.9)	39	0.44
LTH	Peak (CVC)	9.60 (5.73)	8.93 (3.57)	40	0.31
	Plateau (CVC)	11.95 (5.75)	12.19 (3.24)	42	0.28
	Peak ($\%CVC_{plateau}$)	64.64 (19.88)	62.80 (17.52)	**25**	**0.85**
	Peak (%BL)	792.21 (360.44)	801.30 (571.82)	51	0.41
	Plateau (%BL)	1054.47 (768.79)	1073.88 (1115.18)	58	0.44

Data are expressed as raw values, as a function of 44 °C plateau or as a function of baseline. PORH amplitude at D0 and D7 is expressed as median (interquartile) peak cutaneous vascular conductance (Peak CVC, in mV/mmHg), peak CVC minus baseline CVC (PK-BL), peak as a percentage of baseline (PK%BL), area under the curve (AUC, in PU s) and percentage of 44 °C plateau ($\%CVC_{plateau}$). Thermal peak and plateau data at D0 and D7 are expressed as median (interquartile) cutaneous vascular conductance (CVC, in mV/mmHg), as a percentage of 44 °C plateau ($\%CVC_{plateau}$) and as a percentage of baseline (%BL).Variability between D0 and D7 is expressed as intra-subject coefficients of variation (CV, in %). Repeatability is expressed as intra-class coefficients of correlation (ICC). CV<35% were deemed acceptable (Harris et al., 2007). ICC values of <0.40, 0.40 to 0.75 and >0.75 represent poor, fair to good and excellent agreements, respectively (Landis and Koch, 1977). Results appearing in bold fulfill both conditions.

Table 2
Spatial and inter-day reproducibility of local thermal hyperemia (LTH) assessed by laser Doppler imaging (LDI) on the forearm. Baseline skin temperature was maintained at 33 °C.

	Spatial reproducibility		Inter-day reproducibility	
	CV	ICC	CV	ICC
Peak CVC	**16**	**0.56**	39	0.20
Plateau CVC	**16**	**0.68**	**17**	**0.51**
Peak %$CVC_{plateau}$	18	0.35	42	0.04
Peak %BL	**34**	**0.46**	52	0.13
Plateau %BL	**32**	**0.53**	34	0.37

Thermal peak and plateau data are expressed as median (interquartile) cutaneous vascular conductance (CVC, in mV/mmHg) and as a percentage of baseline (%BL). Peak is also scaled to 43°C plateau (%$CVC_{plateau}$). Variability between D0 and D7 (inter-day) or between site 1 and site 2 (spatial) is expressed as intra-subject coefficients of variation (CV, in %). Repeatability is expressed as intra-class coefficients of correlation (ICC). CV<35% were deemed acceptable (Harris et al., 2007). ICC values of <0.40, 0.40 to 0.75 and >0.75 represent poor, fair to good and excellent agreements, respectively (Landis and Koch, 1977). Results appearing in bold fulfill both conditions. Data from day 0 and day 7 were pooled to assess spatial reproducibility.

Protocol 3: Inter-day reproducibility of post-occlusive reactive hyperemia and local thermal hyperemia assessed by LSCI

Fourteen other healthy volunteers were enrolled in protocol 3. Population characteristics were similar to those of protocols 1 and 2, except that 50% were female.

The flux patterns of PORH followed by LTH on the forearm assessed by LSCI were qualitatively similar to those obtained with LDF (Fig. 1).

Inter-day reproducibility of PORH was very good whether expressed as peak CVC or as a function of baseline (PK-BL and PK%BL). However, we observed poor reproducibility of PORH peak expressed as %$CVC_{plateau}$ (Table 3).

Inter-day reproducibility of LTH peak and plateau on the forearm was very good when expressed as CVC (Fig. 2) or as %$CVC_{plateau}$ (for the peak). Reproducibility was acceptable when they were expressed as %BL (Table 3).

Inter-day reproducibility data (expressed as CVs) of PORH and LTH measured by the different techniques are summarized in Fig. 2.

Table 3
Reproducibility of post-occlusive reactive hyperemia (PORH) and local thermal hyperemia (LTH) assessed with laser speckle contrast imaging (LSCI) on the forearm between day 0 (D0) and day 7 (D7).

		D0	D7	CV	ICC
PORH	Peak CVC	11.4 (1.8)	12.46 (3.1)	**8**	**0.76**
	PK-BL	6.89 (1.9)	8.12 (2.5)	**11**	**0.75**
	PK%BL	168.4 (40.9)	187.69 (52.8)	**14**	**0.52**
	%$CVC_{plateau}$	0.91 (0.2)	0.91 (0.3)	35	0.29
LTH	Peak (CVC)	9.5 (2.6)	10.47 (3)	**15**	**0.48**
	Plateau (CVC)	10.11 (2.34)	10.2 (3.16)	**15**	**0.66**
	Peak (%$CVC_{plateau}$)	0.96 (0.1)	0.99 (0.2)	**9**	**0.57**
	Peak (%BL)	186.91 (101.5)	218.06 (34.7)	**21**	**0.59**
	Plateau (%BL)	226.01 (103.6)	239.5 (104.6)	**24**	**0.56**

Data are expressed as raw values, as a function of 43 °C plateau or as a function of baseline.
PORH amplitude at D0 and D7 is expressed as median (interquartile) peak cutaneous vascular conductance (Peak CVC, in mV/mmHg), peak CVC minus baseline CVC (PK-BL), peak as a percentage of baseline (PK%BL), and percentage of 43 °C plateau (%$CVC_{plateau}$). Thermal peak and plateau data at D0 and D7 are expressed as median (interquartile) cutaneous vascular conductance (CVC, in mV/mmHg), as a percentage of 43 °C plateau (%$CVC_{plateau}$) and as a percentage of baseline (%BL).Variability between D0 and D7 is expressed as intra-subject coefficients of variation (CV, in %). Repeatability is expressed as intra-class coefficients of correlation (ICC). CV<35% were deemed acceptable (Harris et al., 2007). ICC values of <0.40, 0.40 to 0.75 and >0.75 represent poor, fair to good and excellent agreements, respectively (Landis and Koch, 1977). Results appearing in bold fulfill both conditions.

Protocol 4: Comparison of post-occlusive reactive hyperemia whether assessed by LSCI or by single-point LDF.

Six of the participants enrolled in protocol 3 were included in protocol 4 (50% were female). Correlations were therefore calculated from 36 points.

We observed significant correlation between simultaneous LDF and LSCI measurements of the PORH peak CVC ($R=0.54$; $p=0.001$) and PK-BL ($R=0.58$; $p<0.001$). However, baseline flux measurements were not correlated ($R=0.05$; $p=0.75$) (Fig. 3).

Post-hoc analysis: Inter-site variability of post-occlusive reactive hyperemia simultaneously assessed with LDF or LSCI on the forearm

We further analyzed data from protocol 4 by calculating within-subject coefficients of variation (CV) between the PORH recorded with the three single-point LDF (skin temperature was homogenized to 33 °C) and the adjacent ROI measured simultaneously with LSCI.

We observed poor inter-site reproducibility of baseline flux when assessed with single-point LDF whereas it was excellent with LSCI. Overall, inter-site reproducibility of PORH was better when assessed with LSCI compared to single-point LDF, except for peak scaled to plateau vasodilation, which was comparable between the two techniques (Table 4).

Discussion

The present study reveals that the inter-day reproducibility of PORH and LTH assessment on the forearm is improved when full-field techniques such as LSCI or LDI are used, compared to single-point LDF. Our data suggest that this is due to lower inter-site variability when measuring wider areas. Indeed, homogenizing baseline skin temperature

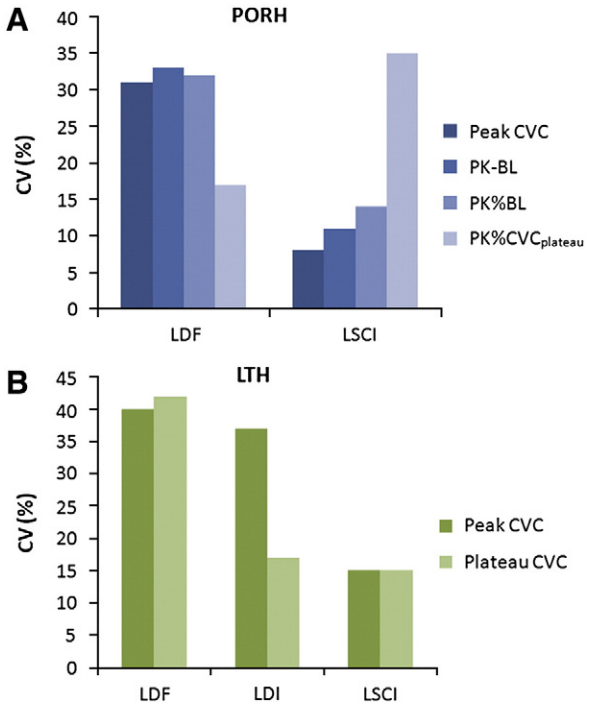

Fig. 2. (A) Inter-day reproducibility of post-occlusive reactive hyperemia (PORH) assessed with laser Doppler flowmetry (LDF) and laser speckle contrast imaging (LSCI). Data are expressed as within subject coefficients of variation (CV, in %) of peak cutaneous vascular conductance (Peak CVC, in mm Hg), peak CVC minus baseline CVC (PK-BL), peak as a percentage of baseline (PK%BL), and percentage of plateau vasodilation (%$CVC_{plateau}$). (B) Inter-day reproducibility of local thermal hyperemia (LTH) assessed with LDF, laser Doppler imaging (LDI) and LSCI. Data are expressed as CV of peak and plateau CVC.

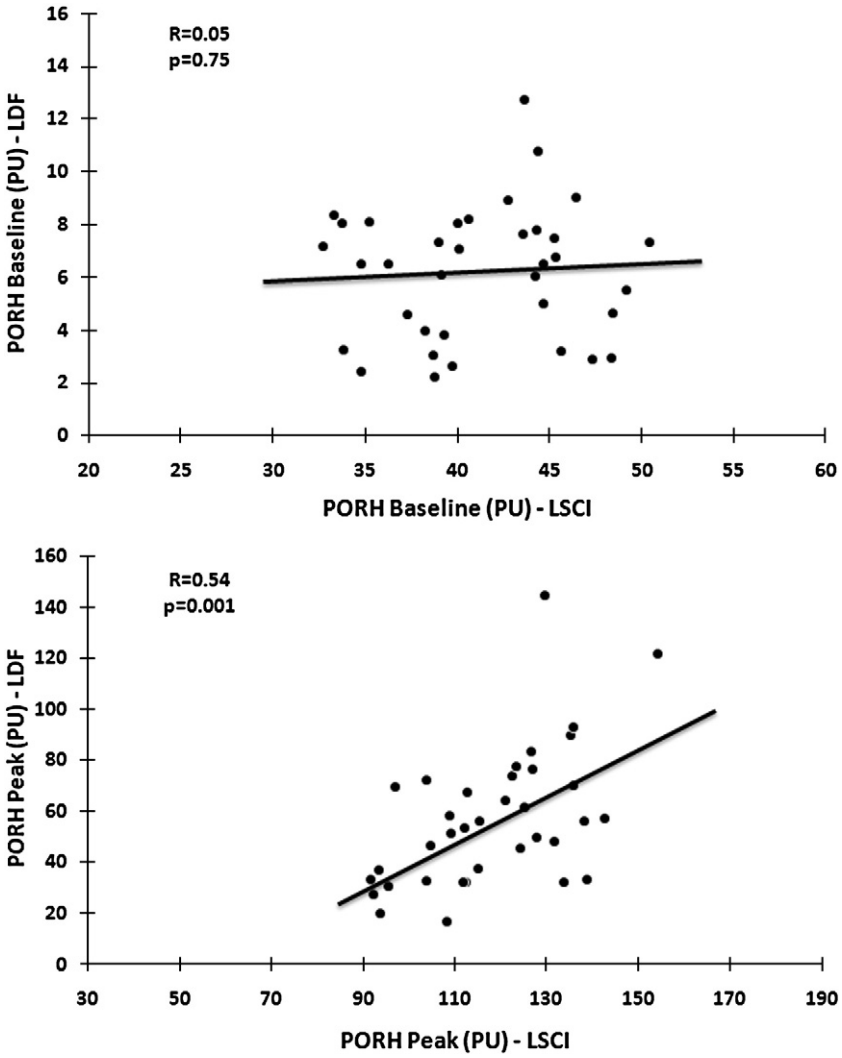

Fig. 3. Correlation between simultaneous assessment of post-occlusive reactive hyperemia (PORH) baseline (up) and peak (down), with laser Doppler flowmetry (LDF) and laser speckle contrast imaging (LSCI) (Pearson correlation test). Data are expressed as blood flux in perfusion units (PU).

when using single-point LDF was not sufficient to compensate for spatial variability, as it only slightly improved the reproducibility of PORH and had no effect on the reproducibility of LTH measurements. Finally, we showed significant correlation between LSCI and single-point LDF when assessing PORH, but not LTH.

Microvascular reactivity is frequently assessed on the forearm using single-point LDF while performing tests such as PORH and LTH. However, we recently showed the poor inter-day reproducibility of these techniques on the forearm, most likely related to spatial variability (Roustit et al., 2010). We therefore considered two options to attempt to improve inter-day reproducibility: the first one was to homogenize resting skin temperature to 33 °C, which had been previously done in order to limit baseline variability (Minson et al., 2001). The present work confirms this hypothesis for PORH assessment, as CVs and ICCs were improved compared to the previous series performed with no skin temperature homogenization (Roustit et al., 2010). Indeed, in the present work we show acceptable reproducibility of PORH assessed by single-point LDF at 33 °C when expressed as PK-BL or PK%BL. As homogeneous resting values are of key importance when expressing results as a function of baseline, these results were expected. In the same way, homogenization of skin temperature drastically improved PORH reproducibility expressed as AUC. Surprisingly, it also improved peak reproducibility expressed as raw CVC. Although CVs and ICCs were also improved for LTH assessed by LDF when baseline skin temperature was homogenized, the overall reproducibility of LTH on the forearm remained poor using single-point LDF, suggesting the need for alternative techniques. Of note, in the present study the CVC was calculated continuously using plethysmography, whereas in the previous study blood pressure had been measured manually at the beginning of each set of experiments (Roustit et al., 2010). This dissimilarity could have played a role in the different inter-study reproducibility results. However, this is unlikely as we found comparable reproducibility results when expressing data as perfusion units (data not shown for clarity).

The second option was to use full-field imaging techniques to scan skin blood flux over wider areas. We therefore conducted similar

Table 4
Inter-site variability of post-occlusive reactive hyperemia (PORH) recorded simultaneously at three sites on the same forearm by LDF and LSCI.

	LDF	LSCI
BL CVC	45	7
Peak CVC	47	12
PK-BL	49	18
PK%BL	30	17
$\%CVC_{plateau}$	23	19

Data are expressed as within-subject coefficient of variation (CV, in %) of baseline (BL) CVC, peak raw CVC, peak expressed as a function of baseline (PK-BL and PK%BL) or as a function of 43 °C plateau ($\%CVC_{plateau}$).

protocols using LDI and the recently developed LSCI technique instead of single-point LDF. Some groups have used LDI to assess PORH over small areas. However, in our experience, LDI is not fast enough to accurately assess the PORH peak (which lasts only a few seconds) over wide areas. We therefore used LDI to assess LTH only. Our results show good inter-day reproducibility of plateau CVC when measured with LDI, whereas peak reproducibility is poor. Again, this discrepancy could be explained by the kinetics of the initial LTH peak, which is only stable over approximately 1 min when recorded with LDF. As, in our protocol, the LDI was set to record one image every minute, our peak data result from a single point. If the scanned image is slightly shifted from the actual peak, it may result in increased variability. The good inter-site reproducibility of peak CVC simultaneously assessed at two sites on the same forearm strengthens this suggestion. As such, lower resolution on smaller areas would probably increase peak reproducibility assessed by LDI.

LSCI is of particular interest as it allows rapid skin blood flow measurements over wide areas, with good resolution. Recent studies have used LSCI to assess cerebral perfusion during neurovascular surgery (Hecht et al., 2009) and skin perfusion in patients with primary or secondary Raynaud's phenomenon (Murray et al., 2009). However, there is scarce data available on LSCI reproducibility. The present study shows very good inter-day reproducibility of PORH assessed with LSCI, whether expressed as raw CVC or as a function of baseline. Interestingly, reproducibility was borderline when $\%CVC_{plateau}$ was used, whereas data expressed in this way showed the best reproducibility when using single-point LDF (Roustit et al., 2010). On the other hand, the LTH peak expressed as $\%CVC_{plateau}$ showed the best reproducibility, as previously shown using LDF (Roustit et al., 2010). This is understandable as recordings were performed at the same site with the same device. Finally, both the LTH peak and plateau measured with LSCI show the highest variability when expressed as % BL, which is consistent with LDF data (Roustit et al., 2010).

Overall, comparing data expressed as $\%CVC_{plateau}$ is difficult between our experiments, as we used different heating protocols to achieve NO-dependent vasodilation, which is a limitation to our study. Indeed, we have heated for 30 min at 42 °C and then 5 min at 44 °C with single-point LDF probes, whereas we have heated at 43 °C for 40 min with the SHP3 heating probes. These differences are justified by the differences in heating rates between the two devices: setting LDF probes to heat from resting skin temperature to 43 °C or 44 °C (1 °C s^{-1}) may lead to pain or a burning sensation in patients with abnormal microvascular function (e.g. systemic sclerosis), whereas heating slowly with probes filled with water (0.07 °C s^{-1}) is well tolerated. Nonetheless, heating to 42 °C allows both the axon reflex-dependent initial peak and NO-dependent late plateau to be observed (Minson et al., 2001).

As a consequence of these findings, the differences observed in the variability of the measurements between LDF and LSCI lead to drastically different numbers of subjects being required to detect any biologically significant difference in skin perfusion. For example, to detect a 20% change in the PORH peak CVC, considering an alpha risk of 0.05 and 80% power, 13 subjects would be needed with LSCI and 75 with LDF. In the same conditions, 17 and 80 subjects would be needed to detect a 20% change in PORH PK%BL assessed by LSCI or LDF, respectively.

To our knowledge, only one group has studied the reproducibility of LSCI measurements in human skin. Murray et al. (2009) showed poor inter-day ICC of skin blood flux on a 35 mm^2 area at the nail-bed of the ring finger. However, it is impossible to compare these low ICC to our results as we focused neither on the same area nor on the same population (Murray et al. studied reproducibility on the nailfold of three healthy volunteers and two primary RP patients). Furthermore, the imager used and the frame rate were also different.

The higher proportion of females in protocols 1 and 2 than in protocols 3 and 4 might explain the lower inter-day reproducibility of microvascular reactivity measurements with LSCI. Indeed, as measurements were made at an interval of 7 days, the young women would have been in different hormonal states, as previously discussed (Roustit et al., 2010). However, this is unlikely to explain the dramatic difference observed between LDF and LSCI measurements. Indeed, we observed poor inter-site reproducibility of PORH simultaneously recorded with LDF at three different sites in protocol 4, while LSCI reproducibility remained excellent. Finally, in order to obtain reproducibility data under the conditions of routine use, variations due to different menstrual phases in women were taken into account in the variability of the response.

A secondary objective of our study was to correlate microvascular reactivity measurements made simultaneously by single-point LDF and by LSCI. We found significant correlation for the PORH peak when expressed as raw CVC or as a function of baseline. However, when expressed as $\%CVC_{plateau}$, PORH measurements were no longer correlated. We explain this discrepancy by the difference in measuring $\%CVC_{plateau}$ between the two techniques. Indeed, we used the same device and exactly the same site for LDF, whereas different probes had to be used to express the PORH peak as a percentage of the LTH plateau when recording with LSCI. We therefore scaled a signal directly recorded on the skin to another recorded through a heating probe containing water. The use of these different devices may therefore increase variability. This is also the reason why we did not test correlation for LTH. Besides these technical considerations, the lack of correlation may be explained by different depths of skin blood flow measured by single-point laser Doppler and LSCI (approximately 1 mm and 300 μm, respectively) (O'Doherty et al., 2009). This raises the issue of the quantitative assessment of skin blood flow with LSCI. Indeed, although PORH and LTH patterns are similar between LSCI and LDF, the quantitative assessment of skin blood flow using LSCI needs to be further studied.

In conclusion, homogenizing baseline skin temperature moderately improved inter-day reproducibility of post-occlusive reactive hyperemia (PORH) but not of local thermal hyperemia (LTH) when assessed by single-point LDF on the forearm. However, full-field techniques improved inter-day reproducibility probably by lowering inter-site variability. Indeed, LTH reproducibility was good on the forearm when assessed with laser Doppler imaging, but its kinetics does not allow accurate assessment of PORH. Encouragingly, the recently developed laser speckle contrast imaging (LSCI) technique showed very good inter-day reproducibility to assess both PORH and LTH on the forearm. Finally, we showed significant correlation between LSCI and single-point LDF when assessing PORH. However, more data are needed to evaluate the linearity between the LSCI signal and skin blood flow.

Conflict of interest

Jean-Luc Cracowski and Matthieu Roustit have received research grants from Actelion, Ampli, Boiron and Pfizer, for other studies.

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Part II. Microvascular reactivity in Raynaud's Phenomenon

As vasospastic attacks are triggered by cold or emotional stimuli, Maurice Raynaud initially thought that central neural mechanisms were mostly involved in the pathophysiology of his eponymous phenomenon [173]. However, experiments by Lewis in 1929 showed that RP can still occur after sympathectomy, suggesting that peripheral modulation of adrenergic nerves was responsible for RP [174]. Raynaud's and Lewis' hypotheses were both correct, although oversimplified. Indeed, central and peripheral neural mechanisms have now been identified, as well as non-neural mechanisms underlying vasospasm.

In accordance with Maurice Raynaud's theory, the improvement of RP after sympathectomy had suggested the involvement of the sympathetic nervous system. Other experiments have highlighted the responsibility of central neural mechanisms in primary RP. However, they suggest that patients show impairment of the process of habituation to stress or to mild cooling [175, 176] rather than hyper-reactivity of the sympathetic system [13].

Local adrenergic modulation has raised considerable attention, particularly through the regulation of postjunctional α_{2c}-adrenoreceptors [177]. Apart from being clustered distally in the digits (i.e. a highly thermosensitive region), their expression at the cell surface is increased during local cooling. Indeed, cooling enhances the translocation of α_{2c}-adrenoreceptors from the Golgi apparatus to the vascular smooth muscle plasma membrane, thus increasing noradrenaline-mediated vasoconstriction [22]. The role of the RhoA/Rho kinase (ROCK) pathway in mediating microvascular vasoconstriction during local cooling has been confirmed *in vivo* in healthy subjects [131].

In vitro, Furspan and colleagues have shown increased cooling-induced α_2-adrenergic constriction of arterioles isolated from patients with primary RP compared to controls, suppressed by protein tyrosine kinase inhibitors [178]. Moreover yohimbine, an α_2-antagonist, prevented vasospasm in patients with primary RP whereas prazosin, an α_1-antagonist did not [179]. Taken together, these data suggest that the ROCK pathway could be involved in the pathophysiology of RP [180], but this remains to be confirmed.

Non-adrenergic neural mechanisms may also play a pivotal role in RP, especially through impaired vasodilation. Indeed, among the pathways responsible for decreased vasodilation, loss of calcitonin gene-related peptide (CGRP) containing nerve fibers has been identified in the digits of patients with primary and, more markedly, secondary RP [181]. Moreover, intravenous administration of CGRP increased skin blood flow in the fingers and the hand of patients with severe RP (most of them had SSc) and improved the healing of digital ulcers [182]. Bunker et al further suggested that CGRP was a contributor to cold-induced vasodilation, and that CGRP release during cooling was enhanced by ET-1 [183].

Besides neural mechanisms, endothelial abnormalities may be involved in the pathophysiology of RP. Although there is growing evidence of endothelial dysfunction in SSc patients, data are conflicting regarding primary RP [14]. The origin of endothelial dysfunction in SSc is not known, viral triggers such as cytomegalovirus (CMV) have been suspected [184]. It may also be secondary to increased expression of Fos-related antigen-2 (Fra-2), a transcription factor of the activator protein-1 family, which induces endothelial cell apoptosis [185]. Whatever the cause, apoptosis further activates the immune-inflammatory system which impairs the microvascular bed, leading to the dysregulation of microvascular tone control and resulting in progressive disorganization of the vascular architecture [184].

Several underlying mechanisms contributing to the imbalance between microvascular vasodilation and vasoconstriction have been proposed. Among them, an increase in ET-1 may

explain in part the excessive vasoconstriction in RP. Indeed, ET-1 is a potent vasoconstrictor peptide produced from a pre-propeptide, big-endothelin, converted to ET-1 by endothelin-converting enzyme (ECE) [186]. Investigators have shown increased levels of ET-1 after mild cooling in RP, whereas it's level remained stable in healthy controls [176]. However results are conflicting regarding basal and cold-induced changes in ET-1 levels in primary and secondary RP [187-190]. Recent data on a larger population has revealed significantly higher ET-1 plasma levels in patients with both primary and secondary RP compared to controls [191]. It is interesting to note that cooling increased ET-1 release from the endothelium at higher temperature in patients with RP than in controls (18°C and 13°C, respectively) [187]. However, no difference was seen in the contractile response of small arteries to endothelin-1 between patients with primary RP and matched controls, either at 37°C or at 24°C [25]. ET-1 may therefore be involved in the pathophysiology of RP, suggesting potential interest of ET-1 receptor antagonists, which we will discuss in the third part of this dissertation.

Vasoconstrictor prostanoids may also be involved, such as thromboxane A_2, prostanglandin H_2 or superoxide anions [13]. The latter may limit the action of NO, whose effect may be altered in RP [14]. Endothelium-dependent and independent vasodilation has been widely assessed in RP, by using Ach and SNP iontophoresis, respectively. Both Ach and SNP-induced vasodilation assessed with LDI were impaired in the fingers of patients with SSc compared to healthy controls and participants with primary RP [192]. However, when skin blood flux was recorded with LDF by the same group, no difference was seen [193]. Another study has shown that Ach-induced vasodilation assessed with LDI was decreased on the dorsum of the hand of SSc patients compared to the healthy controls and to primary RP patients, whereas SNP-induced vasodilation was not different [194]. Finally, Ach-induced vasodilation was decreased on the forearm of SSc patients compared to RP and controls [195], as well as on the finger of primary RP patients compared to controls [196, 197]. The

variability of the test may explain those discrepancies. In conclusion, the limitations of the technique (which are discussed in the first part of the dissertation) and the heterogeneity in methods and skin sites make these results difficult to interpret.

The measurement of several biomarkers has also suggested decreased production of NO in secondary RP. For example, plasma asymmetric dimethylarginine (ADMA), an endogenous inhibitor of endothelial NO synthase (eNOS), is increased in patients with SSc when compared to patients with primary RP [189, 198, 199]. Increase in ADMA has been shown to be correlated to TIMP-1 plasma levels, a biomarker of matrix remodeling, which was negatively correlated to PORH, a global marker of microvascular function [199]. Taken together, these data suggest an association between endothelial dysfunction and matrix remodeling in scleroderma spectrum disorders.

I. Abnormal digital neurovascular response to local heating in systemic sclerosis

As previously detailed in the first part of this dissertation, local thermal challenges, coupled with laser Doppler measurements, are appropriate tests to assess skin microvascular reactivity [34]. In healthy subjects, thermal hyperemia is characterized by an initial peak, mainly depending on sensory nerves, and a late plateau, mostly NO-dependent [83] (please refer to page 44 for details). Previous work from our group has suggested impaired thermal hyperemia in SSc but not in primary RP, affecting both the initial peak and the plateau [200, 201]. The plateau decrease suggests impaired NO-dependent vasodilation, which is consistent with the endothelial dysfunction discussed above. However, the extent to which abnormal neurovascular regulation contributes in microvascular dysfunction has not been clearly established yet. We therefore tested whether sensory nerves were responsible for decreased initial vasodilation induced by local heating in primary and secondary RP.

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Abnormal digital neurovascular response to local heating in systemic sclerosis

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Objectives. To investigate neurovascular dysfunction using the axon reflex-dependent hyperaemia (initial peak of skin local heating response) in fingers of patients with SSc or primary RP.

Methods. Ten healthy subjects were initially enrolled to compare axon reflex-dependent thermal hyperaemia between the finger and forearm cutaneous circulations. Then, 10 patients with primary RP and 16 patients with SSc participated in a similar protocol focusing on the finger circulation only. Lidocaine/prilocaine cream was applied for 1 h to produce local blockade of cutaneous sensory nerves. After lidocaine/prilocaine pre-treatment, laser Doppler probes were heated from skin temperature to 42°C for 30 min, and 44°C for 5 min to achieve maximal skin blood flow. Data were expressed as a percentage of maximal cutaneous vascular conductance.

Results. In healthy volunteers, we observed a significantly higher initial peak on the finger compared with the forearm, with both responses blunted following topical anaesthesia. In primary RP patients, we observed a decreased initial peak following lidocaine/prilocaine pre-treatment in the finger circulation [96.7% (33.4) *vs* 75.9% (29.5) with anaesthesia, $P = 0.02$]. In contrast, pre-treatment did not alter the initial peak in patients with SSc. A minute-by-minute analysis showed no delay of the initial peak.

Conclusions. We show an abnormal digital neurovascular response to local heating in SSc. Thermal hyperaemia could be monitored as a clinical test for neurovascular function in SSc. Further studies are required to test whether the abnormal digital neurovascular response correlates to the degree of peripheral vascular involvement.

Key words: Systemic sclerosis, Microcirculation, Thermal hyperaemia, Neurovascular, Raynaud's phenomenon.

Introduction

Microvascular dysfunction is an early event in the pathogenesis of SSc. SSc microvascular dysfunction involves a decreased endothelium-dependent dilation, and an alteration in neurovascular control [1].

Microvascular function can routinely be studied in humans using non-invasive laser Doppler flowmetry of the skin [2]. The hyperaemic response to local heating of the skin provides an integrated index of neurovascular and nitric oxide-dependent cutaneous blood flow regulation [2, 3]. In healthy subjects, local thermal hyperaemia is characterized by an initial peak within the first 5 min, a subsequent nadir and finally a sustained plateau. The initial peak is axon reflex dependent. This axon reflex is mediated by activation of peripheral C-fibres, which induce antidromic impulse conduction, ultimately resulting in the release of vasodilating neuropeptides [4]. Available data suggest that the neuropeptides involved in the initial peak response are calcitonin gene-related peptide (CGRP) and substance P, whereas the plateau phase is nitric oxide dependent [5].

We, and others, have recently used local thermal hyperaemia as an integrated test to study microvascular function in SSc [6–9]. We have previously shown that local thermal hyperaemia is impaired in patients with secondary RP compared with patients with primary RP [6]. In patients with SSc, the initial peak and the late plateau phase of the response are both impaired. These alterations do not relate to skin fibrosis or to associated macroangiopathy in most cases [7]. In addition, both RP and SSc have been linked to a reduction in the number of CGRP neurons in the finger skin [10]. However, few data are available on dynamic neurovascular regulation in the circulation to the finger pads, which are rich in arterio-venous anastomoses. Indeed, most studies in this area have focused on the non-glabrous skin of the forearm.

Lidocaine/prilocaine, when applied as a cream over the skin of healthy subjects, induces a time-dependent decrease in cutaneous sensitivity that is associated with a blunted axon reflex-mediated vasodilation [11]. As such, lidocaine/prilocaine cream can be used as a pharmacological tool to inhibit the axon reflex. As thermal hyperaemia data on finger pads is lacking, we used this pharmacological tool in the first study to compare the axon reflex-dependent thermal hyperaemia on fingers and forearm of healthy volunteers. Then, we performed a second study to assess whether the axon reflex-dependent thermal hyperaemia is altered in the fingers of patients with SSc or primary RP.

Materials and methods

First study

Study population. Ten healthy subjects were recruited through local newspaper advertisements. Inclusion criteria included an age of 18 yrs or older, and no significant medical history. For all subjects, non-inclusion criteria included any allergies to local anaesthetics and cigarette smoking. Grenoble Institutional Review Board approval was obtained and each subject gave written informed consent before participation.

Study design. This was an open-labelled physiology study performed in a temperature-controlled room (23°C ± 1). Upon arrival of the subject to the laboratory, a subject medical history was taken. Two sites were chosen on the ventral side of the left upper forearm and two sites on finger pads (randomly chosen between index, middle and ring finger). One hour before starting thermal hyperaemia, 1 g of lidocaine/prilocaine cream (5 g tubes containing 125 mg lidocaine and 125 mg prilocaine) was placed on one skin site of the forearm and on one fingertip. The initial application of lidocaine/prilocaine cream covered 1 cm^2 of skin

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surface. Subsequently, an occlusive transparent dressing was placed over the cream on both sites to enhance cutaneous diffusion and cover a larger skin area. The anaesthetized area of skin was larger than the size of the local heating devices. No cream was placed on the control sites. In order to avoid bias, each site was at least 3 cm apart from the other on the forearm. One hour later, the lidocaine/prilocaine cream was removed with a cotton swab. The subject was laid supine for the duration of the whole experiment, and blood pressure was taken manually.

In order to test whether lidocaine/prilocaine application induced a similar anaesthesia on finger pads and forearm, we quantified skin sensitivity of the forearm and the finger tips pretreated for 1 h with lidocaine/prilocaine. We used six different Semmes–Weinstein monofilament sensory evaluators (0.07, 0.4, 2, 4, 10, 300 g Touch-Test, Biomedix IT&M, Lyon, France). We observed a similar loss of sensitivity between healthy controls and patients with SSc on the finger tips (0.07 g before *vs* 0.4 g after lidocaine/prilocaine application). However, on the forearm, the effect was more pronounced (0.4 g before *vs* 10 g after).

Laser Doppler measurements. All the skin sites were instrumented for measurement of skin blood flow using laser Doppler flowmetry (Periflux System 5000, Perimed, Järfälla, Sweden) with integrated local heaters (Probe 457, Perimed). A 5 min baseline was recorded prior to thermal hyperaemia.

Thermal hyperaemia. As previously described, the local heating units were heated from skin temperature to 42°C over 15–22 s and maintained at this temperature for 30 min. After 30 min, the skin sites were heated to 44°C for 5 min to achieve maximal skin blood flow [6].

Second study

Study population. Patients with primary RP and SSc were recruited through local newspaper advertisements and from the Vascular Medicine Department, respectively. Primary RP was diagnosed according to the criteria of LeRoy and Medsger [12]. SSc was classified as limited cutaneous (lcSSc) or diffuse cutaneous SSc (dcSSc) with the criteria of LeRoy and Medsger [13]. Inclusion criteria included an age of 18 yrs or older. Exclusion criteria included any allergies to local anaesthetics, cigarette smoking, diabetes mellitus, hypercholesterolaemia or any associated severe disease (cancer, cardiac and pulmonary failure, myocardial infarction, angina pectoris). Grenoble Institutional Review Board approval was obtained and each subject gave written informed consent before participation.

Study design. Two sites on the finger pads were randomly chosen between the index, middle finger and ring finger. For patients with SSc, fingers with active tip ulcerations were excluded. As in the first study, lidocaine/prilocaine cream was randomly placed on one of the finger pads with an occlusive transparent dressing for 1 h. No cream was placed on the control site. The lidocaine/prilocaine cream was removed with a cotton swab.

Laser Doppler measurements and thermal hyperaemia. The same protocol used in the first study was performed to assess skin blood flow response to local heating.

Data analysis

Data were digitized and stored on a computer and analysed off-line with signal processing software (PeriSoft 2.5.5, Perimed). The amplitude of the thermal hyperaemia was determined by the thermal peak, 10–30 min 42°C thermal plateau and 44°C thermal plateau, expressed as cutaneous vascular conductance (flux in millivolts divided by mean arterial pressure) and scaled to maximal vasodilatation (44°C thermal plateau). Indeed, expressing data as conductance is more of a physiological approach as it takes into account differences and variations in blood pressure [2].

Conductance values for the initial peak were averaged over 1 min between the 150th and 210th seconds, as 3 min is the consistent mean time to peak [6, 7]. Conductance values for the thermal 42°C plateau phase, and the 44°C plateau phase, were averaged over a 3-min period. The day-to-day reproducibility of the thermal hyperaemia has been demonstrated previously [6, 7].

Table 1. Effects of lidocaine/prilocaine cream on the hyperaemia to local heating on the finger pad and the forearm of healthy subjects ($n = 10$)

	Finger pad	Forearm	*P*
Without lidocaine/prilocaine cream			
Peak	104.7 (12.3)	65.5 (18.6)	0.005
Time to peak	180 (35)	180 (15)	NS
42°C plateau	96.1 (15)	92.2 (5.5)	NS
With lidocaine/prilocaine cream			
Peak	73.7* (36.4)	29.4* (23.2)	0.01
Time to peak	181 (91)	175 (29)	NS
42°C plateau	85.2 (18.5)	91.3 (16.3)	NS

Data are expressed as median percentage (interquartile) of maximum conductance (44°C plateau) except for the time to peak, expressed as median time in seconds (interquartile). *$P < 0.05$ *vs* peak without lidocaine/prilocaine cream, Wilcoxon test. NS: non-significant.

Statistical analysis

Quantitative data are expressed as the median and interquartile in parentheses. Qualitative data are expressed as numerical values and percentage in parenthesis. Quantitative data were analysed with the Wilcoxon test for paired analyses, or Mann–Whitney test. The kinetics of the thermal hyperaemia was assessed by performing repeated measures analysis of variance on the minute average data for cutaneous vascular conductance. *P*-values <0.05 were considered statistically significant.

Results

First study: regional variation of the axon reflex induced by thermal hyperaemia in healthy controls

The median age of the 10 healthy volunteers was 51 (3) and median BMI was 21.7 (7). Typical local thermal hyperaemia patterns, including an early initial peak, a nadir and a late plateau, were found on the finger tip as is usually seen on the forearm. However, as shown in Table 1 and Fig. 1, we observed a significantly higher initial peak on the finger pad compared with the forearm. Indeed, the initial peak is higher than the 44°C plateau on the fingertip, whereas it is much lower on the forearm.

Lidocaine/prilocaine cream significantly decreased the initial peak on both finger and forearm sites (Table 1, Fig. 1). However, the decrease was more pronounced on the forearm in comparison with the finger.

Baseline cutaneous conductances without and with lidocaine/prilocaine cream were not significantly different on the finger pad [10.3 (13.3) and 7.9 (1.8) mV/mmHg, respectively] and on the forearm [1.1 (0.4) and 1.4 (2.9) mV/mmHg]. Median maximum conductances (44°C plateau) were not significantly different without and with lidocaine/prilocaine cream on the finger pad [41 (39.6) and 32.6 (11.7) mV/mmHg, respectively] and on the forearm [15.3 (4) and 20.5 (13.9) mV/mmHg].

Second study: effect of lidocaine/prilocaine cream on the axon reflex induced by thermal hyperaemia in primary RP and SSc

The demographic and clinical characteristics of the 26 patients enrolled in the second study are listed in Table 2. Among the

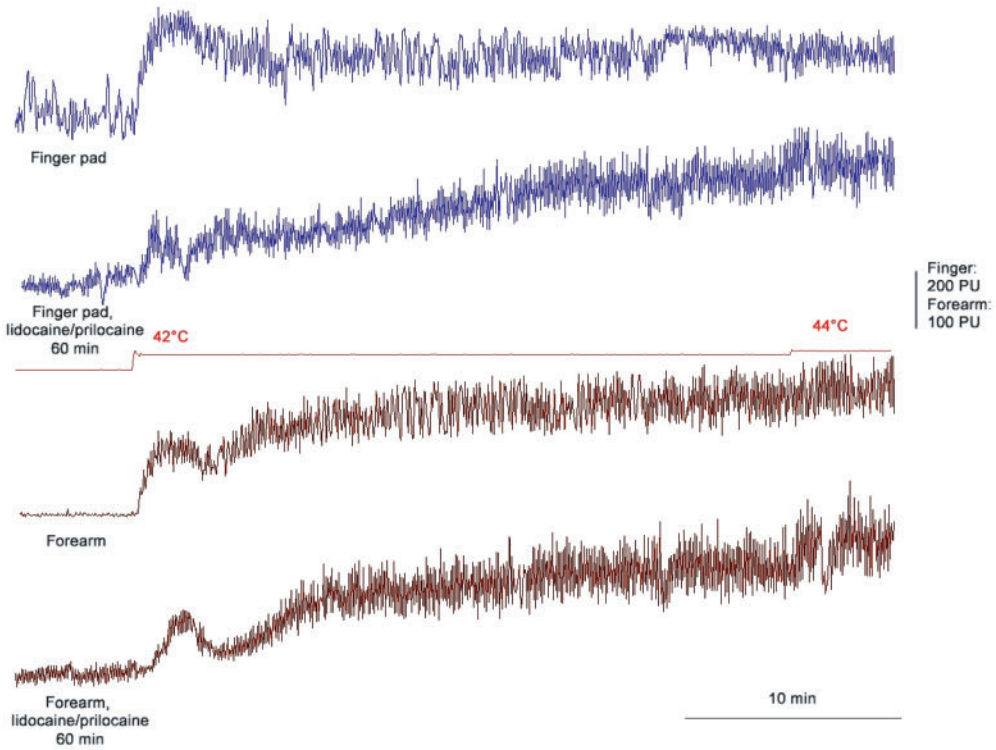

FIG. 1. Tracings of the thermal hyperaemia recorded on the finger pad and forearm of a healthy subject, with and without 1 h topical lidocaine/prilocaine pre-treatment. The initial peak, but not the late plateau, was decreased by the pre-treatment at both sites. PU: perfusion units.

TABLE 2. Demographic and clinical characteristics of patients with primary RP and SSc

	Primary RP ($n=10$)	SSc ($n=16$)
Age (yrs)	51.5 (9)	57 (14)
Female	5 (50)	11 (69)
BMI	23 (1.4)	21.8 (7.5)
RP	10 (100)	15 (94)
RP: duration (yrs)	19.5 (17)	20 (32.7)
RP: number of fingers involved	8 (5)	10 (2)
RP: thumb involved	3 (30)	12 (75)
RP: feet involved	4 (40)	15 (94)
Disease duration (yrs)	NA	6 (7.2)
Digital pitting scars	0 (0)	9 (56)
Sclerodactyly	0 (0)	16 (100)
Rodnan-modified skin score	0 (0)	18 (12.5)
lcSSc/dcSSc	NA	8/8 (50/50)
Pulmonary fibrosis	0 (0)	5 (31)

Quantitative data were expressed as median (interquartile). Qualitative data were expressed as number (percentage). NA: not applicable.

TABLE 3. Effects of lidocaine/prilocaine cream on the hyperaemia to local heating on the finger pad of patients with primary RP or SSc

	Without lidocaine/prilocaine cream	With lidocaine/prilocaine cream	*P*
Primary RP ($n=10$)			
Peak	96.7 (33.4)	75.9 (29.5)	0.02
42°C plateau	86.1 (13.3)	87.7 (11.2)	NS
SSc ($n=16$)			
Peak	85.5 (45.5)	84.2 (41)	NS
42°C plateau	89.7 (10)	91.1 (17.5)	NS

Data are expressed as median percentage (interquartile) of maximum conductance (44°C plateau). NS: non-significant.

16 patients with SSc, 8 were on calcium channel blockers, 3 on colchicine, 3 on angiotensin-converting enzyme inhibitors and 2 on angiotensin II receptor blockers. Among the 10 patients with primary RP, 1 was on calcium channel blockers.

Thermal hyperaemia data are shown in Table 3. We observed a decreased initial peak following lidocaine/prilocaine cream application in patients with primary RP, similar to healthy subjects. In contrast, lidocaine/prilocaine cream application did not alter the initial peak in patients with SSc (Table 3, Fig. 2).

We further tested whether the effect of lidocaine/prilocaine cream could be delayed in SSc patients. The kinetic assessment of median conductance (minute averages), with and without lidocaine/prilocaine cream, is shown in Fig. 3. We observed no significant difference between sites.

Baseline conductances without and with lidocaine/prilocaine cream on the finger pad were not significantly different in patients with SSc [3.3 (6.6) mV/mmHg and 3.7 (8) mmHg, respectively] but statistically different in patients with primary RP [11.5 (7.9) and 1.7 (6) mV/mmHg].

Median maximum conductances (44°C plateau) without and with lidocaine/prilocaine cream on the finger pad were not significantly different in patients with primary RP [38.9 (14.9) mV/mmHg and 37.7 (22) mmHg, respectively] and in patients with SSc [14.6 (20.4) and 12.6 (13.1) mV/mmHg].

Discussion

Our study demonstrates that the axon reflex-dependent thermal hyperaemia is blunted in the finger pads of patients with SSc, but not in those with primary RP.

In the current studies, laser Doppler flowmetry was used to monitor cutaneous perfusion rather than laser Doppler imaging. Indeed, laser Doppler imaging is less well suited for temporal monitoring of cutaneous perfusion whereas laser Doppler flowmetry enables the evaluation of cutaneous blood flow over time (continuous assessment). This aspect makes laser Doppler flowmetry especially useful in monitoring the kinetics of the cutaneous thermal hyperaemia (e.g. initial peak) [2, 3]. Indeed, using our

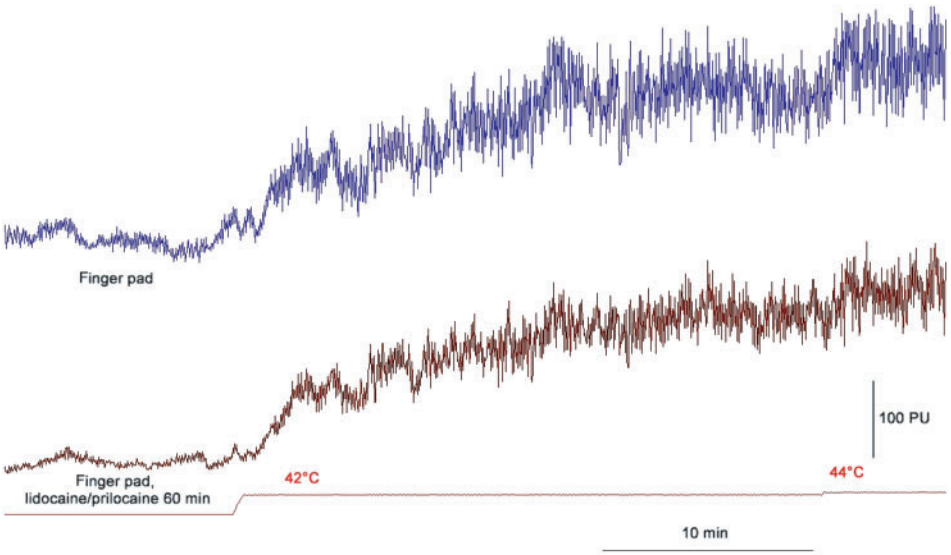

FIG. 2. Tracings of the thermal hyperaemia recorded on the finger pad of a patient with lcSSc, with and without 1 h topical lidocaine/prilocaine pre-treatment. The pre-treatment had no effect on the tracing. PU: perfusion units.

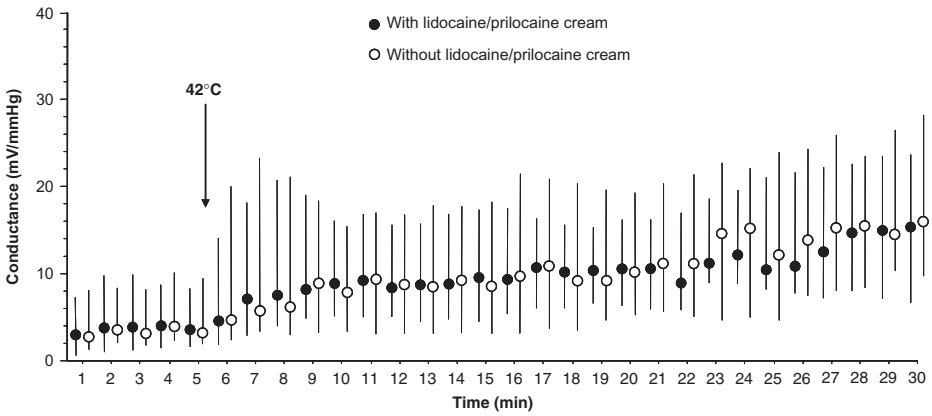

FIG. 3. Median conductance of digital blood flow in patients with SSc averaged over 1-min periods, with and without 1 h topical lidocaine/prilocaine pre-treatment.

heating protocol, the initial peak in cutaneous perfusion is consistently reached 3 min after the onset of the local heating to 42°C. It is followed by a nadir, and a secondary plateau is reached at 20–30 min. In healthy subjects, the initial peak is mediated by a CGRP-dependent axon reflex whereas the plateau is primarily mediated by nitric oxide [5]. We have previously shown a blunted digital vascular response to local heating in SSc [6, 7]. In the current study, the use of laser Doppler flowmetry enabled us to further explore this phenomenon, focusing specifically on the initial peak of the response.

Previously, using laser Doppler imaging, Gunawardena *et al.* [9] demonstrated a blunted local response after 6 min of heating at 44°C on the dorsum of the fingers, suggesting an abnormal digital microvascular regulatory response in patients with SSc. Murray *et al.* [8] used a slower heating protocol (34°C to 40°C in 3 min, then 40°C during 3 min) and showed no significant difference between patients and controls on the dorsum of the hand but did observe an altered digital response to post-ischaemia. Taken together with our results, these data are consistent with an abnormal early digital vascular response to local heating in SSc.

In order to assess the involvement of the axon reflex in the decreased initial peak previously observed in SSc after local heating, we used topical lidocaine/prilocaine to block sensory nerves in a localized region of skin. Indeed, we and others have shown that these topical anaesthetics can be used as pharmacological tools when local heating is started right after cream removal on the forearm [11, 14, 15]. In healthy volunteers, we showed that the amplitude of the initial peak was higher on the finger pad in comparison with the forearm. Indeed, such a peak was even higher than the 44°C late plateau usually used as an index of maximal vasodilation on the forearm [2]. We also observed less of a lidocaine/prilocaine cream effect on the finger pad in comparison with the forearm. This was not due to some intrinsic difference in axon reflex-mediated vasodilatation between skin regions. Rather, there was a decreased aesthetic effect of topical lidocaine/prilocaine on the finger pads, as suggested by the Semmes–Weinstein monofilament sensory test results. We cannot rule out the hypothesis that the effect of topical anaesthesia applied for a longer period of time would have been more pronounced. However, the application time was similar in all primary RP and SSc patients. A potential bias between groups is the fact that most of the SSc patients were under vasoactive medication, whereas this was less frequent in patients with primary RP. However, this does not influence the main objective, given the fact that each subject was its own control, and the drug would affect all sites. Furthermore, while calcium channel blockers may influence baseline blood conductance, there is no pharmacological evidence that they would interfere with the neurogenic response,

which was the objective in the present study. It must also be highlighted that skin fibrosis is unlikely to explain our results, as we previously showed that patients with SSc without skin thickening still present an abnormal thermal hyperaemia on the finger pad [7].

The current findings demonstrate a blunted axon reflex-dependent early vasodilatation during local heating in patients with SSc, for whom topical anaesthesia had less effect than in patients with primary RP and healthy controls. This was not due to a delayed response, as shown by our analysis of response kinetics (no effect of topical lidocaine/prilocaine). Similarly to what was studied in an ageing population, these findings could be consecutive to a decreased sensory component to the change in temperature, or to a reduction in the release and/or effect of neurotransmitters [16]. However, sensory neurological involvement is uncommon in SSc. In contrast, the crucial role of the peripheral nervous system in the control of vascular tone in SSc has been demonstrated previously, suggesting a functional impairment of the perivascular neurofibres [17]. Decreased substance P has been observed in late-stage SSc, while decreased CGRP has been observed both in patients with early and late-stage SSc [10, 17]. As CGRP is the most likely neurotransmitter involved in this axon reflex [18], this suggests a dysfunction of the CGRP neurovascular axis in the pathophysiology of SSc. The current findings indicate that axon reflex-mediated vasodilatation is normal in the digits of patients with primary RP, but not in those with SSc.

Abnormal neurovascular control in SSc may be associated with both functional and structural disorders [17]. Indeed, several studies have described changes in the neural network of affected and non-affected skin in patients with SSc [17, 19]. When using local heating as a test for axon reflex-mediated vasodilation, one cannot discriminate functional from structural changes. Blockade of the cutaneous nerves proximal to the site of local heating does not alter the thermal hyperaemic response, while local anaesthesia does. This shows that the initial thermal hyperaemia is dependent on a local neurovascular mechanism [5].

Our data suggest that monitoring the initial rise in digital skin blood flow following local heating could be a new and easy tool to assess neurovascular control in SSc in a clinical setting. In diabetes, others have used local anaesthesia as a pharmacological tool to assess neurovascular regulation in the skin of the feet [20]. They showed that blockade of C-fibres with lidocaine/prilocaine cream impaired axon reflex-mediated vasodilation in healthy volunteers and non-neuropathic diabetic patients, whereas local anaesthesia had no effect in neuropathic diabetic patients [20]. These findings suggest that neurovascular assessment in diabetic patients can serve as a specific tool to evaluate small fibre integrity that allows a reliable detection of subclinical neuropathy [21]. However, the evaluation of axon reflex-dependent vasodilation to assess neurovascular function in diabetes is not standardized. The current study takes a similar approach in patients with SSc and highlights the need for a standardized tool to assess neurovascular regulation in this patient population. Local heating could serve as an appropriate tool for this purpose because protocols are relatively short (median time to peak is 3 min after the onset of probe heating) and non-invasive. Larger studies are needed to assess the link between neurovascular control (as assessed by local heating) and the pathogenesis and severity of SSc.

In conclusion, we show an abnormal digital neurovascular response to local heating in SSc. The initial peak of the cutaneous vascular response to local heating (i.e. thermal hyperaemia) could be monitored as a clinical test for neurovascular function in SSc. Further studies are required to test whether abnormal digital neurovascular response correlate with the degree of peripheral vascular involvement.

Rheumatology key messages

- The axon reflex-dependent peak is blunted in the finger pad of patients with SSc compared with primary RP, suggesting an abnormal neurovascular response to local heating in SSc.
- Digital thermal hyperaemia could be monitored as a clinical test for neurovascular function in SSc.

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II. Methodological issues in the assessment of microvascular reactivity in primary Raynaud's phenomenon

The previous study suggests that neither the initial peak (axon-reflex dependent) nor the plateau (mainly NO-dependent), are impaired in patients with primary RP. This is not consistent with previous findings by Bunker et al, suggesting decreased CGRP containing fibers in both primary and secondary RP [181]. Indeed, one could expect that such a decrease in CGRP would lead to impaired axon reflexes in primary RP as well. In the same way, NO-dependent vasodilation was normal in primary RP, whereas data have reported an impaired dilator effect of NO [13, 14]. This discrepancy suggests that local heating may not be an appropriate tool to investigate microvascular function in primary RP.

As exposure to cold is in most cases the trigger of vasospasms, we set out to test microvascular reactivity to local cooling. Naturally, the hypothesis of impaired microvascular function in response to cold had been studied before in RP patients, usually by immersing the hand in cold water. However, this technique induces systemic sympathetic activation [129], which may interfere with local microvascular response.

Therefore innovative devices to assess skin blood flux while cooling locally were needed. The following article describes the development of a new LDF probe able to cool locally, inspired by the device made by Johnson *et al* [130]. We further tested the reproducibility of a local cooling test on the skin of healthy volunteers, and assessed whether local cooling affects microvascular reactivity in adjacent and contralateral skin areas.

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Regular Article

Reproducibility of a local cooling test to assess microvascular function in human skin

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ABSTRACT

Objective: In the present study we aimed to assess the reproducibility of skin microvascular reactivity while fast cooling locally with a custom-designed laser-Doppler flowmetry (LDF) probe.
Methods: Twenty-two healthy volunteers underwent local 15 °C cooling on the forearm during 5 (protocol 1, $n=12$) or 30 min (protocol 2, $n=10$). Skin blood flow was concomitantly assessed using LDF. Measurements were repeated after 30 min (protocol 1) or 7 days (protocols 1 and 2). Data were expressed as cutaneous vascular conductance (CVC) and percentage of baseline (%BL). Within subject coefficients of variation (CV) and intra-class correlation coefficients (ICC) were calculated.
Results: Immediate reproducibility of the 5-min cooling was very good, either expressed as CVC or %BL (CV were 8% and 18%; ICC were 0.85 and 0.78, respectively). However, the 30-min cooling was the most reproducible at 1 week, either as CVC or %BL (CV were 26% and 23%; ICC were 0.86 and 0.75, respectively). Local cooling was well tolerated by all volunteers.
Conclusions: We propose in the present work a reproducible 30-min LDF cooling test. Such a tool could be of great interest to assess microvascular reactivity to local cooling in diseases such as Raynaud's syndrome, and to further evaluate drugs for such diseases.

Introduction

Microvascular function can be studied routinely in humans by using non-invasive laser Doppler flowmetry (LDF) on the skin. This technique allows quantifying changes in skin blood flow in response to a given stimulus, e.g. local heating or brachial artery occlusion (Cracowski et al., 2006). We and others have shown that such test results were altered in diseases of the digits, such as primary or secondary Raynaud's phenomenon (RP), suggesting a compromised microvascular function in these patients (Boignard et al., 2005; Murray et al., 2006; Roustit et al., 2008). As RP is often induced by exposure to cold (Herrick, 2005), the assessment of microvascular function, while cooling locally, would be of great interest in such patients.

Cooling of the skin to assess vascular reactivity has been performed for several decades. Takeshita et al. (1982) used ice on the forehead to induce central vasoconstriction, measured on the forearm by plethysmography in hypertensive subjects. Microvascular reactivity has also been assessed with LDF after immersion of the hand or a finger in cold water, to test central and local cold-induced vasoconstriction, respectively (Maver and Strucl, 2000). Other techniques have been used, such as flexible cold packs (Cankar and Finderle, 2003) or a stream of carbon dioxide (Lutolf et al., 1993). Because of its relative ease of use, cold water is the most common cooling test, and it has been performed in patients with RP (Foerster et al., 2007). However, this technique induces systemic sympathetic activation (Victor et al., 1987), which interferes with local microvascular response.

To our knowledge, some authors used a more physiological approach, with a custom-designed metal Peltier cooling LDF probe holder (Alvarez et al., 2006; Hodges et al., 2006; Johnson et al., 2005; Thompson-Torgerson et al., 2007; Thompson et al., 2005). It allowed assessment of skin microvascular response while cooling down to 20 °C, in healthy subjects. This kind of device would be of great interest to assess cutaneous microvascular function in diseases such as primary or secondary RP. It could also be helpful as a test to evaluate the effect of drugs on the digital cutaneous blood flow of such patients. However, the reproducibility of such a technique must be tested prior to be used in the field. In addition, from the perspective of a cold-response test in RP, such a probe should be able to reach lower temperatures (down to 15 °C). Finally, the tool must be easy to use, well tolerated and easily manufactured in order to be used routinely.

We thus designed a cooling LDF probe to assess the reproducibility of a fast local cooling test to safely evaluate skin microvascular reactivity down to 15 °C.

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Methods

Technical data about the cooling probe

Design of the probe

The cooling probe (Probe 415-317, Perimed, Järfälla, Sweden) is a prototype which was designed to perform cold provocation while running LDF measurements. The probe is made of a circular aluminium contact plate (10 mm diameter), with paired optic fibres in the centre (transmitter and receiver). It is fixed to the skin with a thermally conductive and optically neutral tape. The probe is filled with silicone to protect the internal electronics from liquids such as water or sweat.

A Peltier element [thermoelectric cooler (TEC)] is used to perform thermoregulation in the probe. An applied voltage induces a temperature gradient, and hence heat flow, across a junction of two different metals. The direction of the heat flow depends on the polarity of the applied voltage, allowing both heating and cooling of a single surface. As a temperature gradient is induced, cooling the skin will cause the opposite side of the TEC to heat up. The heat is then dissipated using a liquid-cooled system, in order to maintain constant skin temperature. It is made of a sealed cooling chamber integrated into the probe head, and connected to a pump via flexible silicone tubes. A peristaltic pump ensures a constant flow, thus avoiding pulsatile flow-induced artifacts in the LDF measurement. Water was chosen as the liquid medium, instead of other organic liquids, which had better thermal conductivity but were potentially harmful.

User interface and regulation algorithm

The device designed to drive the cooling probe includes a simple analogical circuit for temperature reading, a power stage for driving the TEC and a digital signal processor for temperature control. The temperature is set and controlled by the user through a simple interface, between 12 °C and 45 °C. Moreover, it is monitored and recorded on the computer using signal processing software (PeriSoft 2.5.5, Perimed, Järfälla, Sweden). Finally, the rate of cooling/heating can be manually set by the user, from −0.33 to −16 °/min.

Risk analysis

Risk analysis was performed and the risk of burning was analyzed. Only a double fault condition (software plus hardware) may cause damage. If the software detects temperatures outside of the allowable range (0–46 °C), on either side of the TEC, cooling/heating is immediately stopped. The hardware protection operates similarly, however it utilizes a completely separate circuit and set of temperature sensors.

Since contact with the human body is only superficial for this equipment, allergic reaction was the only biocompatible risk taken into account. The risk likelihood was found to be very unlikely and its severity marginal.

Evaluation of the local cooling

Study population

Twenty-two healthy subjects were recruited through local newspaper advertisements. Inclusion criteria included an age of 18 years or older, and no significant medical history. For all subjects, cigarette smoking was a non-inclusion criterion. Grenoble Institutional Review Board approval was obtained and each subject gave written informed consent before participation.

Study design and skin blood flow measurements

This was an open labeled physiology study. Upon arrival to the laboratory, subjects were placed in a temperature-controlled room (23 ± 1 °C) and blood pressure was taken.

All measurements of skin blood flow were carried out using laser Doppler flowmetry (Periflux System 5000, Perimed, Järfälla, Sweden). The local cooling site was chosen on the ventral side of the left upper forearm and equipped with the prototype probe (Probe 415-317, Perimed, Järfälla, Sweden). Before recording, the arm was immobilized with a vacuum cushion to ensure subject positioning (Fig. 1). In our practice, the use of this cushion decreases artifacts associated with arm movement. After a 20-min resting period, a 5-min baseline was recorded at 33 °C.

Local cooling protocols

First protocol. In order to test the reproducibility of a short local cooling at different temperatures, skin was first cooled at 24 °C for 5 min, and then at 15 °C for 5 min (day 0, first cooling). The cooling rate was set to −16 °C/min (maximal rate allowed by the probe). After a 10-min resting period, the probe temperature was set at 33 °C for 5 min and a second series of measurements were carried out (at 24 °C and 15 °C, respectively), to assess short-term reproducibility (day 0, second cooling). The same protocol was performed 7 days later to assess long term reproducibility (day 7, first and second cooling).

In order to test whether local cooling would affect other sites, two temperature-controlled probes (Probe 457, Perimed) were placed 5 cm apart the cooling probe and on the right upper forearm (contralateral site) of six subjects. They were set at 33 °C during the whole measurement.

Second protocol. Local cooling was performed over an extended period of time (30 min) at 15 °C on the forearm, in order to assess the kinetics of cutaneous blood flow variations. The second protocol was

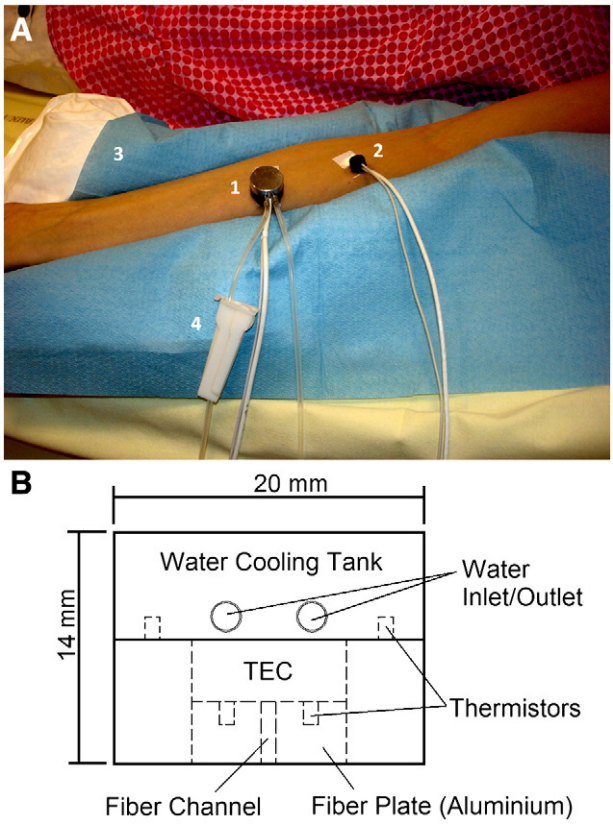

Fig. 1. (A) LDF cooling probe prototype (1) and 5-cm control site probe (2). A vacuum cushion (3) was used to decrease artifacts associated with arm movement. The liquid cooling system is made of silicon tubes with a cutting wheel (4) to regulate water flow. (B) Schema of the cooling probe prototype. TEC: thermoelectric cooler.

repeated at day 7. As in the first protocol, the cooling rate was set to −16 °C/min.

Data analysis

The data were stored on a computer and analyzed offline with signal processing software (PeriSoft 2.5.5, Perimed). Skin blood flow was expressed as cutaneous vascular conductance in mV/mm Hg (CVC, flux in millivolts divided by mean arterial pressure), averaged over 3 min at baseline after each probe temperature change (protocol 1), and averaged over 5 min at baseline and between the 25th and the 30th minute of cooling at 15 °C (protocol 2). Expressing data as conductance is more of a physiological approach, as it takes into account differences and variations in blood pressure (O'Leary, 1991). In order to take into account baseline flux variations, we also expressed data as a percentage of baseline flux, as previously described (Johnson et al., 2005). A minute by minute analysis of median CVC (interquartile range) was performed to assess the kinetics of the response in the second protocol.

Finally, we analyzed mean temperature of the probe over time to verify the cooling rate and to assess temperature stability during a 30-min cooling.

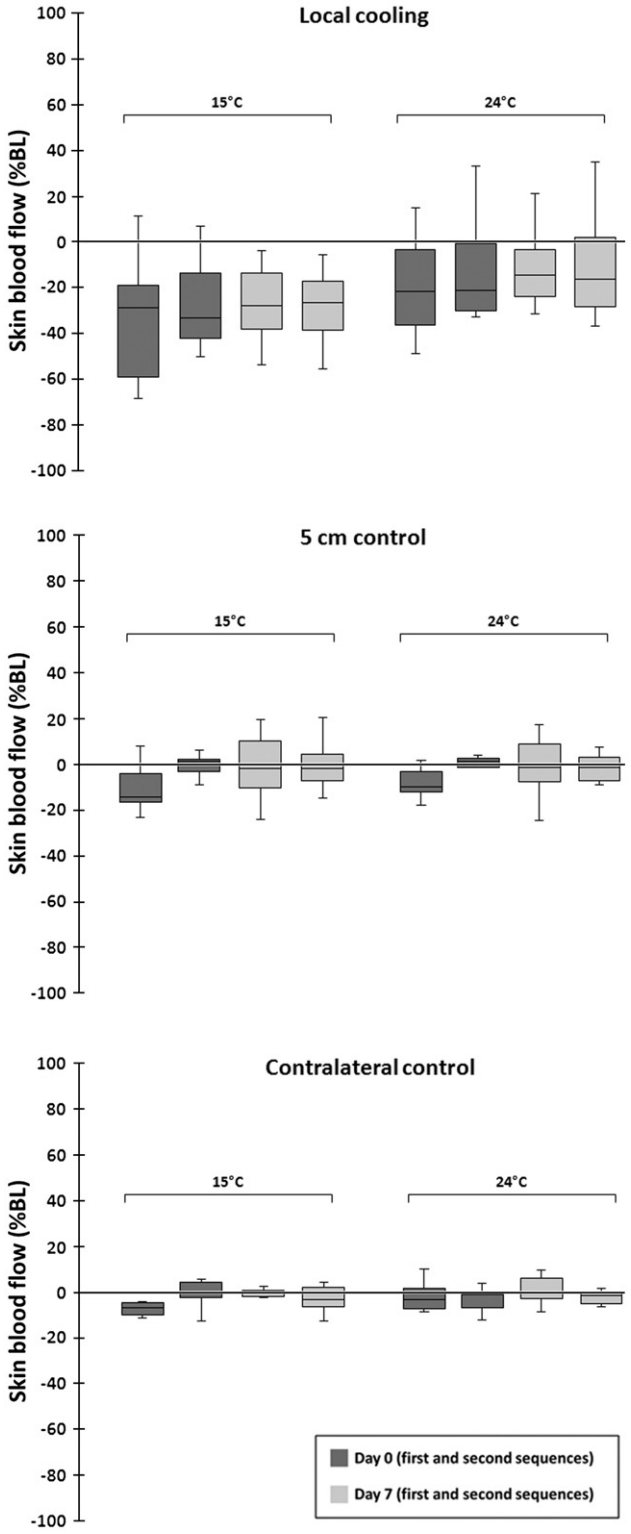

Fig. 2. Effect of a 15 °C and a 24 °C local cooling at day 0 (dark plots, 2 sequences) and at day 7 (light plots, 2 sequences), with ipsilateral (5 cm) and controlateral control sites. Data are expressed as median percentage of baseline (%BL).

Statistical analysis

Quantitative data are expressed as the median and interquartile range in parenthesis. Quantitative data were analyzed with the Friedman test and with the Wilcoxon test for paired analyses, with each subject serving as his/her own control. We considered P values <0.05 as significant, corrected by Bonferroni's method for multiple comparisons. Short-term and 7-day reproducibility was expressed as within subject coefficients of variation (Bland, 2000; Donald et al., 2008) and repeatability was expressed as intra-class correlation coefficients (ICC) (Bland, 2000). ICC values of <0.40, 0.40 to 0.75 and >0.75 represent poor, fair to good and excellent agreements, respectively (Landis and Koch, 1977). Bland–Altman plots were used to assess the agreement between day 0 and day 7 measurements (Bland, 2000).

Results

First protocol: reproducibility of a 5-min local cooling

Twelve subjects were enrolled in the first protocol. Among them, data from 11 volunteers were analyzed (we encountered technical problem in one case). In all series, we observed as expected a significant positive relationship between skin temperature and skin blood flow with minimum skin perfusion at 15 °C (P<0.01 for all series) (Fig. 2). Baseline CVC were not significantly different between each series (P=0.1). Median (interquartile) percentages of baseline at 15 °C were not significantly different either: −28.7 (44.6) (first cooling) and −33.3 (34.6) (second cooling) at day 0, and −27.7 (27.4) (first cooling) and −26.3 (24.6) (second cooling) at day 7 (P=0.2).

Short-term reproducibility was good to excellent at 15 °C when data were expressed either as CVC or as a percentage of baseline (Table 1). At 1 week however, we observed a loss of the correlation when data were expressed as CVC whereas reproducibility was fair

Table 1
Short term and 7-day reproducibility of skin local cooling expressed as cutaneous vascular conductance (CVC, in mV/mm Hg) or as a percentage of baseline flux (%BL).

		15 °C local cooling			
		CVC		%BL	
		ST	7-day	ST	7-day
5 min cooling (n=11)	CV	8	27	18	26
	ICC	0.85	−0.04	0.78	0.64
30 min cooling (n=10)	CV	NA	26	NA	23
	ICC	NA	0.86	NA	0.75

Reproducibility results are expressed as within subject coefficients of variation (CV) and repeatability is expressed as intra-class correlation coefficient (ICC) (Bland, 2000). ICC values of <0.40, 0.40 to 0.75 and >0.75 represent poor, fair to good and excellent agreements, respectively (Landis and Koch, 1977). ST: short-term reproducibility; 7-day: 7-day reproducibility; NA: not applicable.

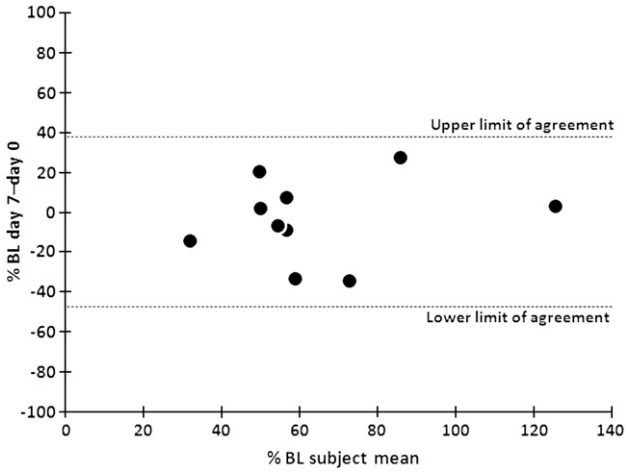

Fig. 3. Bland–Altman plots of the percentage of baseline flux (% BL) after a 30-min cooling at 15 °C. Horizontal dash lines represent the 95% limits of agreement.

to good when data were expressed as a percentage of baseline (Table 1).

Short term reproducibility at 24 °C was good when expressed as CVC (CV = 12%, ICC = 0.75). Repeatability was poor however when expressed as a percentage of baseline (ICC = −0.15). In the same way, we observed poor reproducibility at day 7, expressed either as CVC or as a percentage of baseline (CV = 122 and 110%, respectively).

Regarding control sites, we observed no CVC variation between baseline and after cooling, neither at the 5 cm control site nor on the contralateral arm (Fig. 2).

Second protocol: 1 week reproducibility of a 30-min local cooling

Ten subjects were enrolled in the 30-min cooling protocol. Median percent of baseline while cooling at 15 °C were −43.3 (24) at day 0 and −47 (10) at day 7 (CVC were 0.46 (0.33) mV/mm Hg and 0.48 (0.33) mV/mm Hg, respectively). Seven day reproducibility of this parameter was good, either expressed as CVC or as a percentage of baseline (Table 1). Bland–Altman plots show a good comparability of the measurements at day 0 and day 7, as all subjects appear within the 95% limits of agreement (Fig. 3).

The minute by minute analysis showed a high variability of the first 10 min of the tracing. Indeed, we first observed a quick decrease of CVC after the cooling onset, followed by an inconstant vasodilation after 5 to 10 min of cooling, and finally, a prolonged decrease which stabilizes after approximately 20 min (Fig. 4A).

Analysis of the probe temperature over time showed the fast rate of cooling and revealed the excellent stability of temperature during a 30-min local cooling (Fig. 4B).

Tolerance

In both protocols, none of the subjects complained of pain associated with the local cooling. No adverse effects were observed and notified to the safety committee.

Discussion

We showed that our prototype of a LDF probe coupled to a Peltier element and a liquid-cooled system allowed prolonged, stable, and safe local skin cooling at 15 °C for 30 min. One week reproducibility of the concomitantly assessed microvascular vasoconstriction was good. We observed no effect on ipsilateral and contralateral control sites.

In this study, we showed as expected a positive relationship between local skin temperature and skin blood flow. Indeed, vasoconstriction was more pronounced at 15 °C than at 24 °C (about −30 versus −20% of baseline decrease, respectively). These results are lower than that found by Hodges et al. (2006) while cooling at 24 °C, but the cooling protocols are not comparable. Indeed, Hodges et al. held skin temperature at 34 °C for baseline assessments, whereas we held it

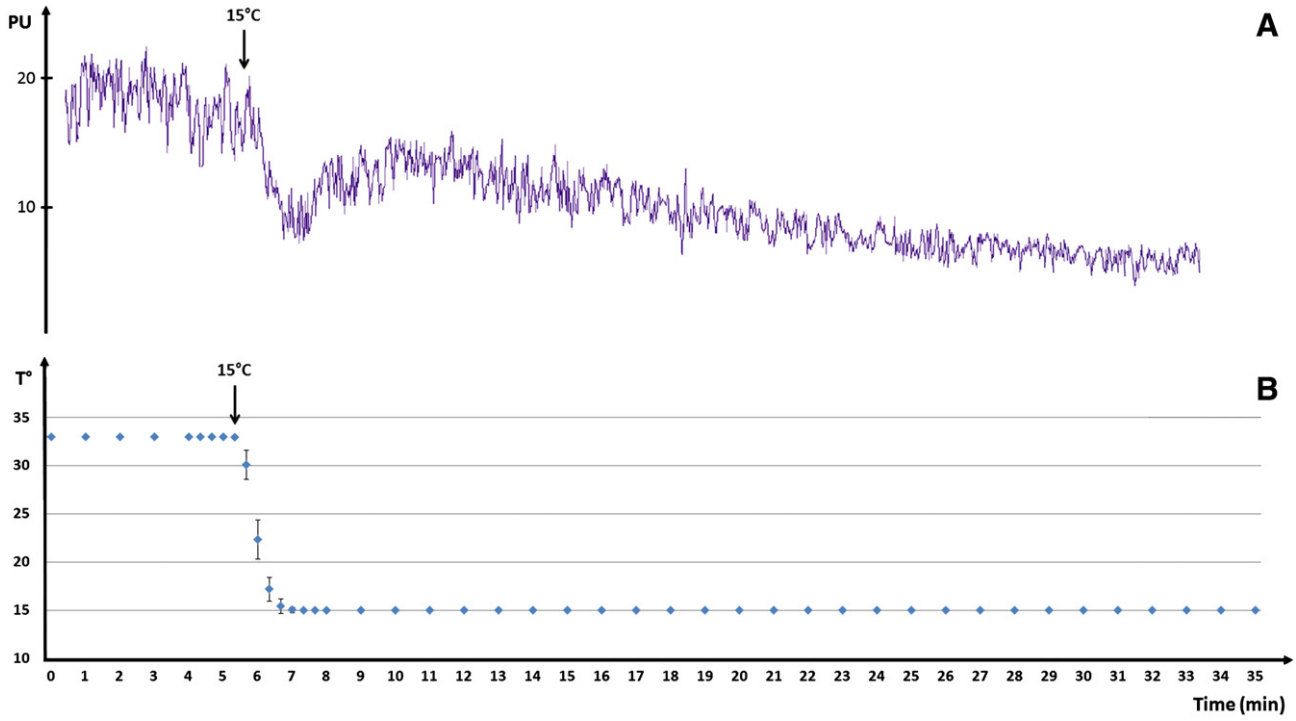

Fig. 4. (A) Typical tracing of a 30-min local cooling on the forearm at 15 °C. An inconstant cold-induced vasodilation is observed within the first 10 min. Data are expressed as perfusion units (PU). (B) Mean (standard deviation) temperature (T°) of the probe during a 30-min cooling at 15 °C. Day 0 and day 7 data were pooled for all subjects enrolled in the second protocol.

at 33 °C in the present work. Moreover, the larger cooling area used in their study may be playing a role in the increased vasoconstriction. The 30-min cooling at 15 °C further decreased CVC (more than −40% of baseline). Despite the cooling velocity, local cooling was not painful. In addition, we observed no side effects in any subject.

We used within subject coefficients of variation (CV) to express reproducibility of our data, as previously described (Bland, 2000; Donald et al., 2008; Harris et al., 2007). In order to assess the reliability of the measurement, we also calculated intra-class correlation coefficients (ICC) (Bland, 2000). Considering that CV <35% were deemed acceptable and that ICC >0.75 represent excellent agreements (Harris et al., 2007), we showed an excellent reproducibility of short term 5-min cooling at 15 °C. Indeed, CVs were lower than that obtained for the long term (7 days) reproducibility. As we did not use markers to identify recording sites on the skin, this discrepancy may be explained by the spatial variation due to the wide disparity in skin capillary density (Cracowski et al., 2006; Johnson et al., 1984). Our results further confirm this hypothesis as expressing data as a percentage of thermoneutral baseline (33 °C) improves 7-day reproducibility of a short cooling. Despite higher CV, 7-day reproducibility and repeatability remain very good considering that the probes were not applied on the same skin sites. This is of interest as identification of skin sites to perform measurements precisely on the same area over time is not easy in a routine use.

Although reproducibility was good at 15 °C, we obtained after the first protocol a high variability of the response to a 24 °C local cooling. When looking at detailed data, we observed in some subjects a cold-induced vasodilation in the 5 min following the cooling onset. This phenomenon had already been described when cooling locally from 34 °C to 24 °C (Yamazaki et al., 2006). According to the work of Yamazaki et al. (2006), it may be explained by the rate of cooling. Indeed, fast cooling (−4 °C/min) is associated with an early vasodilation whereas slow cooling (0.33 °C/min) does not induce such dilation. In our study, the rate of cooling was even faster (−16 °C/min), which may explain the poor reproducibility of the 24 °C local cooling. However, when we cooled down from 24 to 15 °C this vasodilation was less obvious and the reproducibility was better.

These observations strengthened the interest of a prolonged cooling to assess the kinetics of such response and to optimize reproducibility. In the second protocol, we then observed in some but not all volunteers the typical pattern described by Yamazaki et al. These findings agree with our hypothesis of a high variability of the 5-min cooling due to early cold-induced vasodilation. Indeed, this phenomenon did not affect the 7-day reproducibility of blood flux measurement after a 30-min cooling at 15 °C, which was good whether expressed as CVC or as a percentage of baseline. Considering these data, we strongly suggest that prolonged cooling should be preferred as a cooling test to short cooling when measuring skin blood flux in such conditions (especially regarding the cooling rate).

The microvascular reactivity during local skin cooling involves different mechanisms, including sympathetic adrenergic function and local control (Johnson, 2007). As a consequence, in order to test whether local cooling with our probe could induce reflex cutaneous vasoconstriction at distant sites, we simultaneously recorded CVC 5 cm from the cooling site and on the contralateral arm. No effect was observed on ipsilateral and contralateral control sites, neither at 24 °C nor at 15 °C. This suggests that using our prototype, there is no apparent systemic cutaneous vascular response, unlike the cold water immersion test or the use of cold packs (Cankar and Finderle, 2003).

The prototype we used to assess the reproducibility of the local cooling test offers many possibilities concerning the temperature and the rate of cooling. In the present paper however, our objective was not to perform exhaustive test of the device. Indeed, we aimed to design a local cooling test and to assess its reproducibility, with the perspective of using it to assess microvascular reactivity in patients with RP. As a consequence, we had to choose cooling parameters close to those able to induce RP, i.e. temperature below 24 °C and fast cooling. Moreover, the fast cooling was mandatory to be able to detect whether there is abnormal cold-induced vasodilation in such patients. Indeed, this hypothesis should be tested in a future study.

Cooling tests have aroused great interest to test drug activity in diseases affecting microcirculation (Cracowski et al., 2005; Foerster et al., 2007; Hahn et al., 1995; Maricq et al., 2000). However, there is no method standardization. Furthermore, most of the techniques used, such as immersion in cold water, are too complex for routine use and may be painful in such patients and induce systemic response. Laser Doppler flowmetry coupled to local cooling could offer a simple alternative to avoid these issues.

In conclusion, we propose in the present work a reproducible 30 min LDF cooling test at 15 °C, expressing data as CVC or as a percentage of thermoneutral baseline. This local cooling test was well tolerated by all volunteers. Such a tool could be of great interest to assess cutaneous vascular response to local cooling in diseases such as Raynaud's syndrome. It could therefore be a promising test for the evaluation of drugs in such diseases.

Acknowledgments

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III. Impaired transient vasodilation and increased vasoconstriction to digital local cooling in primary Raynaud's phenomenon

The local cooling test described in the previous study was well tolerated and reproducible [20]. We also observed the typical pattern of microvascular skin blood flux when cooling locally on the forearm, i.e. initial vasoconstriction followed by transient vasodilation and prolonged vasoconstriction [123]. The ROCK pathway is involved in cooling-induced vasoconstriction [131], but local cooling also exerts a significant portion of its vasoconstrictive effect through inhibition of the NO system [123]. Moreover, sensory nerves are involved in the transient vasodilation [202].

The objective of the next part of our work was to compare microvascular reactivity to local cooling in participants with primary RP compared to matched controls. We hypothesized that vasoconstriction was increased in primary RP, and aimed at assessing which phase(s) of the response was involved. In order to assess the role of sensory nerves, we pre-treated one skin site with lidocaine/prilocaine, as previously described [19].

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Impaired transient vasodilation and increased vasoconstriction to digital local cooling in primary Raynaud's phenomenon

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Roustit M, Blaise S, Millet C, Cracowski JL. Impaired transient vasodilation and increased vasoconstriction to digital local cooling in primary Raynaud's phenomenon. *Am J Physiol Heart Circ Physiol* 301: H324–H330, 2011. First published may 13, 2011; doi:10.1152/ajpheart.00246.2011.—Raynaud's phenomenon (RP) is defined as episodic ischemia of the extremities in response to cold. Although the structure of skin capillaries is normal in primary RP, some data suggest impairment of microvascular function. We aimed at testing whether digital skin blood flow was lower in RP than in controls while cooling locally. We further evaluated the contribution of sensory nerves in the response. We recruited 21 patients with primary RP and 20 healthy volunteers matched on age and gender. After a 10-min baseline at 33°C, skin temperature was cooled at 15 or 24°C during 30 min on the forearm and the finger while monitoring perfusion with a custom-design laser Doppler flowmetry probe. Perfusion was also assessed after topical anesthesia. Blood flow was expressed as cutaneous vascular conductance (CVC). Data were subsequently expressed as area above the curve (AAC_{0-30}) of the percentage decrease from baseline CVC (%BL). CVC on the dorsum of the finger was lower in RP patients compared with controls at 15°C (AAC_{0-30} were 106,237.2 and 69,544.3%BL·s, respectively; $P = 0.02$) and at 24°C (AAC_{0-30} were 86,915 and 57,598%BL·s, respectively; $P = 0.04$) whereas we observed no significant difference on the finger pad and the forearm. Topical anesthesia increased CVC in patients with RP ($P = 0.05$), whereas it did not affect reactivity in controls ($P = 0.86$). Our study shows exaggerated skin microvascular vasoconstriction to local cooling on the dorsum of the finger in primary RP compared with controls. Part of this abnormal response in primary RP depends on sensitive nerves.

microcirculation; skin blood flow

FIRST DESCRIBED BY MAURICE RAYNAUD in 1862 (25), Raynaud's phenomenon (RP) is defined as episodic ischemia of the extremities in response to cold or emotional stimuli, often accompanied by pain. It presents clinically as pallor, cyanosis, and often rubor of the skin. Pallor indicates vasospasm and decreased blood flow, and cyanosis is a sign of deoxygenation of the static venous blood, while rubor implies reactive hyperemia (2). RP can be primary (i.e., idiopathic) or secondary to a connective tissue disease. The pathophysiology of primary RP is multifactorial and complex, with both vascular and neural abnormalities (7, 15). Although available evidence suggests that microvascular function is impaired in primary RP, the exact mechanism of such dysfunction remains poorly understood.

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Several features of primary RP pathophysiology that could explain decreased vasodilation have been suggested, including a loss of calcitonin gene-related peptide (CGRP) containing nerve fibers in the digits (5) or increased endothelin-1-dependent vasoconstriction in RP (15). Another mechanism could involve postjunctional α_{2C}-adrenoreceptors, which are clustered distally, whereby increasing translocation of α_{2C}-adrenoreceptors from cytosol to cell surface occurs during cooling, thus enhancing contraction (7). In vitro, Furspan et al. (13) showed increased cooling-induced α_2-adrenergic constriction of arterioles isolated from patients with primary RP compared with controls, suppressed by protein tyrosine kinase inhibitors. The role of the RhoA/Rho kinase (ROCK) pathway in mediating vasoconstriction after local cooling in healthy subjects has been confirmed in vivo (31). Indeed, cold-induced activation of ROCK may initiate constriction, mediated by both α_{2C}-adrenoreceptors (through their mobilization from the Golgi apparatus to the vascular smooth muscle plasma membrane) and direct calcium sensitization (10). Therefore, an impaired signal transduction pathway could be involved in the pathophysiology of RP (10), but this remains to be confirmed.

Local cooling of the skin induces an initial vasoconstriction followed by a transient vasodilation and, finally, a prolonged vasoconstriction (18). The initial vasoconstriction would be mainly dependent on norepinephrine and mediated by the ROCK pathway (by translocating α_{2C}-adrenoreceptors; Ref. 31), whereas the prolonged vasoconstriction probably involves both the ROCK and the nitric oxide (NO) pathways (18). On the other hand, sensory nerves could play a role in the transient vasodilation, which is less well understood (18). Such transient vasodilation is more obvious when the cooling is rapid (35).

Assessment of the microvascular response to cooling in RP is of interest so as to better understand its pathophysiology. However, it is challenging as most tests (i.e., cold pressure test, systemic cooling) induce a tremendous increase in the sympathetic response, overshadowing the local reactivity. Therefore, to study microvascular reactivity due to local cooling in RP, we developed a local cooling test (26) using a cooling laser Doppler flowmetry (LDF) probe inspired by the device made by Johnson et al. (19). This test was reproducible, well tolerated, and did not induce distant sympathetic activation of the skin of healthy subjects (26).

We hypothesized that skin microvascular response to local cooling would be exaggerated in patients with primary RP compared with controls. The primary objective of this study was therefore to compare cutaneous vascular conductance (CVC) in patients with primary RP and matched controls by using a 30-min local cooling test on the dorsum of the finger and the finger pad. In addition, we aimed at assessing which phase would be affected in primary RP (i.e., increased initial

vasoconstriction, impaired transient vasodilation, and/or exaggerated prolonged vasoconstriction). As transient vasodilation is mediated through sensory nerves, we also compared conductance after local anesthesia. Finally, we tested the safety of our device in patients with primary RP.

METHODS

Study population. All the participants enrolled in this study were recruited through local newspaper advertisements and included between February 2009 and February 2010. All subjects were 18 yr of age or older. Patients with primary RP were diagnosed according to the criteria of LeRoy and Medsger (20). Subjects taking calcium-channel blockers were instructed to stop medication 1 wk before enrolment in the study.

Noninclusion criteria included cigarette smoking and any associated chronic disease. Additional noninclusion criteria for patients with RP included abnormal capillaroscopic pattern (8). Antinuclear autoantibodies were assessed for all participants. In case of positive antinuclear autoantibodies (>80 UI/ml), specific autoantibodies against topoisomerase I (Scl–70) or centromere-associated proteins were sought. Positive autoantibodies against topoisomerase I (Scl–70) or centromere-associated proteins were a noninclusion criterion.

The investigation conforms with the principles outlined in the Declaration of Helsinki. Grenoble Institutional Review Board approval was obtained on August 2, 2008, and each subject gave written informed consent before participation.

Study design. All subjects were fasted and all experiments were performed in a temperature-controlled room (24 ± 1°C). After clinical examination, the participants remained supine for the whole duration of the experiments, with forearms resting at heart level. Blood pressure was recorded continuously by using digital photoplethysmography (Nexfin monitor; Bmeye B.V., Amsterdam, The Netherlands) during skin blood flow measurements. Before recording started, the arm was immobilized with a vacuum cushion to ensure stable positioning (Fig. 1).

Five skin sites were randomly chosen on the fingers between the index, the middle, and the ring finger. Among them, two sites were chosen on the finger pad (*sites 1* and *2*, on 2 distinct fingers of the left hand), and three on the dorsum of the finger [middle phalanx, 1 on the left hand (*site 3*) and two on distinct fingers of the right hand (*sites 4* and *5*)]. For patients with primary RP, all sites were chosen on fingers affected by RP. Maricq color charts (22) were used to confirm the diagnosis of RP among the index, the middle, and the ring finger and to specify its topography. When the three fingers were equally affected by RP, two of them were randomly chosen. Another site (*site 6*) was selected on the ventral side of the left upper forearm, more than 5 cm from the elbow and the prominence of the wrist, avoiding visible veins. Local cooling started after a 30-min resting period for acclimation.

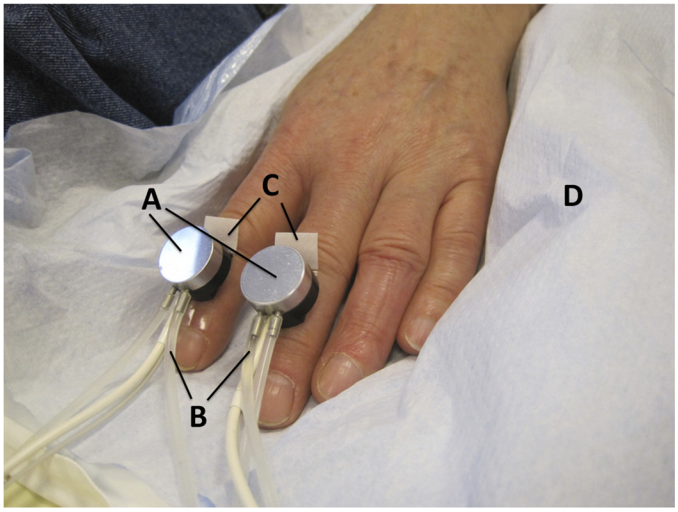

Fig. 1. Single-point laser Doppler probes (*A*) with a liquid cooling system made of silicon tubes (*B*). *C*: probes were fixed with double-sided tape. *D*: a vacuum cushion was used to decrease artifacts associated with arm movements.

Local cooling and skin blood flow measurements. Cutaneous blood flow was assessed by LDF (Periflux System 5000; Perimed, Järfälla, Sweden) by using custom-designed LDF cooling probes (Probe 415-317; Perimed; Ref. 26). Risk analysis was performed and was fully compatible with human use.

Baseline skin temperature was maintained at 33°C, and blood flow was recorded over 10 min. We standardized baseline skin temperature because we had previously observed a tendency to lower baseline skin blood flow in patients with primary RP compared with healthy controls (28, 30). Afterwards, local cooling tests were sequentially performed as follows: *sequence 1*: local temperature was decreased to 15°C (*sites 1* and *6*); *sequence 2*: cooling at 24°C (*sites 2* and *3*); and *sequence 3*: cooling at 15°C (*sites 4* and *5*). This design allowed study of microvascular reactivity at both 15 and 24°C on the finger pad and on the dorsum of the finger. On the forearm, the temperature was decreased to 15°C only. All the tests were performed on distinct skin sites, and the reproducibility of the test has been previously demonstrated (26).

In *sequence 3*, local cooling was performed after topical anesthesia (*site 5*) to assess whether local digital neurovascular control was affected in patients with primary RP. Before the local cooling was started, 2 g of lidocaine/prilocaine cream (5-g tubes containing 125 mg lidocaine and 125 mg prilocaine; Anesderm, Pierre Fabre, Boulogne, France) was placed on *site 5* over a 1-cm^2 skin surface. Subsequently, an occlusive transparent dressing was placed over the cream to enhance cutaneous diffusion. No cream was placed on the other sites. After 1 h, lidocaine/prilocaine cream was removed with a cotton swab and the cooling probe was positioned on *site 5*. The anesthetized skin area was larger than the size of the local cooling probe.

Data analysis. Data were digitized, stored on a computer, and analyzed off-line with signal processing software (PeriSoft 2.5.5; Perimed). Skin blood flow was expressed as CVC (flux in millivolts divided by mean arterial pressure). Expressing data as conductance is more of a physiological approach, as it takes into account differences and variations in blood pressure (24). To take into account baseline (BL) flux variations, data were subsequently expressed as a percentage decrease from baseline CVC, as previously described (19). Baseline CVC was averaged over 5 min just before cooling onset. Then, a minute-by-minute analysis of CVC was performed to assess the kinetics of the response (CVC was averaged over 20 s, providing 3 points/min), and data were expressed as area above the curve over the 30-min cooling period (AAC_{0-30}, in %BL·s) as the primary outcome. We subsequently analyzed the three phases of the response: *1*) initial vasoconstriction: CVC was averaged over 1 min around the lowest flux value within the first 5 min; *2*) transient vasodilation: CVC was averaged over 1 min around the highest flux value within the first 15 min; and *3*) late prolonged vasoconstriction: CVC was averaged over the last 3 min of the measurement.

Statistical analysis. Quantitative data are expressed as the mean (SD). Qualitative data are expressed as number and percentage. Repeated-measures ANOVA were used to compare evolution of CVC over time between the two groups. Mauchly's test of sphericity was used to assess equality of variance in the data. When significant (i.e., inequality of variance cannot be excluded), Greenhouse-Geisser adjustment was used. We tested the effect of the phase, of the group, as well as the interaction between phase and group. To compare the difference in phases between the two groups, we performed a post hoc analysis of covariance as previously described (33). Paired *t*-tests

Table 1. *Demographic and clinical characteristics of patients with primary RP and controls*

	Controls (n = 20)	Primary RP (n = 21)
Age, yr	42.8 (16.2)	43 (18.5)
Female	15 (75)	16 (76)
Body mass index	22.6 (3.4)	21.1 (2.3)
Blood pressure (MAP), mmHg	94.3 (10.2)	98.3 (20.5)
RP: duration, yr	NA	14.2 (11.3)
RP: number of fingers involved	NA	7.9 (1.6)
RP: thumb involved	NA	5 (24)
RP: other locations (feet, ears, or nose)	NA	3 (14)

Quantitative data are expressed as mean (SD). Qualitative data (female, thumb involvement, and other locations) are expressed as number (percentage). RP, Raynaud's phenomenon. MAP, mean arterial pressure; NA, not applicable.

were used to analyze the effect of lidocaine/prilocaine in each participant. Two-sided significance tests were used throughout. P values of <0.05 were considered significant.

RESULTS

Population characteristics. The demographic and clinical characteristics of the 41 participants enrolled in the study are listed in Table 1. One of the participants initially included in the control group was diagnosed with primary RP and was therefore switched to the other group, thus explaining the difference in sample size between the two groups. Mean serum creatinine was 70.4 (10.3) μmol/l, glycemia was 4.5 (0.8) mmol/l, and total cholesterol was 1.9 (0.4) g/l. There was no significant difference between the groups.

Three participants (2 patients with primary RP and 1 control) had positive antinuclear antibodies. None of them had positive autoantibodies against topoisomerase I (Scl-70) or centromere-associated proteins; clinical examination and anamnesis did not argue for a connective-tissue disease. The nailfold videocapillaroscopy pattern was normal in all subjects.

Seven women were taking oral contraceptives (3 with primary RP and 4 in the control group). Women were matched in terms of hormonal status (menopause and hormonal phase) between controls and patients with primary RP.

Baseline CVC. Before cooling at 15°C, CVC on the finger pad of participants with primary RP and controls was 13.6 (9.9) and 10.86 (8.2) mV/mmHg, respectively (P = 0.36). On the dorsum of the finger, baseline CVC was 4.39 (3.6) in primary RP patients compared with 3.06 (1.51) mV/mmHg in controls (P = 0.14). On the forearm, baseline CVC at 33°C was 0.95 (0.5) in participants with primary RP and 0.64 (0.3) mV/mmHg in controls (P = 0.03).

Baseline CVC on the finger pad before cooling at 24°C was 11.8 (10.4) mV/mmHg in participants with primary RP and 10.1 (7.2) mV/mmHg in controls (P = 0.55). On the dorsum of the finger, it was 3.52 (2.9) mV/mmHg in primary RP patients compared with 1.96 (1.9) mV/mmHg in controls (P = 0.07).

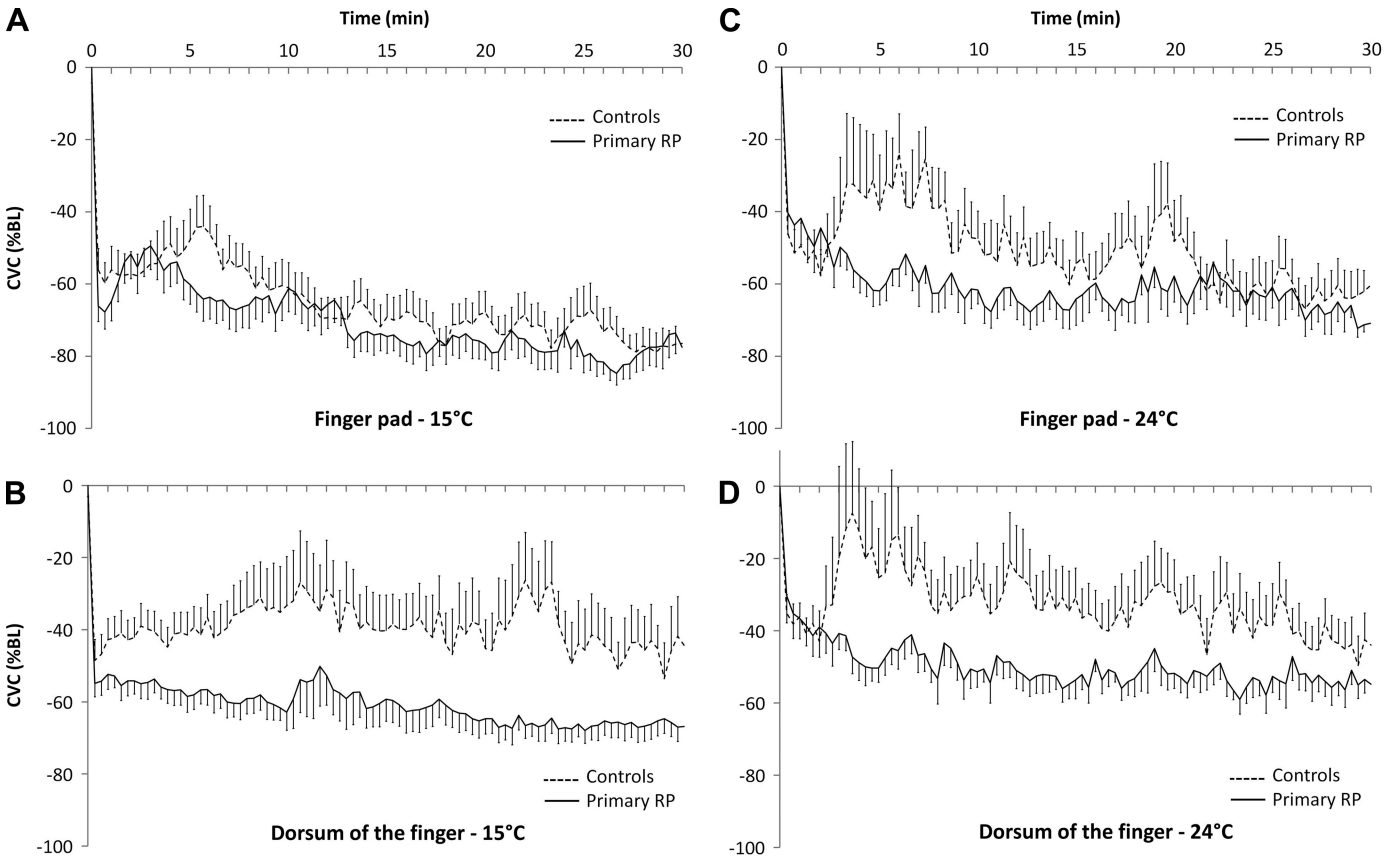

Fig. 2. Mean (SE) cutaneous vascular conductance (CVC; expressed as a percentage decrease from baseline CVC) in participants with primary Raynaud's phenomenon (RP; plain lines) and controls (dash lines). Local cooling to 15°C (*A* and *B*) and 24°C (*C* and *D*) was performed on the finger pad (*A* and *C*) and the dorsum of the finger (*B* and *D*).

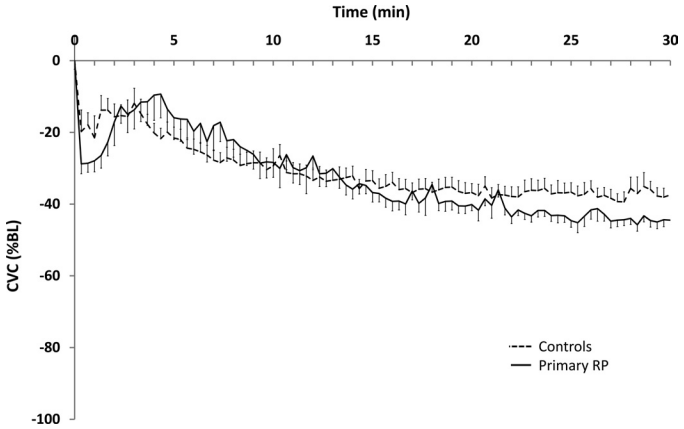

Fig. 3. Mean (SE) CVC (expressed as a percentage decrease from baseline CVC) in participants with primary RP (plain line) and controls (dash line) while cooling to 15°C on the forearm.

Microvascular reactivity to local cooling. Qualitatively, we observed a similar pattern of skin blood flow during local cooling on the fingers of healthy subjects (Fig. 2) to that described on the forearm, i.e., initial vasoconstriction, followed by transient vasodilation and prolonged vasoconstriction (Fig. 3).

On the dorsum of the finger, overall vasoconstriction ($AAC_{0–30}$) was more pronounced in patients with RP than in controls, both at 15 and 24°C (Table 2). At 15°C, the interaction between group and phase is not significant, suggesting that the difference affected in the same way all the phases of the response (Table 2). However, the interaction between group and phase is significant at 24°C, suggesting that both groups behave differently over time. Post hoc analysis showed that the difference between groups is significant for transient vasodilation (Table 2).

On the finger pad at 15°C, the cooling-induced decrease in CVC was comparable between patients with primary RP and controls (Table 2). Qualitatively, the pattern of response at 24°C was different between the two groups, with a blunted initial transient vasodilation in patients with RP (Fig. 2). Again, the interaction between group and phase being significant, both groups behave differently over time. Post hoc analysis showed that the difference between groups is significant for transient vasodilation (Table 2).

Vasoconstriction while cooling at 15°C on the forearm was similar between controls and patients with RP (Table 3; Fig. 3).

Effect of lidocaine/prilocaine on microvascular reactivity during local cooling at 15°C. Local anesthesia with lidocaine/prilocaine cream did not affect microvascular reactivity to local cooling on the dorsum of the finger in controls (Table 4; Fig. 4). However, in patients with primary RP, pretreatment with lidocaine/prilocaine suppressed the exaggerated vasoconstriction of the initial phase of the vasomotor response during cooling locally at 15°C, with AUC values similar to those of controls (Table 4; Fig. 4).

Safety. None of the subjects complained of pain associated with the local cooling and local cooling did not induce any symptoms of RP among the patients. No adverse effects were observed requiring notification to the safety committee. Blood pressure did not change significantly during the measurements.

DISCUSSION

The present study shows decreased microvascular perfusion in response to local cooling on the dorsum of the finger of patients with primary RP compared with controls, using an original local cooling test. Both transient vasodilation and prolonged vasoconstriction were affected when cooling down to 15°C. Moreover, local anesthesia suppressed the increased vasoconstriction during the initial phase seen in patients with RP.

Knowledge about the mechanisms underlying the microvascular response to local cooling has greatly improved in the past few years, especially through the work by the Johnson, Kel-

Table 2. *Digital skin microvascular reactivity to local cooling to 15 and 24°C in participants with primary RP and controls*

	Group		*P* Value		
	Controls	Primary RP	Group	Phase	Interaction
Finger pad 15°C					
Overall ($AAC_{0–30}$)	117,550 (39,226)	124,594 (32,193)	0.45		
Initial VC	−63.50 (24.8)	−66.44 (18.5)	0.4	<0.001	0.62
Transient VD	−30.73 (41.5)	−45.12 (29.3)			
Prolonged VC	−78.38 (21)	−80.94 (18.6)			
Dorsum 15°C					
Overall ($AAC_{0–30}$)	69,544 (66,481)	106,237 (33,068)	0.02		
Initial VC	−52.46 (19.6)	−62.23 (14.4)	0.04	<0.001	0.35
Transient VD	−18.63 (47.4)	−44.15 (44.3)			
Prolonged VC	−49.70 (39.1)	−70.09 (17.8)			
Finger pad 24°C					
Overall ($AAC_{0–30}$)	90,558 (49,477)	105,483 (33,439)	0.19		
Initial VC	−64.43 (20.8)	−65.08 (18.2)	0.08	0.001	0.05
Transient VD	−4.19 (82.9)	−50.88 (26.8)*			
Prolonged VC	−65.59 (23.1)	−68.77 (19.3)			
Dorsum 24°C					
Overall ($AAC_{0–30}$)	57,598 (59,045)	86,915 (27,301)	0.04		
Initial VC	−50.49 (17.9)	−50.60 (17.1)	0.15	0.02	0.02
Transient VD	2.21 (111)	−39.31 (19.6)*			
Prolonged VC	−43.81 (30.5)	−54.19 (19.5)			

Data are expressed as area above the curve of CVC decrease from baseline (in %BL/s) over the 30-min cooling (AAC_{0-30}). Each phase of the response, i.e., initial vasoconstriction (VC), transient vasodilation (VD), and prolonged VC, is expressed as CVC decrease from baseline (in %BL). Data were analyzed with repeated-measures ANOVA between groups over time. *$P < 0.05$ vs. controls, post hoc analysis of covariance.

Table 3. *Skin microvascular reactivity to a 15°C local cooling on the forearm of participants with primary RP and controls*

	Group		*P* Value		
	Controls	Primary RP	Group	Time	Interaction
Overall ($AAC_{0–30}$)	55,472 (18,977)	57,183 (19,464)	0.67		
Initial VC	−24.05 (13.2)	−31.22 (10)	0.34	<0.001	0.4
Transient VD	−10.47 (20)	−7.79 (31.3)			
Late VC	−41.28 (11.4)	−47.95 (8.8)			

Data are expressed as area above the curve of CVC decrease from baseline (in %BL/s) over the 30-min cooling (AAC_{0-30}). Each phase of the response, i.e., initial VC, transient VD, and prolonged VC, is expressed as CVC decrease from baseline (in %BL). Data were analyzed with repeated-measures ANOVA between groups over time. *$P < 0.05$ vs. controls, post hoc analysis of covariance.

logg, Flavahan, and Kenney groups (18, 31). Briefly, direct cooling of the skin (on the forearm) first induces an initial vasoconstriction followed by a transient vasodilation, within the first 10–15 min after cooling onset, followed by prolonged vasoconstriction. This involves inhibition of the NO system, postsynaptic upregulation of α_{2C}-adrenoreceptors through the ROCK pathway and cold-sensitive afferents (18).

In the present work, we did not observe any difference in the initial vasoconstriction between patients with primary RP and controls. On the contrary, Lütolf et al. (21) showed significant difference in digital skin blood flux recorded with single-point LDF while cooling locally. However, the cooling protocols were different as they used a stream of CO_2 at −10°C on the nailfold during 60 s.

By using a simple local cooling test with a custom-designed LDF probe inspired from the work of these groups, we observed increased long-term vasoconstriction on the dorsum of the finger at 15°C in participants with primary RP compared with controls. This could support the involvement of the ROCK pathway in the pathophysiology of primary RP. Indeed, this would be consistent with previous findings that showed, in vitro, increased cooling-induced α_2-adrenergic constriction of arterioles isolated from patients with primary RP compared with controls, reversed by protein tyrosine kinase inhibitors (13). There is a similar tendency at 24°C, but it does not reach significance, probably because of a lack of power in our study. The potential involvement of the ROCK pathway remains to be explored in a pharmacological study using ROCK inhibitors in patients with primary RP. Moreover, the involvement of ROCK, NO, and sensory nerves in skin microvascular reactivity to local cooling has been studied on the forearm; we therefore extrapolate that similar mechanisms are involved on the finger, which is to be confirmed.

A more striking observation is the difference in the transient vasodilation between the two groups. Indeed, in most cases such vasodilation was blunted in patients with RP compared with controls. Although the mechanisms underlying the transient vasodilation are not fully understood, it is limited by intact sensory nerves (16). This is interesting as in healthy subjects, local anesthesia does not affect transient vasodilation to local cooling, whereas it is unmasked by the blockade of norepinephrine release or adrenergic receptors (18). These results are consistent with the previously suggested impaired cold-induced digital vasodilation in primary RP, indirectly assessed with skin temperature (17). More surprisingly, cold-induced vasodilation was also blunted or decreased on the forearm of five out of six patients with primary RP (3), which we did not observe in the present study. However, experimental conditions were different from ours as the authors used a 2-min cooling at 4–6°C and recorded skin blood flux before and after cooling.

In primary RP, several neural abnormalities have been described. Bunker et al. showed the loss of CGRP containing nerve fibers (5), with decreased skin blood flow response to CGRP when fingers were exposed to cold (using an environmental chamber; Ref. 4). In the present study, the use of local anesthesia on the dorsum of the finger partially restored the transient vasodilation in participants with primary RP when cooling locally at 15°C. These results suggest an abnormal

Table 4. *Effect of lidocaine/prilocaine on the reactivity to local cooling to 15°C on the dorsum of the finger of patients with primary RP and controls*

	Without Lidocaine/ Prilocaine Cream	With Lidocaine/ Prilocaine Cream	*P* Value
Controls			
Overall ($AAC_{0–30}$)	69,544 (66,481)	72,821 (60,380)	0.86
Initial VC	−52.46 (19.6)	−50.23 (26.7)	0.82
Transient VD	−18.63 (47.4)	−21.15 (50.3)	0.71
Prolonged VC	−49.70 (39.1)	−52.74 (30.4)	0.76
Primary RP			
Overall ($AAC_{0–30}$)	106,237 (33,068)*	82,569 (56,880)	0.05
Initial VC	−62.23 (14.4)	−48.46 (26.2)	0.13
Transient VD	−44.15 (44.3)	−27.95 (44.7)	0.04
Prolonged VC	−70.09 (17.8)	−61.43 (21.3)	0.15

Data are expressed as area above the curve of CVC decrease from baseline (in %BL/s) over the 30-min cooling ($AAC_{0–30}$). Each phase of the response, i.e., initial VC, transient VD, and prolonged VC, is expressed as CVC decrease from baseline (in %BL). Paired-*t*-tests were used to analyze the effect of lidocaine/prilocaine in each participant. *$P < 0.02$ vs. controls, repeated-measures ANOVA.

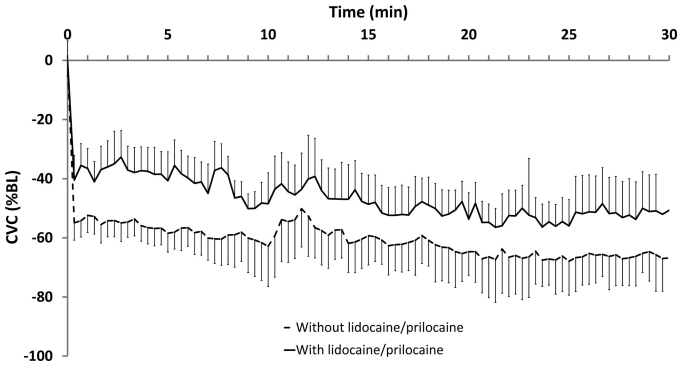

Fig. 4. Mean (SE) CVC (expressed as a percentage decrease from baseline CVC) in participants with primary RP while cooling to 15°C on the dorsum of the finger with (plain line) and without (dash line) lidocaine/prilocaine pretreatment.

neural response in primary RP depending on cold-sensitive nerves. The nonspecific effect of lidocaine/prilocaine does not permit to explain the exact mechanism of the transient vasodilation, but available evidence suggests that sensory nerves play a key role in transient vasodilation (16). Of interest, when using local thermal hyperemia as an integrated test to study microvascular function, the initial axon-reflex vasodilator response to heating (which is thought to be mediated through CGRP and substance P; Ref. 23) is not affected in primary RP (1, 29). Therefore, the mechanism through which microvascular dysfunction to local cooling is mediated in primary RP differs from that involved during local thermal hyperemia. Indeed, response to local cooling and heating are triggered by different transient receptor potential proteins (TRPs). In response to local warming, the response is temperature dependently mediated by TRPV4 (27–34°C), TRPV3 (33–39°C), and TRPV1 (43°C), the latter being responsible for heat nociception. In contrast, local cooling mostly activates TRPM8 (23–28°C) while TRPA1 (17°C) is mostly responsible of cold nociception (32).

It is interesting that we observed no difference in CVC between participants with RP and controls when cooling to 15°C on the finger pad. This is probably due to the marked vasoconstriction observed in both groups, suggesting that local cooling to 15°C on the finger pad induces maximal vasoconstriction both in controls and in participants with primary RP. There are little published data concerning digital skin blood flow of patients with primary RP while cooling locally, but another group (12) came to similar conclusions when cooling down to 8°C on the finger pad, comparing their results to the same protocol previously performed in healthy controls. In the same way, Jobe et al. (17) observed little difference in skin temperature between primary RP patients and controls when immersing the finger in a 5°C water bath, whereas differences were more pronounced at higher temperatures. Another potential explanation is that the TRP activated during local cooling differs between 24 and 15°C, the latter being putatively TRPA1 dependent.

The originality of the test used in this study is that it cools locally, close to the area where blood flux is measured. We have previously shown that this local cooling test does not induce a systemic cutaneous vascular response (26), unlike cold water immersion, which is the most common cooling test because of its relative ease of use (11, 34). Our method therefore allows study of microvascular response with limited systemic sympathetic interference.

The choice of baseline skin temperature raises a methodological issue. Indeed, patients with primary and secondary RP have lower baseline CVC compared with controls (4, 14, 28). As temperature plays a key role in baseline flux, standardizing baseline skin temperature when performing microvascular reactivity improves reproducibility, especially when expressing data as a function of baseline (26, 27). Therefore, in the present study, we decided to set baseline temperature to skin thermoneutrality (i.e., 33°C), as previously described (23). We observed a slightly higher baseline CVC in participants with primary RP than in controls. However, these data should be considered with caution due to the high variability of baseline flux on the forearm when recording with a single-point LDF probe (27). Moreover, the comparable profile of microvascular response to local cooling on the forearm between the two groups strengthens the argument that data should be expressed as a function of baseline while cooling locally, as already suggested by previous work (26).

Moreover, basal cutaneous blood flow has been shown to be lower in the fingers of young women than in young men, which appears to be due to a basal increase in sympathetic tone (6). This may reflect the influence of estrogen in the prevalence of RP, higher in women than in men, and decreasing in postmenopausal women. Of interest, a recent study (9) has shown that estrogen can increase the expression of α_{2C}-adrenoreceptors and also increased α_2-adrenoreceptor-mediated constriction during exposure to cold. In the present study, we were not able to observe any difference between women according to their hormonal status, but the sample size was too small to address this issue. Nonetheless, these findings strengthen the fact that women should be matched in terms of hormonal status when studying skin microvascular function, especially when participants with RP are involved.

In the present work, standardizing baseline skin temperature further suggests that decreased digital CVC in RP patients compared with controls while cooling locally is not due to decreased baseline skin temperature but rather to an abnormal reactivity.

Finally, local cooling did not induce any paroxystic ischemia among the patients, suggesting that microvascular dysfunction in primary RP preexists in the absence of the syndrome.

In conclusion, our study shows with a noninvasive and original test that microvascular reactivity to local cooling is impaired on the dorsum of the finger in primary RP, with exaggerated vasoconstriction compared with controls. The underlying mechanism may involve the ROCK pathway, but this remains to be confirmed. This work further shows that the initial transient vasodilation was blunted on the dorsum of the finger and that part of this abnormal response in primary RP depends on cold-sensitive nerves.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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Part III. The cutaneous microcirculation as a pharmacological target

Despite grey area in the exact mechanism of RP, pathophysiology studies using the reactivity tests detailed in the first chapter have helped to better understand the nature of the imbalance between vasoconstriction and vasodilation. Indeed, the investigation of the cutaneous microvasculature in RP, whether primary or secondary to SSc, has revealed abnormalities. Although the results reported in the second part of this dissertation confirm that there are disparities in the mechanisms involved in microvascular dysfunction between SSc and primary RP, the cutaneous microvasculature could be an interesting therapeutic target in both cases.

I. The nitric oxide pathway

In primary RP, we have been able to show exaggerated vasoconstriction during local cooling [21], which is partly due to inhibition of the NO pathway [123]. Of note, resting microvascular function does not seem to be altered. This suggests that optimal preventive therapy against vasospasm could consist in "as required" treatment, i.e. single dose before exposure to cold. In the following study we therefore tested the effect of single-dose oral sildenafil, a phosphodiesterase 5 (PDE5) inhibitor, on cutaneous vascular conductance before and during local cooling on the finger of patients with primary RP. Indeed, NO is a potent vasodilator by generating cyclic guanosine-5-monophosphate (cGMP), which induces vascular smooth muscles relaxation. It is hydrolyzed by phosphodiesterases, and particularly by the cGMP-specific PDE5.

Sildenafil increases digital skin blood flow during all phases of local cooling in primary Raynaud's Phenomenon

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Submitted manuscript

1. Introduction

Raynaud's phenomenon (RP) is defined as episodic ischemia of the extremities in response to cold or emotional stimuli, often accompanied by pain. RP can be primary (i.e. idiopathic) or secondary to a connective tissue disease. The pathophysiology of primary RP is multifactorial and complex, with both vascular and neural abnormalities [13, 14]. Although the morphology of skin capillaries is normal in primary RP, available evidence suggests that skin microcirculation is impaired [178, 183, 203]. By using a local cooling test we recently showed increased digital skin vasoconstriction in participants with primary RP compared to healthy controls [21].

Local cooling of the skin induces an initial vasoconstriction followed by a transient vasodilation and finally, a prolonged vasoconstriction [123]. Cooling exerts a significant portion of its vasoconstrictor effects through inhibition of the nitric oxide (NO) system [123]. Indeed, NO is a potent vasodilator as it generates cyclic guanosine-5-monophosphate (cGMP), which induces vascular smooth muscle relaxation leading to vasodilation. cGMP is hydrolyzed by phosphodiesterases, and particularly by cGMP-specific phosphodiesterase 5 (PDE5).

PDE5 inhibitors potentiate the effect of endothelial nitric oxide (NO) by decreasing cGMP metabolism, therefore leading to enhanced vasodilatation and increased blood flow. They have been studied in RP mostly as continuous therapy in secondary RP patients [204]. However, the continuous use of PDE5 inhibitors may induce adverse drug reactions, counterbalancing the potential benefit of such drugs in primary RP. Single dose PDE5 inhibitors (e.g. before exposure to cold) could be an interesting therapy in the prevention of ischemic attacks in primary RP. However, little data are available, and the effect of tadalafil and vardenafil on skin blood flow in response to cooling gave conflicting conclusions [205, 206]. Moreover, neither of these two studies used a reactivity test able to detect impaired

microvascular function in primary RP. Although local cooling cannot be used as a surrogate outcome for RP, it could be a useful experimental test to explore the effect of drugs on the different phases of microvascular reactivity to local cooling in primary RP. Moreover it is safe and reproducible [20].

We therefore hypothesized that sildenafil, a PDE5 inhibitor, may reverse the exaggerated microvascular response to local cooling in primary RP. The primary objective of this study was to assess the effect of a single oral dose of sildenafil (50 mg and 100 mg) on cutaneous vascular conductance (CVC) in the fingers of patients with primary RP, while cooling locally at 15°C or 24°C. We also assessed the effect of sildenafil on resting CVC and skin temperature, as well as on the perfusion gradient between the fingers and the hand. The safety of sildenafil in primary RP patients was also assessed.

2. Methods

2.1. Study population

All the participants enrolled in this study were recruited through local newspaper advertisements and included between February 2011 and June 2011. All subjects were 18 years of age or older. Primary RP was diagnosed according to the criteria of LeRoy and Medsger [207], and had to affect more than 2 fingers on a hand. Subjects taking calcium-channel blockers were instructed to stop medication one week before enrolment in the study. The Raynaud's Condition Score (RCS) was assessed as previously described [208].

Non-inclusion criteria included pregnancy (urine pregnancy tests were performed at the beginning of each visit), cigarette smoking, any associated chronic disease, and an abnormal capillaroscopic pattern [209]. Antinuclear autoantibodies were determined for all participants. In cases with positive antinuclear autoantibodies (>80 UI/mL), specific autoantibodies against topoisomerase I (Scl-70) or centromere-associated proteins were

sought. Positive autoantibodies against topoisomerase I (Scl-70) or centromere-associated proteins were exclusion criteria.

The investigation conforms to the principles outlined in the Declaration of Helsinki. Grenoble Institutional Review Board (IRB n°6705) approval was obtained and each subject gave written informed consent before participation.

2.2. Study design

This was an open-label pharmacology study. Three consecutive visits were planned for each volunteer, separated by 7 days +/- 3 at the same time of the day. Upon arrival at the center subjects were placed in a temperature-controlled room (23+/-1°C). They remained supine for the whole experiment.

Visit 1 included a clinical examination, followed by a local cooling test during which digital skin blood flux was continuously recorded. Blood pressure was also recorded continuously by using digital photoplethysmography (Nexfin monitor, Bmeye B.V., Amsterdam, The Netherlands). Before recording started, the arm was immobilized with a vacuum cushion to ensure stable positioning, as previously described [21].

The procedure was repeated at visit 2 and visit 3, one hour after the oral administration of sildenafil citrate (purchased from Pfizer France, Paris, France). Patients were being given 50 mg sildenafil on visit 2 and 100 mg sildenafil on visit 3.

2.3. Local cooling and skin blood flow measurements

Cutaneous blood flow was assessed by LDF (Periflux System 5000, Perimed, Järfälla, Sweden). Two skin sites were equipped with custom-designed LDF cooling probes (Probe 415-317, Perimed, Järfälla, Sweden) [20]. Risk analysis was performed and was fully compatible with human use. The skin sites were chosen on the dorsum of two fingers affected

by RP between the index, the middle and the ring finger (left hand). Maricq color charts [210] were used to confirm the diagnosis of RP and to specify its topography. When the three fingers were equally affected by RP, two of them were randomly chosen. Local cooling started after a 30-minute resting period for acclimation.

Resting skin blood flux was recorded for 10 minutes. Skin temperature was then maintained at 33°C and blood flux was recorded during the following 10 minutes to obtain baseline flux (visit 1). In visits 2 and 3, skin temperature homogenization was done one hour after taking sildenafil. After baseline, local cooling was maintained over 30 min at 15°C on one skin site and at 24°C on the other.

2.4. Perfusion gradient measurements

Skin perfusion of the dorsal surface of the hand (right hand) was recorded before cooling with laser Doppler imaging (PeriScan PIM 3, Perimed, Järfälla, Sweden). Regions of interest (ROI) were defined on the distal, middle and proximal phalanges of three fingers (index, middle and ring fingers), as well as on the dorsal surface of the hand (without the fingers). Mean CVC was calculated for all ROIs. Data from the 3 fingers were averaged.

2.5. Data analysis

Data were digitized, stored on a computer, and analyzed off-line with signal processing software (PeriSoft 2.5.5, Perimed, Järfälla, Sweden). Skin blood flow was expressed as cutaneous vascular conductance (CVC) in mV/mmHg (i.e. flux in millivolts divided by mean arterial pressure). Expressing data as conductance is a more physiological approach, as it takes into account differences and variations in blood pressure [166]. Then, a minute by minute analysis of CVC was performed to assess the kinetics of the response and data were expressed as area under the curve over a 5-min baseline and the 30-min cooling

period (AUC_{0-35}, in mV.s/mm Hg) as the primary outcome. We subsequently analyzed the 3 phases of the response as previously described: 1. initial vasoconstriction: CVC was averaged over 1 min around the lowest flux value within the first five minutes; 2. transient vasodilation: CVC was averaged over 1 min around the highest flux value within the first fifteen minutes; 3. late prolonged vasoconstriction: CVC was averaged over the last 3 minutes of the measurement [21]. In order to take into account baseline (BL) flux variations, data were expressed as a percentage change from baseline CVC as a secondary outcome.

2.6. Statistical analysis

Categorical data were reported as frequency and percentage, and continuous data as mean and standard deviation. They were analyzed by repeated measures ANOVA, and paired t tests for 2x2 comparisons. Mauchly's test of sphericity was used to assess equality of variance in the data. When significant (i.e. inequality of variance cannot be excluded) a Greenhouse-Geisser adjustment was used. When the conditions of application of parametric tests were not respected, nonparametric tests were used (Friedman test, and Wilcoxon test for paired comparisons). Two-sided significance tests were used throughout. We considered p values <0.05 as significant, corrected by Bonferroni's method for multiple comparisons. Statistical analysis was performed with SPSS 13.0 for Windows (SPSS Inc, Chicago IL, USA).

3. Results

3.1. Population characteristics

The demographic and clinical characteristics of the study population are summarized in Table 3. One patient had elevated triglycerides (3.31 mmol/L, untreated) and another

patient was taking rosuvastatin for a mild dyslipidemia corrected by the treatment. All subjects had normal glycemia and serum creatinine.

One participant had positive antinuclear antibodies, but negative autoantibodies against topoisomerase I (Scl-70) or centromere-associated proteins; clinical examination and anamnesis did not argue for a connective-tissue disease. The nailfold videocapillaroscopy pattern was normal in all subjects.

One postmenopausal woman was taking hormonal substitutive treatment, and three of the premenopausal women were taking oral contraceptives. Among the premenopausal women, 4 were enrolled during the follicular phase and 4 during the luteal phase of the cycle.

Table 3. *Demographic and clinical characteristics of the study population (N=15).*

Age (years)	48.5 (17.9)
Female	11(73)
Postmenopausal	3 (27)
Premenopausal	8 (73)
BMI (kg/m²)	21.8 (3.4)
Arterial blood pressure	
Systolic (mm Hg)	124.5 (14.4)
Diastolic (mm Hg)	75.3 (8.5)
Glycaemia (mmol/L)	4.8 (0.5)
Serum creatinine (µmol/L)	76.9 (14.6)
Raynaud's phenomenon	
Duration (years)	18.8 (16.2)
Number of fingers involved	8.1 (1.4)
Thumb involved	5 (33)
Other locations (feet, ears or nose)	10 (67)
Raynaud's Condition Score	4.1 (1.8)

Quantitative data are expressed as mean (standard deviation). Qualitative data (female, thumb involvement and other locations) are expressed as number (percentage). BMI: body mass index.

3.2. Effect of sildenafil on resting CVC and skin temperature

Oral 50 mg sildenafil significantly increased skin temperature from 28 ± 3.3°C before administration to 29.3 ± 3.1°C 50 min later (P=0.02, Wilcoxon rank test). In the same way, 100 mg sildenafil significantly increased skin temperature from 28.9 ± 3.3°C to 30.7 ± 2.7°C (P=0.008, Wilcoxon rank test). There was a significant difference in skin temperature over time between 50 mg sildenafil and 100 mg sildenafil (P=0.048, two-way ANOVA) (Figure 10A).

Resting CVC was 4.1 ± 2.7 mV/mm Hg before and 4 ± 2.8 mV/mm Hg 50 min after administration of 50 mg sildenafil (P=0.34, Wilcoxon rank test). On the other hand, CVC was 4.8 ± 3.9 mV/mm Hg before and 7 ± 5.3 mV/mm Hg 50 min after administration of 100 mg sildenafil (P=0.001, Wilcoxon rank test). There was a significant difference in CVC over time between 50 mg sildenafil and 100 mg sildenafil (P=0.035, two-way ANOVA) (Figure 10B).

3.3. Effect of sildenafil on cutaneous vascular conductance during local cooling

Sildenafil at 100 mg, but not 50 mg, significantly lessened the 15°C local cooling-induced skin blood flux decrease compared to control (Table 4, Figure 11A). This difference is not uniquely due to the change in baseline, as the $AUC_{5\text{-}35}$ (i.e. excluding baseline CVC) were also significantly different (6856.7 ± 5977.4 and 2248.7 ± 1778.6, respectively; P=0.04).

This difference is observed at baseline and for all phases of local cooling, i.e. initial vasoconstriction, transient vasodilation and prolonged vasoconstriction (Table 2). When data were expressed as a percent change from baseline however, there was no significant difference between sildenafil at 100 mg, 50 mg and control (Table 2).

Similar results were observed when the cooling temperature was set at 24°C (Table 2, Figure 11B). Again, the difference in skin blood flux between 100 mg sildenafil and control was not uniquely due to the change in baseline ($AUC_{5\text{-}35}$ were 5592.1 ± 3434.2 and 2518.5 ±

920.9, respectively; P=0.008). The difference between 100 mg sildenafil and control was significant at baseline and for all phases of local cooling when expressed as CVC (Table 2), but not as a function of baseline.

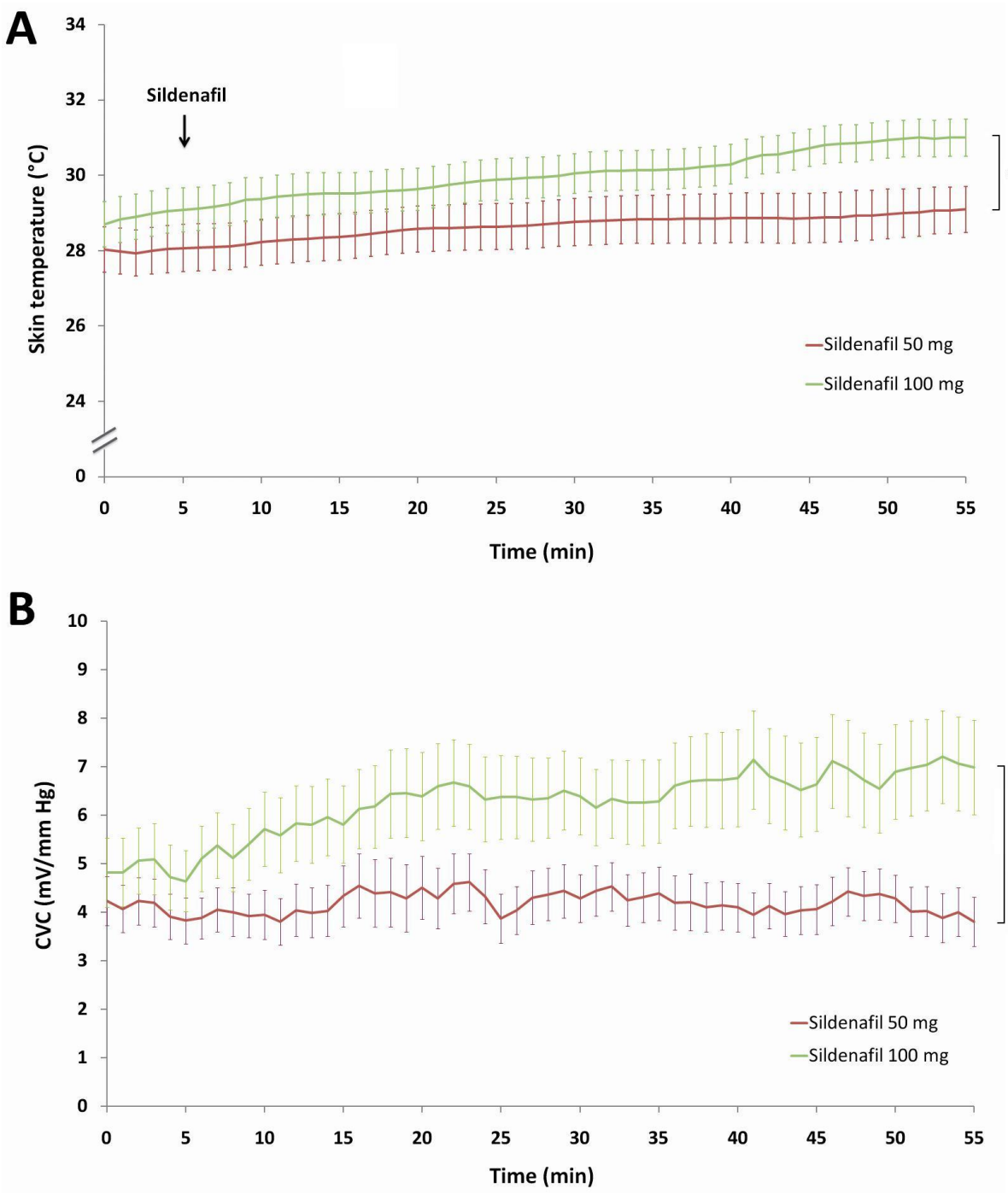

Figure 10. *Single oral dose of 50 mg (red line) and 100 mg (green line) sildenafil: both increased resting skin temperature in a temperature-controlled room (A). 100 mg Sildenafil, but not 50 mg, also increased resting cutaneous vascular conductance (CVC) (B). * P<0.05*

Table 4. Effect of 50 mg and 100 mg sildenafil on cutaneous vascular conductance during local cooling on the finger of primary RP patients.

			Sildenafil		P-value
		Control	50 mg	100 mg	
15°C	AUC_{0-35} (mV.s/mm Hg)	3581.41 (2688.12)	5326.08 (3349.08)	9360.25 (6681.89)*	0.02
	Baseline (mV/mm Hg)	3.54 (2.61)	6.33 (3.46)*	8.35 (4.07)*	0.001
	Initial VC (mV/mm Hg)	1.16 (0.71)	2.49 (2.16)	4.04 (3.46)*	0.031
	Transient VD (mV/mm Hg)	1.86 (1.75)	3.44 (2.33)	5.05 (3.99)*	0.026
	Prolonged VC (mV/mm Hg)	1.1 (1.06)	1.16 (0.78)	2.93 (2.44)*	0.017
	Initial VC (%BL)	-68.21 (10.85)	-63.13 (18.28)	-51.91 (26.44)	0.077
	Transient VD (%BL)	-50.17 (26.08)	-43.82 (27.86)	-39.35 (29.44)	0.45
	Prolonged VC (%BL)	-68.78 (27.34)	-80.46 (10.38)	-63.72 (24.03)	0.069
24°C	AUC_{0-35} (mV.s/mm Hg)	3609.76 (1507.41)	5559.08 (3349.08)	7546.98 (3976)*	<0.001
	Baseline (mV/mm Hg)	3.64 (2.1)	5.34 (2.4)	6.52 (2.81)*	0.001
	Initial VC (mV/mm Hg)	1.51 (1.16)	2.08 (1.15)	3.28 (2.14)*	0.003
	Transient VD (mV/mm Hg)	2.08 (1.75)	2.85 (1.83)	4.09 (2.42)*	0.007
	Prolonged VC (mV/mm Hg)	1.21 (0.37)	2.1 (1.63)	2.55 (1.67)*	0.009
	Initial VC (%BL)	-54.39 (19.55)	-59.51 (14.65)	-49.14 (23.21)	0.29
	Transient VD (%BL)	-35.9 (27.23)	-44. 1 (23.56)	-34.43 (29.6)	0.94
	Prolonged VC (%BL)	-59.68 (15.5)	-59.77 (18.82)	-58.33 (21.56)	0.92

Data are expressed as mean (SD) cutaneous vascular conductance in mV/mm Hg or as a percentage of baseline CVC (%BL). Area under the curve (AUC_{0-35}) includes the 5-min baseline and the 30-min cooling, and is expressed as mV.s/mm Hg. VC: vasoconstriction; VD: vasodilation.* P<0.025 vs control.

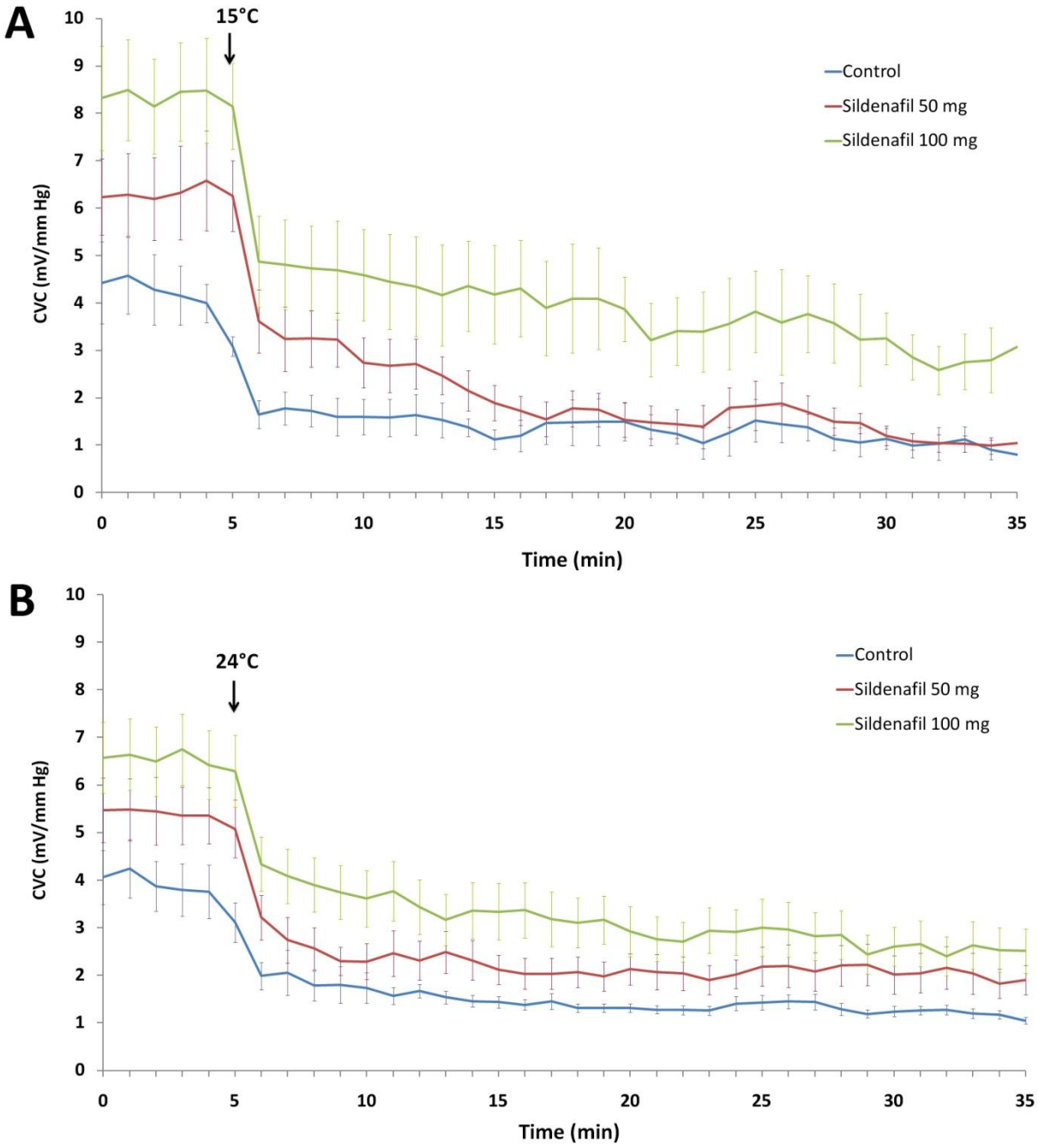

Figure 11. *Effect of a single dose of 50 mg sildenafil given orally (red line) and 100 mg (green line) compared to control (blue line) on cutaneous vascular conductance (CVC). Local cooling was started one hour after oral administration and set at 15°C (A) and at 24°C (B).*

3.4. Effect of sildenafil on the skin perfusion gradient

At each visit we observed a perfusion gradient between the distal, middle and proximal phalanges and the dorsum of the hand: 15.9 ±13.4, 11.5 ± 8.3, 10.2 ± 6.9 and 7.9 ±

4.5 mV/mm Hg respectively for control (P=0.008); 13 ± 9.9, 10.6 ± 7.1, 9.4 ± 6.1 and 8.1 ± 4.7 mV/mm Hg respectively for 50 mg sildenafil (P=0.004); and 22.3 ± 13.5, 18 ± 13.4, 16.2 ± 13.8 and 10.3 ± 5.7 respectively for 100 mg sildenafil (P=0.001). The difference between 100 mg sildenafil, but not 50 mg, and control is significant for each phalange and for the hand (Figure 12).

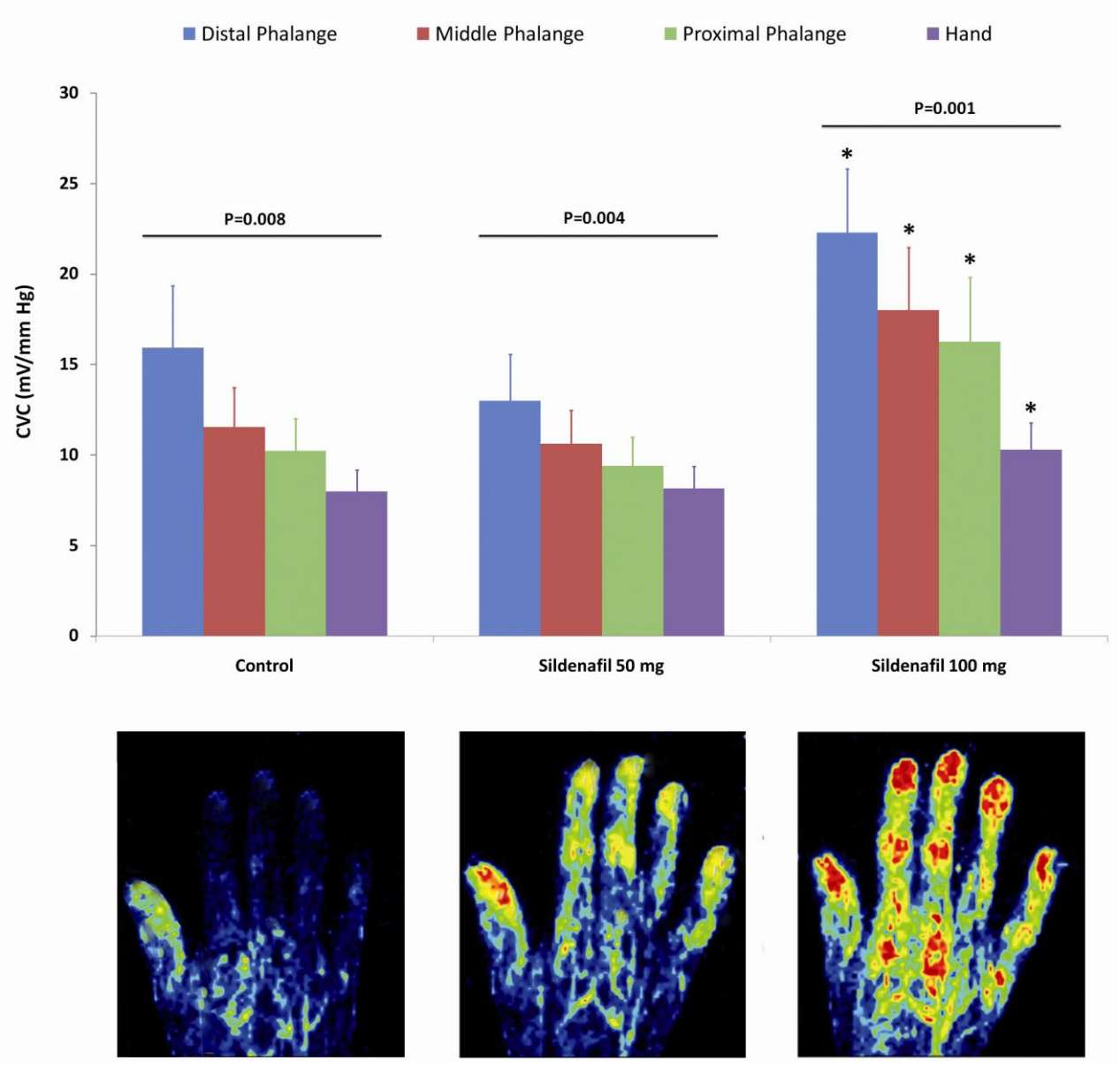

Figure 12. *Effect of sildenafil on the gradient of skin perfusion between the distal, middle and proximal phalanges, and the dorsum of the hand. * P<0.05 vs control. Below are typical images of skin perfusion in the same patient at each visit (control, 50 mg sildenafil and 100 mg sildenafil, respectively).*

3.5. Safety

None of the subjects complained of pain associated with the local cooling and it did not induce any symptoms of RP. No adverse event was reported during visit 1 (no treatment). At visit 2 (50 mg sildenafil), four patients experienced headache: 3 were mild and regressed spontaneously, the fourth one was moderate and ceased after taking 1 g acetaminophen. Mild flushing was observed in 6 patients. At visit 3 (100 mg sildenafil), one patient complained of moderate headache and was given 1 g acetaminophen. Mild flushing was observed in 4 patients, one of them also reporting nasal congestion.

Mean arterial blood pressure before 50 mg sildenafil (visit 2) and 100 mg (visit 3) were 90 ± 10.9 and 93 ± 10.8 mm Hg, respectively. The nadir were reached 37 and 43 min after sildenafil administration, and were 85.9 ± 9.6 (P=0.07 vs baseline) and 83.5 ± 14.6 mm Hg (P=0.01 vs baseline), respectively.

4. Discussion

Our data show that a single dose of 100 mg sildenafil significantly increases cutaneous vascular conductance during the three phases of localized digital cooling in patients with primary RP. This effect was observed within 1 hour after sildenafil intake, at 15°C and 24°C. PDE5 inhibition had a dose-dependent effect on baseline CVC. Finally, we observed a non significant trend towards increased CVC while cooling with 50 mg sildenafil. None of the patients experienced a serious adverse event.

Microvascular reactivity impairment in primary RP is not yet fully understood. A loss of CGRP containing nerve fibers in the digits has been suggested [181], as well as increased endothelin-1 dependent vasoconstriction [14]. Another mechanism could involve postjunctional α_{2c}-adrenoreceptors, which are clustered distally, whereby their translocation from cytosol to cell surface is enhanced by cooling, thus leading to vasoconstriction [13]. In

vitro, cooling-induced α_2-adrenergic constriction of arterioles isolated from patients with primary RP was increased compared to controls, which was suppressed by protein tyrosine kinase inhibitors [178].

Local cooling is a reproducible test to assess skin microvascular reactivity [20]. Most of its vasoconstrictor effect depends on inhibition of the NO system and on adrenergic function [211], particularly through the translocation of α_{2c}-adrenoreceptors mediated by RhoA-Rho kinase (ROCK) [131]. We recently showed that local cooling-induced vasoconstriction on the fingers of patients with primary RP was increased compared to healthy controls [21].

This study further demonstrates that sildenafil, a selective PDE5 inhibitor, increases digital skin blood flow both at baseline and during local cooling in primary RP. However, sildenafil did not restore the typical pattern of skin blood flow during local cooling, i.e. initial vasoconstriction followed by a transient vasodilation and finally, a prolonged vasoconstriction [123]. Indeed, in most cases the transient vasodilation is blunted in patients with RP compared to controls, and partially restored by local anesthesia, suggesting an abnormal neural response in primary RP depending on cold sensitive nerves [21]. Sildenafil induced a shift towards higher flux values at all phases, explaining why its effect was not significant when data were expressed as a percentage of baseline. Moreover, we observed at each visit a perfusion gradient between the phalanges and the dorsum of the hand. Sildenafil at 100 mg, but not 50 mg, significantly increased skin blood flux at all sites. All together, our data suggest a non specific effect of sildenafil on baseline skin blood flow and on the microvascular response to local cooling.

Friedman *et al* have studied the effect of another PDE5 inhibitor, tadalafil, on cold-induced skin vasoconstriction in 18 patients with primary RP and 2 with secondary RP [205]. However, they did not show any significant change in skin blood flow at baseline and during

local cooling between tadalafil 10 mg (single dose) and placebo. The discrepancy between their findings and the present study may be explained by the differences between the PDE5 inhibitors. Although both drugs have the same pharmacodynamic properties, tadalafil has a longer time to onset of action. Nevertheless this was taken into account as the cooling was started 90 min after drug administration. Of note, continuous treatment with tadalafil did not show any difference from placebo in the Raynaud condition score (RCS) or on the frequency and duration of attacks in secondary RP [212]. Another explanation could be the difference in methods between the studies. Indeed, in the study of Friedman *et al* cooling was regional (whole hand) and flux was recorded on the finger pad [205], whereas in the present work cooling and measurements were restricted to the dorsum of the finger. Finally the cooling protocol used by Friedman *et al* was not directly compared between primary RP patients and matched controls [205]. Their methodology might be inappropriate to detect impaired microvascular reactivity to cooling in primary RP, thus making it more difficult to show a difference after tadalafil administration. The test we used in the present study allowed us to demonstrate microvascular dysfunction to local cooling in primary RP compared to healthy controls [21].

An open-label study has assessed the effect of vardenafil (10 mg bid) on skin blood flow in basal conditions and during exposure to cold, in 7 patients with primary RP and 33 with secondary RP [206]. Caglayan *et al* showed a significant increase in skin blood flow after vardenafil administration during exposure to cold at 4°C, but not at room temperature. The effect was also visible after a single dose and after 2 weeks [206].

Another small open-label study has reported significant improvement in digital temperature in response to a mild cold challenge (immersion in water at 15°C) after 50 mg sildenafil in 3 out of 5 patients with secondary RP [213]. We did not assess the effect of local

cooling on skin temperature (as the temperature was set at 24°C or 15°C), but we observed a dose-dependent effect of sildenafil on resting skin temperature and cutaneous blood flow.

In order to allow for the variability of baseline skin blood flow (i.e. after sildenafil administration but before cooling) we homogenized baseline skin temperature to 33°C, as previously described [83]. Homogenizing skin temperature considerably decreased inter-individual variability (data not shown for clarity) although baseline CVC remained significantly increased by sildenafil.

Gender is another concern when studying microvascular function. The effect of the phase of the menstrual cycle or of oral contraception on microvascular function has been explored, but with conflicting results. Resting cutaneous blood flux and conductance are affected by sex, females having lower values than males [150]. Finally, healthy females show greater vasoconstriction on local cooling than males, the response being more pronounced during the luteal phase than the follicular phase of the cycle [158]. Despite its small sample size, the population of this study is well balanced in terms of menstrual phase, postmenopause or participants on oral contraception. Moreover, as each participant was his/her own control, a gender bias is unlikely.

A limitation of this study is that it was not randomized and double-blind. Indeed, we were unable to obtain a placebo for sildenafil from the manufacturer. We therefore chose objective measurements as the primary outcome (i.e. cutaneous vascular conductance), and on the recommendation of the ethics committee, we sequentially tested 50 mg sildenafil followed by 100 mg, as 100 mg given orally could have induced more side effects in our mostly young female population. This pilot pharmacology study suggests that in controlled conditions, a single dose of 100 mg sildenafil improves microvascular reactivity to local cooling in primary RP. This treatment could therefore be used “as required” before exposure

to cold in such patients. This should be confirmed in real-life conditions by a randomized double-blind controlled trial with clinical outcomes.

Several controlled studies have shown improved outcomes (i.e. RP duration, frequency of attacks, RCS or a visual analogue scale score) in secondary Raynaud's phenomenon when treated with sildenafil [214, 215]. Herrick *et al* recently reported a greater decrease in attack frequency with modified-release sildenafil (100 mg followed by 200 mg once daily) than placebo [215]. Another double-blind randomized cross-over trial showed improvement in frequency or RP, duration of RP and RCS with add-on treatment with 20 mg tadalafil once daily as compared with placebo [216]. In contrast, tadalafil (20 mg once daily) did not improve the same outcomes when patients were asked to discontinue concomitant medications such as calcium channel blockers [212]. However, these studies enrolled patients with secondary RP (particularly patients with systemic sclerosis) and assessed the continuous use of PDE5 inhibitors rather than single doses.

Finally, sildenafil was well tolerated; none of the participants experienced a serious adverse event. The mild adverse drug reactions observed in this study are similar to those observed with PDE5 inhibitors in the treatment of erectile dysfunction. The frequencies of headache and flushing were comparable to that previously reported [217]. The decrease in mean arterial pressure was maximal at around 40 min, and was -10% and -16% from baseline after administration of 50 mg sildenafil and 100 mg sildenafil, respectively.

In conclusion, this study shows that a single dose of 100 mg sildenafil significantly increases skin blood flow within one hour in patients with primary RP, both at baseline and during all phases of digital local cooling. Sildenafil administration did not induce any serious adverse drug event. This pilot pharmacology study suggests that 100 mg sildenafil could be used “as required” before exposure to cold in primary RP. This should be confirmed in a randomized double-blind controlled trial.

II. Therapeutic iontophoresis: targeting the skin microvasculature

1. Definition

As previously mentioned in the first part of this dissertation, iontophoresis is a method for non-invasive transdermal drug delivery under the influence of electricity. Two mechanisms are involved in iontophoretic transport. Electromigration (or electrorepulsion) is the movement of ions across a membrane (i.e. the skin) under direct influence of the electric field. Negatively charged drugs are therefore repelled into the skin under the cathode, whereas the transfer of positively charged drugs occurs under the anode (Figure 13). The second mechanism is called electro-osmosis, which can be schematized as the volume flow induced by the current flow. As the isoelectric point (pI) of the human skin in around 4-4.5, which is below the pH in physiological conditions, the skin will be charged negatively. Therefore, the flow will be directed in the anode-to-cathode direction, facilitating the transport of cations (Figure 13) [26].

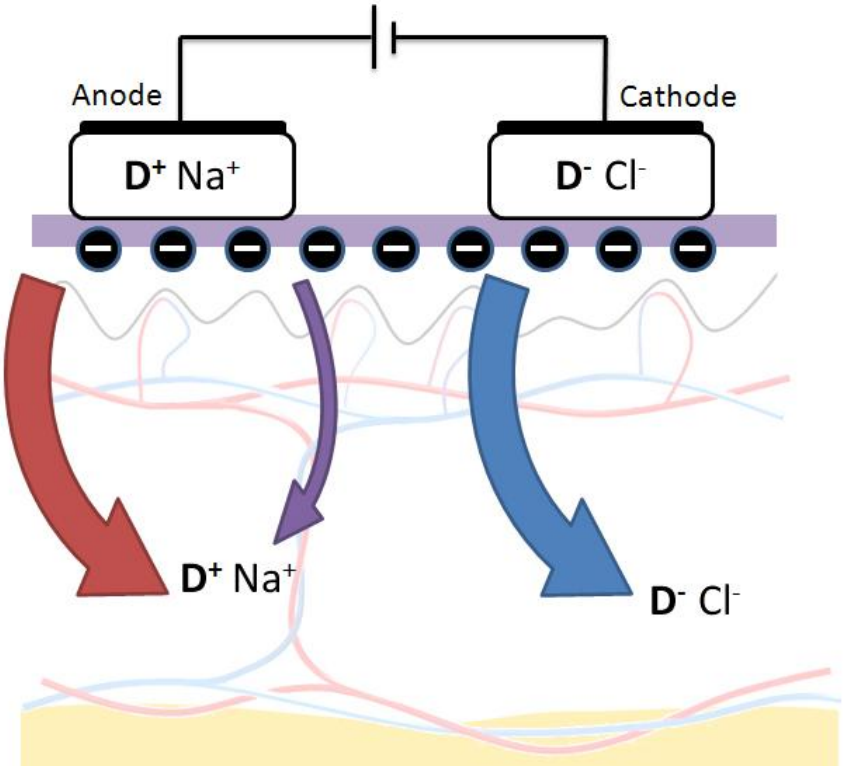

***Figure 13**. Schematic representation of iontophoretic transport. Positively charged drugs (D+) migrate under the anode whereas negatively charge drugs (D-) migrate under the cathode. The red and blue arrows represent anodal and cathodal electromgration, respectively. The purple arrow represents electro-osmosis.*

Electro-osmosis also allows the diffusion of neutral molecules with anodal iontophoresis. The respective part of the transfer explained by electromigration or electro-osmosis highly depends on the physicochemical properties of the molecules.

The iontophoretic transport depends on the current density, i.e. the intensity divided by the cross-sectional skin area in contact with the electrode (usually expressed as $mA.cm^{-2}$) [26]. The time of application also influences the dose delivered. Therefore the quantity of charge (expressed in coulomb, i.e. A.s) per unit of skin area should be considered. Drug concentration is one of the factors influencing iontophoretic transfer. Although in some cases there is an almost linear relationship between concentration and flux, the flux often reaches a plateau as the concentration increases [26]. The quantity of charge, the skin area and the drug concentration are experimental variables that can be adjusted to modify the electrophoretic transfer.

Other parameters such as the physicochemical properties of the molecule cannot be easily influenced by the operator. The size, the partition ratio and of course the charge of the molecule are of primary importance [218]. The charge depends of the pH of the solution and the pI of the compound. In general, small and hydrophilic molecules are transported at a faster rate than larger, lipophilic molecules [218].

Finally, the integrity of the skin surface, its thickness, and whether it is glabrous or not will influence iontophoretic transfer [26]. There are significant regional variations, which may be related to differences in local blood flow and therefore differences in drug clearance [104].

2. Applications

Iontophoresis of acetylcholine (Ach) and sodium nitroprusside (SNP), when combined with laser Doppler, have been used as markers of microvascular endothelium-dependent and independent vasodilation, respectively [80, 85] (please refer to page 34 for details). Before

being used as reactivity test, iontophoresis had known therapeutic applications for several decades, particularly in physical therapy and dermatology [219].

One of the first experiences of medication transfer by electricity may be attributed to Hermann Munk, as early as 1879. Indeed, after a 20-25 minute exposure to an electrified strychnine solution, Munk observed spontaneous cramps in the rabbits [220]. About forty years later, Stéphane Leduc described the methods to administrate salicylic acid using an electric current to accelerate wound healing [221].

Iontophoresis as a route of drug delivery presents several theoretical advantages, among which non-invasiveness, faster administration and better control of the delivered dose than usual passive transdermal administration. Moreover, depending on the properties of the molecule, systemic administration can be achieved without first-pass metabolism [219]. Recent technological advances have allowed the miniaturization of delivery systems, opening the way to clinical perspectives.

Two drugs have been marketed in such devices. Lidocaine combined with epinephrine (Lidosite®, Vyteris Inc.) has been used as a local anesthetic. The technique allows faster anesthesia than with lidocaine/prilocaine cream (about 10 min vs 60 min, respectively). Epinephrine is used to decrease skin blood flow, thus limiting the clearance of the drug [222]. The second medication is an iontophoretic device containing fentanyl, an opioid analgesic, approved in 2006 in Europe (Ionsys®, Janssen-Cilag) for the management of acute moderate to severe postoperative pain. Unlike lidocaine, fentanyl is not used as a topical but systemic drug, and iontophoresis allows on-demand administration [222]. However, corrosion of a component within the system has been found in one batch, leading to a suspension of the marketing authorization in January 2009. Indeed, this defect could result in fentanyl release without activation by the patient. This could expose patients to fentanyl overdose, with a risk of severe respiratory depression.

Other drugs in the pipeline could lead to new marketed applications within the next few months, e.g. zolmitriptan [223] and sumatriptan (Zelrix®, NuPathe Inc.) [224] for the treatment of migraine, or terbinafine in the treatment of onychomycosis [225]. New devices (e.g. EyeGate® delivery system) have recently permitted drug delivery to both the anterior and posterior segments of the human eye. Recent controlled studies suggest that dexamethasone administered through ophthalmic iontophoresis may be an effective treatment of drye eye. Finally, it is interesting to note that tap water iontophoresis has been successfully used without drugs in the treatment of hyperhidrosis [226]. In the same way, the current itself may be beneficial in wound healing [227].

3. Perspectives in the treatment of microvascular dysfunction

As previously mentioned, when Raynaud's phenomenon occurs secondary to SSc, it may progress to irreversible tissue ischemia with scarring, ulceration and sometimes gangrene [87]. Iloprost, a prostacyclin (PGI2) analogue used intravenously, is the only drug approved for the treatment of existing digital ulcers [228]. However, the therapeutic effect of prostaglandin analogues is counterbalanced by potentially serious systemic side effects related to their potent vasodilator properties, such as severe headaches, flushing, tachycardia and systemic hypotension [28].

Iontophoresis of vasodilator drugs could be an appropriate answer to microvascular dysfunction in RP, especially secondary to SSc [86]. Indeed, it could permit to reach significant drug concentration locally, while limiting systemic exposure, the decreasing the risk of adverse drug events.

Preliminary data on rats have revealed a sustained increase in skin blood flux after cathodal iontophoresis of two PGI_2 analogues, treprostinil and iloprost [28] (this reference is available as an annex at the end of this chapter). In a subsequent clinical study, we have

confirmed that iontophoresis of 250 µM treprostinil induces a large and sustained increase in cutaneous blood flow on the forearm of healthy volunteers. No effect was observed when applied without current, which confirms the validity of iontophoresis as a local route of administration for treprostinil. However, iloprost caused large erythema in the first healthy volunteer, which led us to stop the experiment for safety reasons (Blaise *et al*, submitted manuscript).

III. Iontophoresis of endothelin receptor antagonists

Increased plasma levels of ET-1 have been associated with primary RP and with SSc (please refer to page 83 for details). Moreover, increased ET-1 receptors have been found in the skin of patients with SSc [40]. Since this observation, several studies have suggested the involvement of ET-1 in scleroderma vascular disease [184, 229]. These findings have led to clinical trials showing efficacy of bosentan, a non specific endothelin receptor antagonist (ERA), in the prevention of digital ulcers in SSc patients at risk [41, 42], but not on ulcer healing [41, 42] or on SSc-related interstitial lung disease [230].

Bosentan is administered orally. Its main adverse effect is a dose-dependent elevation of liver aminotransferases. The annual rate of hepatotoxicity induced by bosentan has been estimated at 10.1%, which leads to therapy discontinuation in more than one third of cases [231]. In order to avoid this toxicity while maintaining therapeutic concentrations in the finger cutaneous microvasculature, local administration of ERAs may be beneficial.

The following article describes an experimental study in rats to assess the effect of iontophoretically-administered ERAs on cutaneous blood flux, as well as their toxicity. We subsequently tested iontophoretically-administered sitaxentan in humans.

Iontophoresis of endothelin receptor antagonists in rats and men

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Running title: Iontophoresis of endothelin receptor antagonists

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1. Introduction

Systemic sclerosis (SSc) is a rare disease affecting digital microcirculation, leading to finger ulcers and in some cases to amputation [37]. Therapy of SSc-related ulcers is challenging. Bosentan, a non specific endothelin receptor antagonist (ERA), has been indicated to prevent digital ulcers in patients at risk, but it has no efficacy on existing ulcers [41]. Elevated aminotransferase levels is the main adverse effect of bosentan, with an annual rate of 10.1%, leading to therapy discontinuation in 39% cases [231]. Prostacyclin (PGI_2) analogues are used intravenously [228], but their therapeutic effect is counterbalanced by potentially serious vasodilatation-induced side effects (e.g. severe headaches, flushing, tachycardia and hypotension).

The topical administration of these drugs may be a way of getting around the toxicity of systemic treatments. Iontophoresis is a simple, non-invasive transdermal drug delivery method using a low-intensity electric current [26]. Some authors have highlighted the potential interest of iontophoresis of vasodilating drugs as a treatment for digital ulcers in SSc [86, 87] and previous work from our laboratory has suggested that PGI_2 analogues are appropriate candidates [28]. Iontophoresis of ERAs could also be interesting but, to our knowledge, it has never been tested either in animals or in humans.

We conducted a laboratory and a clinical study to address this question. The main objective of the animal study was to assess whether iontophoretically-administered ERAs, bosentan and sitaxentan, increase cutaneous blood flux in rats. As a secondary objective, we tested the toxicity of the iontophoresis of ERAs. In a second study, we tested the effect of the iontophoresis of sitaxentan on human skin blood flux as well as its cutaneous and systemic tolerance.

2. Methods

2.1. Animal study

Animals

Thirty-two male Wistar rats (eight-weeks old, 295-380g; CERJ, Le Genest-St-Isle, France) were housed in controlled conditions conforming to the current French legislation and provided with standard rat chow. The protocol was approved by the Rhone Alpes Region Animal Ethics Committee (number 309). Rats were kept in a day/night cycle of 12h/12h with food and water at will. Preparation of the animals for iontophoresis has been previously described [28].

Drugs

Bosentan sodium salt (Actelion Pharmaceuticals, Allschwil, Switzerland) (MW 573.6 $g.mol^{-1}$) and sitaxentan sodium salt (Pfizer Inc, Groton, CT, USA) (MW 476.9 $g.mol^{-1}$) were used for iontophoresis, and isotonic sodium chloride (NaCl 0.9%) (Aguettant, Lyon, France) was used as a control. Solutions were prepared extemporaneously by diluting 20 mg of bosentan or sitaxentan in 3.6 mL and 4.4 mL of NaCl 0.9%, respectively, to obtain 10^{-2} M solutions. The pH of these solutions was determined before iontophoresis using a microprocessor-based pH meter (pH 210, Hanna Instruments, Woonsocket, RI, USA). The solutions were pH 5.5 to 6.5, which is suitable for epidermal application. Endothelin-1 (Sigma-Aldrich, Saint-Quentin Fallavier, France) (MW 2491.9 $g.mol^{-1}$) was diluted in water for injection to obtain 0.5 10^{-5} M, 0.5 10^{-6} M, 0.5 10^{-7} M, 0.5 10^{-8} M and 0.5 10^{-9} M solutions. In order to assess the concentration of ET-1 needed to decrease skin blood flux, dose-response was assessed with 0.5 10^{-9} to 0.5 10^{-5} M solutions (n=3). Only ET-1 at 0.5 10^{-5} M decreased skin blood flux. This concentration was then used throughout the study.

Laboratory experimental procedures

The rats were anesthetized with sodium pentobarbital (50 $mg.kg^{-1}$ i.p.) and were maintained in the prone position for the duration of the whole experiment, with the back uppermost. Experiments were performed in a temperature controlled room, and the rats were placed on a thermal pad, the temperature being maintained at 37.5°C, adjusted using a rectal probe connected to the thermal pad (Harvard apparatus).

Experiment 1: Iontophoresis of bosentan, sitaxentan and NaCl (20 min, 100 µA) were simultaneously performed using cathodal or anodal current (n=9 for each series). Mean arterial blood pressure was measured by plethysmography using the tail cuff method, before and immediately after iontophoresis. Before iontophoresis each rat was inspected to ensure that the hairless skin in the back and the hind legs was intact. Photographs were taken before iontophoresis, immediately after and 3 days later. A cutaneous score was used to assess skin tolerance, based on the International Contact Dermatitis Research Group (ICDRG) scoring [232]. Negative reactions were coded grade 0; weak reactions (grade 1) are characterized non-vesicular erythema. Strong positive reactions (grade 2) are characterized by erythema associated with vesicles. Extreme positive reactions (grade 3) are bullous reactions. Irritant reactions (that we coded grade 4) are characterized by necrosis.

Experiment 2: Catheters were inserted in both carotid arteries in order to record arterial pressure (Powerlab, ADInstrument) and to administer ET-1 (5 $nmol.kg^{-1}$ i.a. bolus followed by 0.5 $\mu L.min^{-1}$ infusion) was administered through the second catheter. Iontophoresis of bosentan, sitaxentan and NaCl (20 min, 100 µA) were then simultaneously performed using cathodal (n=8) or anodal (n=6) currents. Histopathologic examination of full-thickness skin biopsies from bosentan, sitaxentan, NaCl and from one non-treated skin area was realized in experiment 2 (n=9: 5 after cathodal and 4 after anodal iontophoresis). The thirty-six biopsies were fixed in AFA fluid (5% acetic acid, 75% absolute ethyl alcohol, and

18% water; Carlo Erba) paraffin-embedded and stained with hematoxylin, eosin, and safran. In order to evaluate the effect of the treatment on the skin, various features were sought:

- hyperkeratosis and epidermolytic aspects. In the stratum corneum, the degree of hyperkeratosis was observed, and the presence of any parakeratosis was noted. The granular layer was evaluated for perinuclear vacuolar changes, cytolysis, and the appearance of keratohyaline granules. The spinous layer was evaluated for any extension of these features.

- Vasculitis, a histological diagnosis defined as inflammation targeting blood vessel walls and compromising their function, leading to haemorrhagic and⁄or ischaemic events.

- Inflammation with neutrophilic, lymphocytic or mast cell infiltration at the dermo-epiderma interface.

Skin blood flux measurement and data analysis

Three 1.2 cm^2 circular electrodes (PeriIont System, Perimed, Järfälla, Sweden) containing bosentan, sitaxentan or NaCl were placed on the hairless skin of the lower back/hind legs. Passive probes were placed on the back of the neck. Skin blood flux was continuously recorded with laser Doppler imaging (PeriScan PIM 3, Perimed, Järfälla, Sweden). The laser head was placed 20 cm above the skin. The resolution was 2 mm step length and LDI scans were taken every minute.

In experiment 1, iontophoresis was started after a 5-min baseline recording. Skin blood flux was initially expressed as arbitrary perfusion units (PU) during iontophoresis and during the 20 min following the end of iontophoresis. Baseline (BL) flux was averaged over 5 minutes. In order to take into account inter-individual BL variations, data were subsequently expressed as a percentage change from BL (%BL). Then, a minute by minute analysis was performed to calculate the area under the curve during iontophoresis (AUC_{0-20}, in %BL.s) and during the whole recording (AUC_{0-40}).

In experiment 2, the skin blood flux was recorded for 5-min under resting conditions. Five min after ET-1 infusion started (time for skin blood flux to decrease and reach a plateau) iontophoresis was performed. Data were expressed as percentage change between baseline (i.e. 5 min after infusion of ET-1 was started) and the iontophoresis plateau (averaged over 10 min) (expressed as %BL). Then, $AUC_{0\text{-}20}$ was calculated as described above and expressed as %BL.s. In order to take into account ET-1-induced variations in arterial blood pressure, we also expressed data as cutaneous vascular conductance (CVC), i.e. flux divided by arterial pressure (in mV.mm Hg^{-1}) [80, 166], to subsequently calculate $AUC_{CVC\ 0\text{-}20}$ (expressed as %BL.s).

2.2. Clinical study

Study population

We recruited healthy men through the Clinical Research Center database. Inclusion criteria were an age of 18-65 years with no significant medical history. Non-inclusion criteria included any allergies to local anaesthetics, cigarette smoking or dermatologic disease on the arm or forearm. Any abnormal liver enzyme level was also an exclusion criterion. Grenoble Institutional Review Board (IRB n°6705) approval was obtained in September 2009 and each volunteer gave written informed consent before participation.

Study design

This was an open-label pharmacology study. Upon arrival at the centre volunteers were placed in a temperature-controlled room (23+/-1°C) during 1 hour for acclimatization. They remained supine for the whole experiment.

Three consecutive visits were planned for each volunteer, separated by 7 days +/- 3. Visit 1 was the initial enrolment visit. During visit 2, iontophoresis of sitaxentan 10^{-2} M and

NaCl 0.9% were performed. Volunteers arrived fasted for at least 6 hours and were allowed to eat only after the end of the experiment. Visit 3 was a safety monitoring clinical visit. Skin photographs were taken before the start of iontophoresis and immediately after iontophoresis (visit 2), and 7 days later (visit 3). Liver enzymes levels were assessed at visits 1 and 3.

Iontophoresis protocol and skin blood flux measurement

Four iontophoresis skin sites were randomly chosen on the ventral side of the right forearm, more than 5 cm from the elbow and the prominence of the wrist, avoiding visible veins. Two of them were pre-treated with 1 g of lidocaine/prilocaine cream (5 g tubes containing 125 mg lidocaine and 125 mg prilocaine, Anesderm®, Pierre Fabre, Boulogne, France) in order to attenuate axon reflex-induced hyperaemia [101]. An occlusive transparent dressing was placed over the cream to enhance cutaneous diffusion. Each anesthetized area was larger than the area of the iontophoresis chambers.

One hour after lidocaine/prilocaine application (corresponding to the acclimatization period) the cream was removed with a cotton swab. The two pre-treated skin sites were then equipped with 1.2 cm^2 circular iontophoresis chambers (PeriIont System, Perimed, Järfälla, Sweden) containing sitaxentan or NaCl. After a 5-min baseline recording, cathodal iontophoresis was simultaneously performed for 20 minutes with a current intensity set at 20 µA. After iontophoresis, skin blood flux was recorded for an additional 20 min. During the experiment, the two remaining skin sites were pre-treated with local anaesthetic cream for one hour. The same protocol as described above was then performed on these skin sites, the only difference being the current intensity set at 100 µA.

Skin blood flux was recorded throughout with laser-Doppler imaging (PeriScan PIM 3, Perimed, Järfälla, Sweden). The resolution was 1 mm step length and LDI scans were taken every 30 seconds. Before recording, the arm was immobilized with a vacuum cushion to

decrease artefacts related to arm movement. Baseline and maximal skin blood flux were averaged over 5 min. Data were subsequently expressed as a percentage change from BL (%BL) and AUC_{0-40} were calculated (expressed as %BL.s).

Statistical analysis

Categorical data were reported as frequency and percentage and continuous data as mean and standard deviation. They were analyzed by repeated measures ANOVA, and paired t tests for 2x2 comparisons. When the conditions of application of parametric tests were not respected, nonparametric tests were used (Friedman test, and Wilcoxon test for paired comparisons). Two-sided significance tests were used throughout. We considered p values <0.05 as significant, corrected by Bonferroni's method for multiple comparisons. Statistical analysis was performed with SPSS 13.0 for Windows (SPSS Inc, Chicago IL, USA).

Table 5. *Effect of cathodal and anodal iontophoresis of bosentan and sitaxentan on skin blood flux recorded on the back and the hind legs of rats.*

Polarity		Bosentan	Sitaxentan	NaCl	P-value
Cathodal	AUC_{0-20}	5061.8 ± 39161.6	13553.2 ± 33368.5	9598.3 ± 23693.3	0.46
(n=9)	AUC_{0-40}	2717.6 ± 64048.1	32534.3 ± 74077.3	15142.4 ± 48007.6	0.24
Anodal	AUC_{0-20}	1090.1 ± 40367.4	20851.5 ± 39909.7	15123.2 ± 26678.7	0.27
(n=9)	AUC_{0-40}	4215.6 ± 71472.5	20286.7 ± 64462.7	32436.5 ± 55790	0.27

Data are expressed as area under the curve of the percentage change from baseline (expressed as %BL.s) during the 20-min iontophoresis (AUC_{0-20}) and during the whole recording (AUC_{0-40}).

3. *Results*

3.1. Animal study

Effect of iontophoresis of bosentan and sitaxentan on skin blood flux

Neither cathodal nor anodal iontophoresis of bosentan or sitaxentan induced any significant change in skin blood flux compared to NaCl (Table 5; Figure 14). Mean arterial blood pressure was 87.5 ± 17.9 mmHg before iontophoresis and 90.6 ± 10.2 mmHg after iontophoresis (P=0.7).

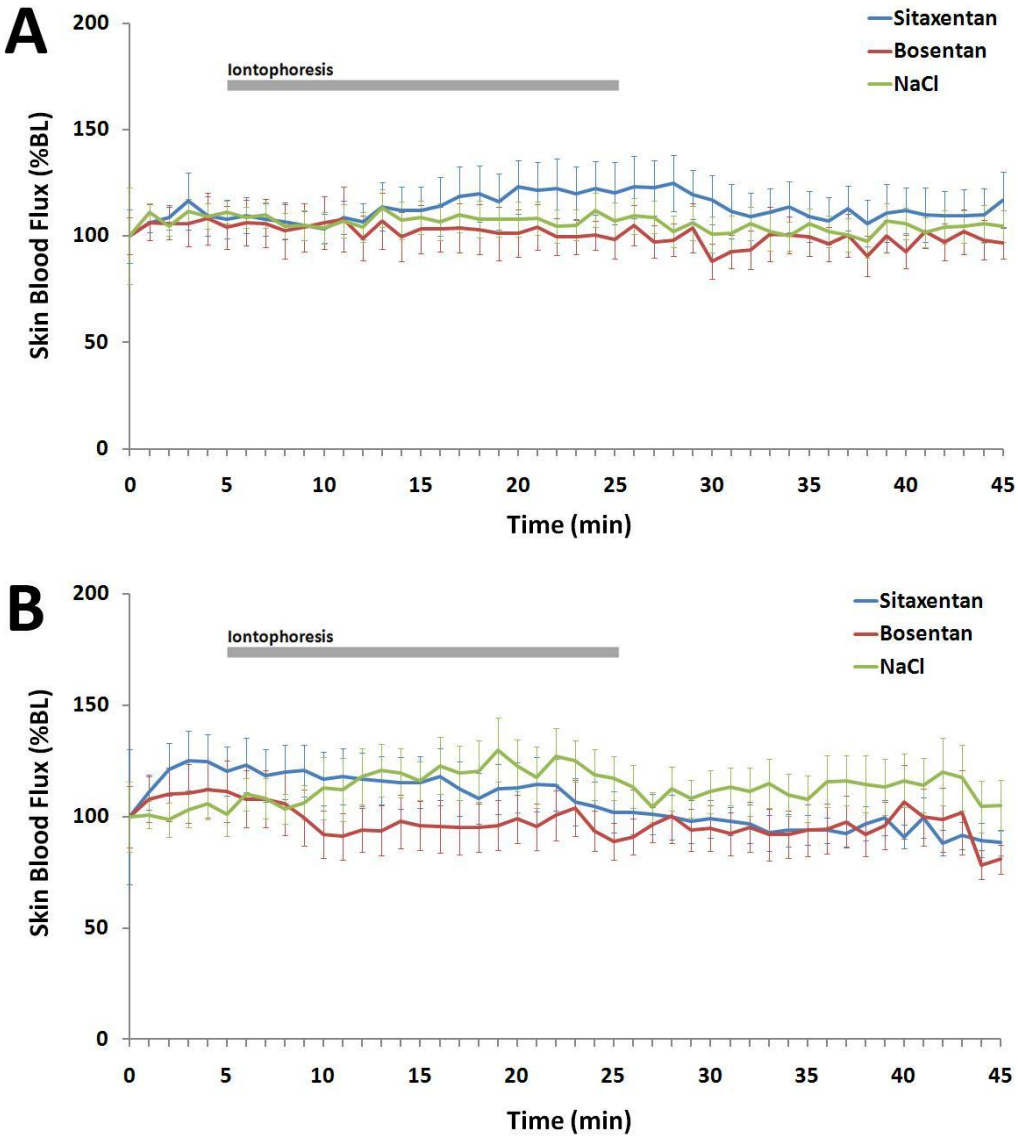

Figure 14: *Effect of iontophoresis of bosentan 10^{-2} M, sitaxentan 10^{-2} M and NaCl 0.9 % on skin blood flux, expressed as a percentage change from baseline (%BL), in the cathodal direction (A) and the anodal direction (B).*

Effect of cathodal and anodal iontophoresis of bosentan and sitaxentan after administration of ET-1

Baseline skin blood flux before cathodal iontophoresis at bosentan, sitaxentan and NaCl skin areas were 91.5 ± 25 PU, 116.7 ± 48.8 PU and 102.2 ± 32.1 PU, respectively (P=0.2). ET-1 decreased skin blood flux to 57.8 ± 17.4 PU, 65.1 ± 31.6 PU and 59.4 ± 17.7 PU, respectively (P=0.7). We observed an initial transient decrease in blood pressure immediately after ET-1 infusion was started, followed by a sustained increase (Figure 15). Cathodal iontophoresis of sitaxentan significantly increased skin blood flux compared to NaCl. However, we observed no significant difference between bosentan and NaCl (Table 6; Figure 16A).

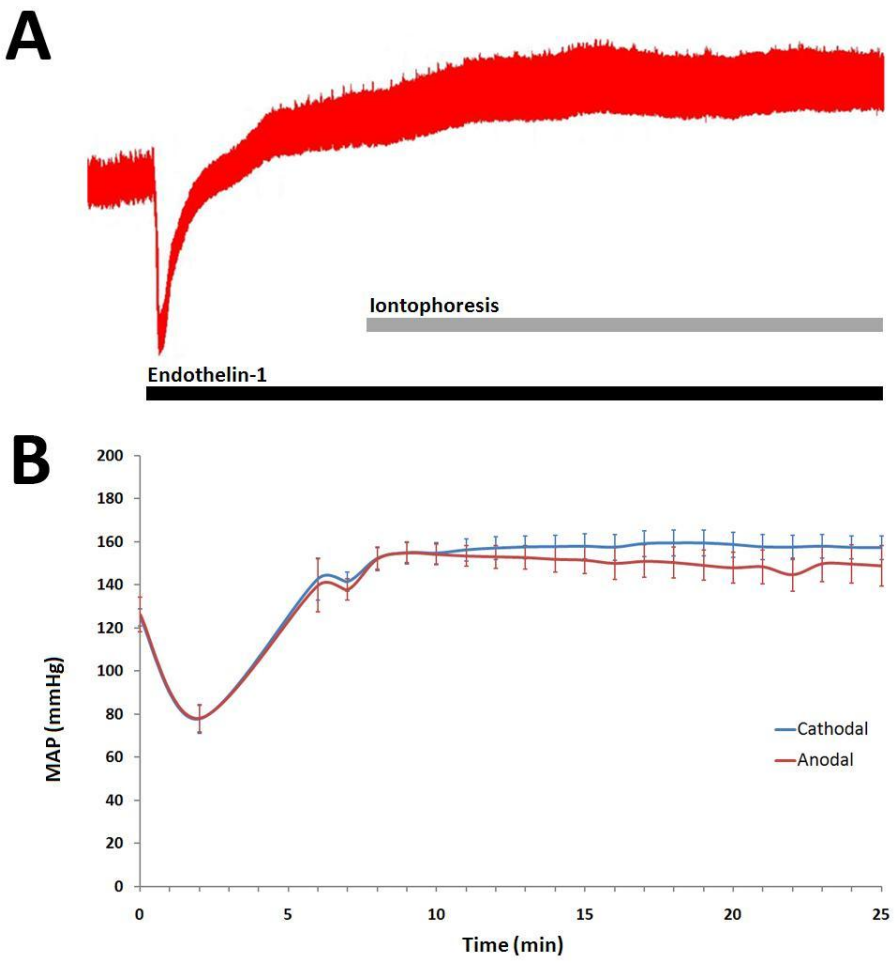

Figure 15: *Typical tracing of the effect of ET-1 0.5 10^{-5} M on arterial pressure: the initial transient decrease of arterial pressure is followed by a sustained increase (A). Mean arterial pressure during ET-1 perfusion in the cathodal (blue line) and anodal (red line) directions (B).*

Table 6. Effect of cathodal and anodal iontophoresis of bosentan and sitaxentan on skin blood flux recorded on the back and the hind legs of rats during ET-1 infusion.

		Bosentan	Sitaxentan	NaCl	P-value
Cathodal (n=8)	%BL	19.3 ± 23.1	34.3* ± 15.5	8.21 ± 20	0.02
	AUC_{0-20}	27569.8 ± 25533	44032.2* ± 12277	14957.5 ± 23818.8	0.01
	$AUC_{CVC\ 0-20}$	12370.5 ± 24147.3	27835.7* ± 18050.8	3687.9 ± 26073	0.008
Anodal (n=6)	%BL	21.4 ± 26.2	26.4 ± 24.2	18.7 ± 24.4	0.84
	AUC_{0-20}	31898 ± 30426.7	24747.1 ± 28687.4	29513.6 ± 34899.4	0.85
	$AUC_{CVC\ 0-20}$	19063.5 ± 16564.9	13405.8 ± 34335.9	19792.2 ± 37485.5	0.85

Data are expressed as percentage change between baseline and iontophoresis plateau (%BL), and as the area under the curve (AUC_{0-20}) of the percentage change from baseline (expressed as %BL.s), whether calculated from arbitrary perfusion units (PU) or from cutaneous vascular conductance (CVC) in order to take into account variations in arterial blood pressure. * $P<0.05$ vs NaCl.

Baseline skin blood flux before anodal iontophoresis for bosentan, sitaxentan and NaCl skin areas were 88.3 ± 16.1 PU, 106.9 ± 39.1 PU and 98.1 ± 21.7 PU, respectively (P=0.3). ET-1 infusion decreased skin blood flux to 59.7 ± 33.8 PU, 55.1 ± 26.5 PU and 47.5 ± 18.8 PU, respectively (P=0.5). Anodal iontophoresis of bosentan or sitaxentan did not significantly increase skin blood flux compared to NaCl (Table 6; Figure 16B).

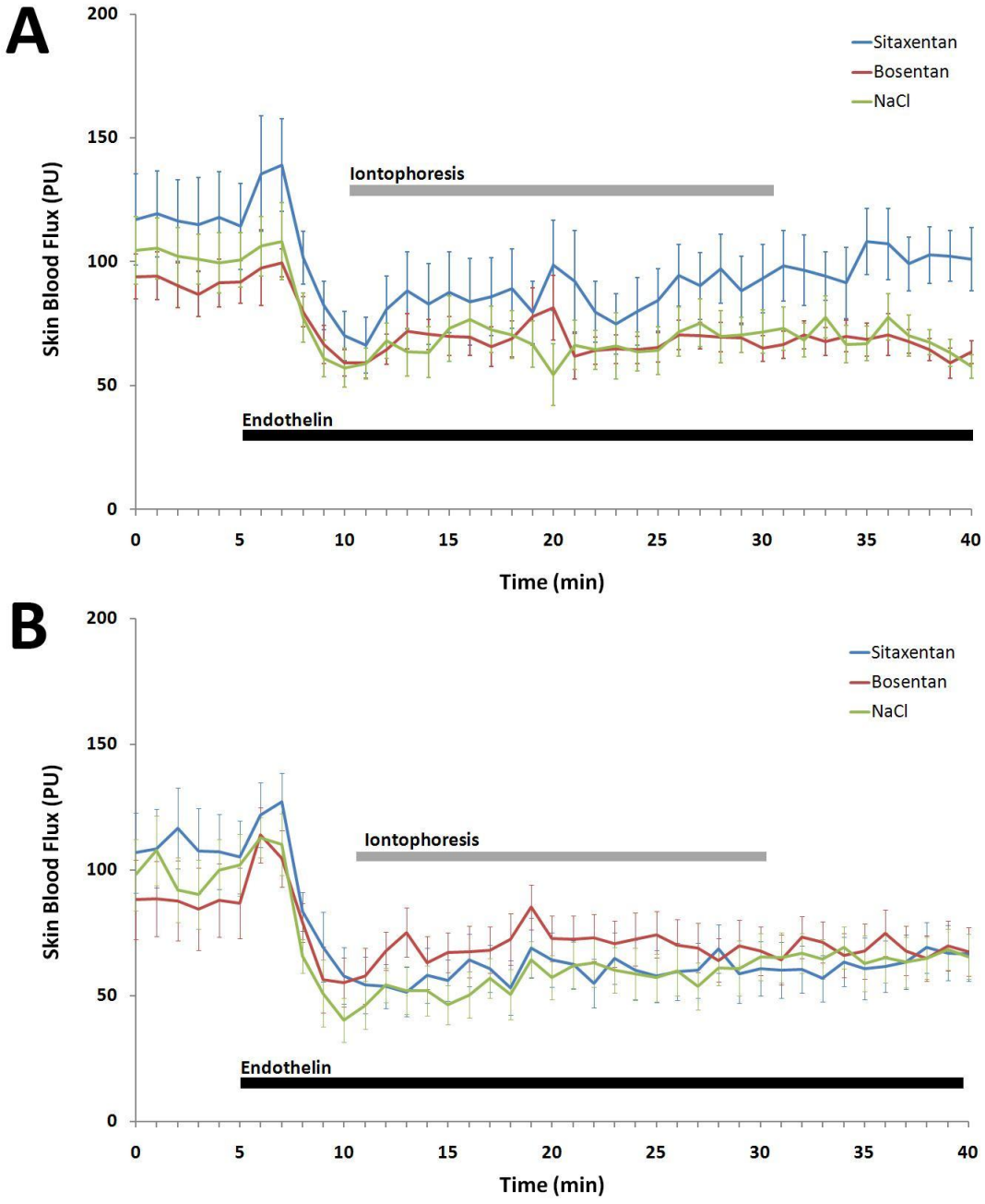

Figure 16: *Effect of iontophoresis of bosentan 10^{-2} M, sitaxentan 10^{-2} M and NaCl 0.9 % on skin blood flux during infusion of ET-10.5 10^{-5} M, expressed as arbitrary perfusion units, in the cathodal direction (A) and anodal direction (B).*

Skin tolerance of the iontophoresis of bosentan and sitaxentan

No skin side-effects were observed. In experiment 1, all the cutaneous scores were 0 both immediately after the procedure and at day 3. In experiment 2, none of the various histopathologic features listed in the Methods section were found in any of the skin biopsies.

3.2. Clinical study

Study population

Six male volunteers were enrolled in the study. One of them did not attend visits 2 and 3 and was excluded. The characteristics of the population were: mean age 22.8 ± 1.9 years and body mass index 22.1 ± 1.5 kg/m^2. Mean systolic/diastolic arterial pressure at inclusion was 115 ± 7.4 / 77 ± 10.9 mm Hg.

Effect of cathodal iontophoresis of sitaxentan on skin blood flux

Neither sitaxentan nor NaCl increased skin blood flux when the iontophoresis current intensity was 20 µA (AUC_{0-40} were -2785.3 ± 35811.4 %BL.s and -3315.1 ± 42523.1 %BL.s, respectively; P=0.69). Cathodal iontophoresis of sitaxentan induced an inconstant increase in skin blood flux when the current intensity was set at 100 µA (AUC_{0-40} was 289409.4 ± 348171 %BL.s). However, a similar increase was observed with NaCl (AUC_{0-40} was 236430.4 ± 348333.1 %BL.s; P=0.22) (Figure 17A).

Tolerance

No side-effect occurred. We observed no significant change in mean arterial pressure during iontophoresis of sitaxentan (Figure 17B). No squamous, vesicular or bullous lesions were observed at the end of the experiment or at the safety visit. Erythema was observed in one participant. It was localized to the area surrounding the anesthetized area and was attributed to hypersensitivity to the adhesive of the dressing. Aspartate transaminase

(AST)/alanine transaminase (ALT) at visit 1 and visit 3 for each volunteer were: 34/17 and 36/14 $UI.L^{-1}$, 18/27 and 21/21 $UI.L^{-1}$; 16/15 and 20/12 $UI.L^{-1}$; 30/25 and 31/25 $UI.L^{-1}$; 30/18 and 26/29 $UI.L^{-1}$.

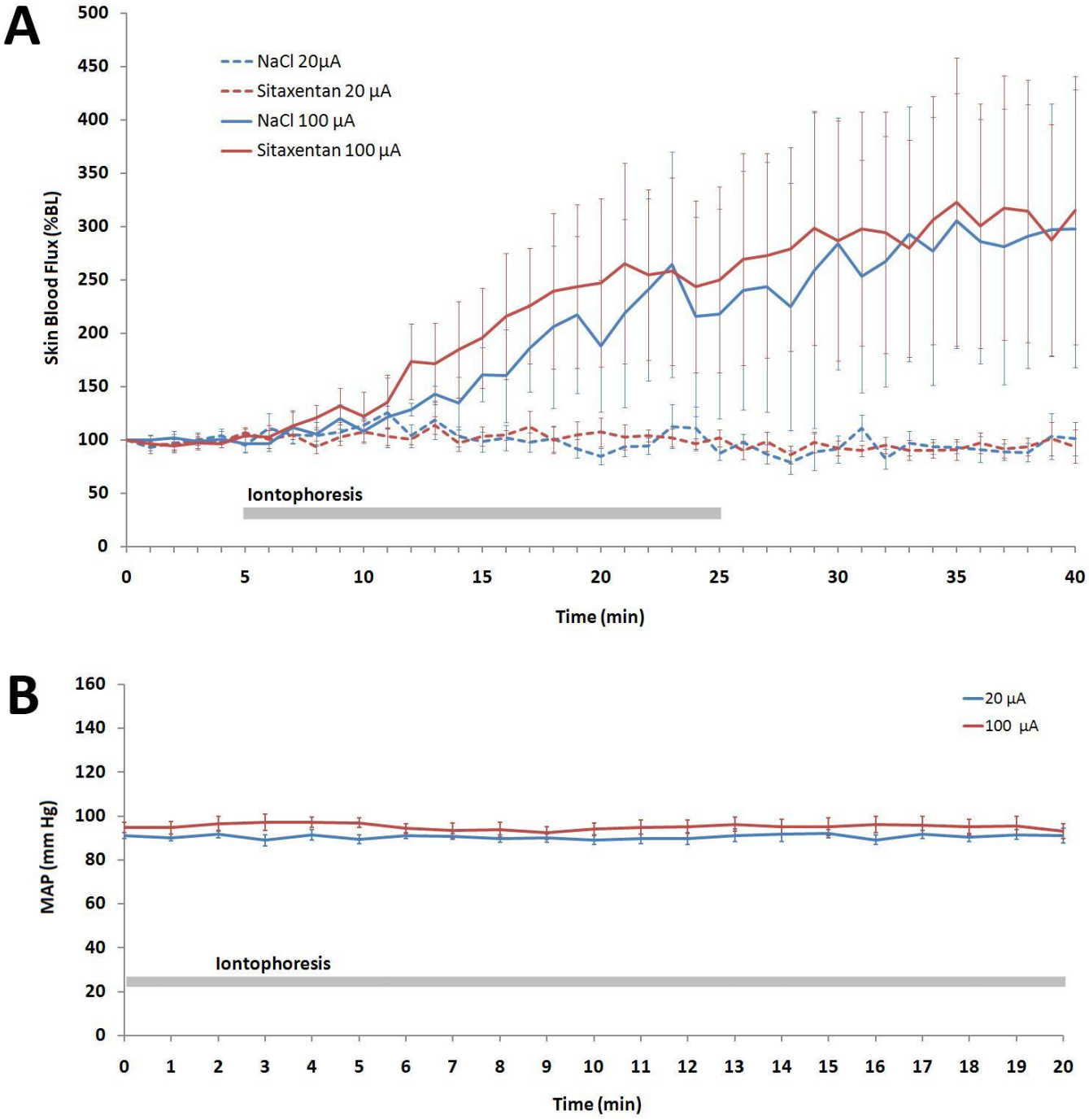

Figure 17: *Effect of iontophoresis of sitaxentan 10^{-2} M (red lines) and NaCl 0.9 % (blue lines) on skin blood flux in healthy volunteers' forearm skin (A). Current intensity was set at 20 µA (dashed lines) or 100 µA (solid lines). Iontophoresis did not induce a significant change in mean arterial pressure (MAP) (B).*

4. Discussion and conclusion

This study showed that in basal conditions iontophoresis of bosentan (in rats) or sitaxentan (in rats and humans) did not increase skin blood flux. However, after ET-1 infusion, iontophoresis of sitaxentan significantly increased cutaneous blood flux in rats, suggesting that the absence of effect initially observed was due to low ET-1-dependent basal skin microvascular tone rather than the absence of diffusion of the antagonist into the skin. However, no effect was seen with bosentan. Iontophoresis of ERAs was well tolerated both in animals and humans.

Iontophoretic transport is influenced by several parameters, including drug concentration, molecular weight, ionization and solution pH or current strength and skin hydration and resistance [218]. Bosentan and sitaxentan are small molecules with isoelectric points (pI) at 2.64 and 4.57, respectively. These chemical properties make these ERAs appropriate candidates for cathodal iontophoresis, being negatively ionized at neutral pH. However, we were not able to see any effect of the iontophoresis of bosentan or sitaxentan on skin blood flux in rats. In order to test whether the absence of effect was related to low ET-1-dependent vascular tone in the skin of healthy rats or to the absence of iontophoretic transport into the dermis, we repeated the experiment while infusing ET-1 (experiment 2). The results of this series suggest that the cathodal iontophoresis of sitaxentan enabled the drug to be locally administered in sufficient concentration to partially reverse the sustained effect of ET-1 on the skin microcirculation. However, such an effect was not observed with bosentan, suggesting that we were unable to reach sufficient skin concentration to reverse ET-1 vasoconstriction.

It is of interest that we obtained similar results when data were expressed as cutaneous vascular conductance, to take into account the effect of ET-1 on arterial blood pressure. The effect of ET-1 on blood pressure was comparable to that previously described: an initial

transient decrease in blood pressure immediately after ET-1 infusion was started was followed by a prolonged increase [233].

The relationship between the pharmacodynamic effect of ERAs on microvascular function and their effect in the prevention of digital ulcers in patients at risk remains uncertain. Two groups have studied the effect of the systemic administration of bosentan on skin blood flux in patients with SSc [234, 235]. Hettema *et al* did not show any difference in acetylcholine and sodium nitroprusside iontophoresis as pharmacological tests of microvascular endothelium-dependent and endothelium-independent function, respectively [235]. On large vessels however, bosentan increased flow-mediated dilation (FMD), a marker of endothelial macrovascular function [236]. Conversely, Rosato *et al* showed an increase in skin perfusion 8 weeks after bosentan therapy was started in SSc patients with pulmonary hypertension. In SSc patients without pulmonary hypertension however, there was no significant difference [234]. These discrepancies could be due to insufficient diffusion of ERAs into the skin. Indeed, when administered directly onto the dermis BQ-123 (an ET_A receptor antagonist) significantly increased skin blood flux in human skin, suggesting that ET-1 contributes to the maintenance of basal vascular tone [237]. On the other hand, intradermal injection of BQ-123 and bosentan (non specific ET_A and ET_B antagonists) did not alter basal skin blood flow in rats but reversed the effects of ET-1 [238], which is consistent with our findings. We note that intradermal BQ-788 (an ET_B receptor antagonist) unexpectedly increased skin blood flow in humans [237], suggesting that both ET_A and ET_B contribute to ET-1-mediated basal microvascular tone in the human skin.

Therefore, iontophoresis probably does not allow sufficient concentrations of ERAs to be reached in human skin. We did not use intradermal injections or microdialysis as these methods would be too far removed from the initial therapeutic objective, i.e. iontophoresis in physiological conditions.

In healthy participants, we observed an unexpected increase in skin blood flux for both sitaxentan and NaCl when the current intensity was 100 µA. Indeed, one of the main issues when performing iontophoresis is the non specific effect of the current itself, where the amplitude depends on the delivered electrical charge (i.e. the product of current intensity by duration of application) [94]. This may explain why we did not observe any reaction when the current intensity was only 20 µA. As a neural axon reflex has been suggested to be involved in current-induced vasodilation [94], we pre-treated the skin sites with a local anaesthetic (lidocaine/prilocaine). However, this did not abolish current-induced vasodilation in all subjects when the current intensity was 100 µA. This could be explained by the role of prostaglandins, which are likely to be essential for the axon reflex-related vasodilatation [96], mainly through the COX-1 pathway [97].

In humans the iontophoresis of sitaxentan and NaCl were both highly variable at 100 µA. Indeed, analysis of individual data reveals major current-induced vasodilation in two participants, whereas in the three other subjects we only observed a weak or even no effect at all (data not shown for clarity). This difference could be due to inter-individual differences in skin resistance, which was not recorded at this early stage. Nonetheless, this is unlikely to have a major impact on the conclusion of these experiments as each subject was his own control. Indeed, the iontophoresis were simultaneously performed at both sites with the same distance between electrodes.

The clinical study was prematurely stopped because the manufacturer of sitaxentan decided to discontinue all ongoing clinical trials with this compound and to withdraw the commercial drug, because of several cases of fatal liver injury [27]. Nonetheless, despite the small sample size, there was no clear-cut effect of sitaxentan compared to control in any of the five participants.

It should be noted that the iontophoresis of sitaxentan was well tolerated both in animals and humans. No change in arterial blood pressure was observed and skin tolerance was excellent. Moreover, it did not induce any significant increase in aspartate transaminase or alanine transaminase in humans.

In conclusion, this study shows that cathodal iontophoresis of sitaxentan but not bosentan partially reverses endothelin-induced skin vasoconstriction in rats, suggesting that sitaxentan diffuses into the dermis. We observed no effect of sitaxentan on basal skin microvascular tone in rats or humans.

Annex 1. Cathodal iontophoresis of treprostinil and iloprost induces a sustained increase in cutaneous flux in rats.

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RESEARCH PAPER

Cathodal iontophoresis of treprostinil and iloprost induces a sustained increase in cutaneous flux in rats

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iontophoresis; prostaglandin I2; prostaglandin analogue; treprostinil; iloprost; microcirculation; rat

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BACKGROUND AND PURPOSE

The treatment of scleroderma-related digital ulcers is still a therapeutic challenge. The most effective drugs are prostacyclin analogues. However, their usage is limited to an intravenous route of administration and by their frequent side effects. The objective of this study was to test whether treprostinil, iloprost and epoprostenol can induce sustained vasodilatation in rats when delivered locally using cutaneous iontophoresis.

EXPERIMENTAL APPROACH

Treprostinil, iloprost and epoprostenol were delivered by cathodal and anodal iontophoresis onto the hindquarters of anaesthesized rats (n = 8 for each group). Skin blood flow was quantified using laser Doppler imaging and cutaneous tolerance was assessed from day 0 to day 3.

KEY RESULTS

Cathodal but not anodal iontophoresis of treprostinil (6.4 mM), iloprost (0.2 mM) and epoprostenol (1.4 mM) induced a significant and sustained increase in cutaneous blood flow. The effects of treprostinil and iloprost were significantly different from those of treprostinil vehicle. Only weak effects were observed when both drugs were applied locally without current. Skin resistance was unchanged in areas treated with prostacyclin analogues. Finally, skin tolerance was good, with no evidence of epidermal damage.

CONCLUSIONS AND IMPLICATIONS

Cathodal iontophoresis of treprostinil and iloprost increases cutaneous blood flow with a good local tolerance. The effects of cathodal iontophoresis of these drugs should be investigated in humans, as they could have potential as new local therapies for digital ulcers in patients with scleroderma.

Abbreviations

PGI_2, prostacyclin; SNP, sodium nitroprusside; SSc, systemic sclerosis

Introduction

Systemic sclerosis (SSc) is a rare disease affecting the microcirculation and its treatment poses a difficult therapeutic challenge. Most SSc patients suffer from severe Raynaud's phenomenon, and 30–50% of them exhibit cutaneous ulcers on their fingers, which can lead to amputation (Herrick, 2000). Indeed, despite long-term treatment with calcium channel blockers they are liable to serious handicap. Nitrates have been used for decades for the treatment of Raynaud's phenomenon, but not digital

ulcers (Franks, 1982). More recently, bosentan, a non-specific endothelin receptor antagonist, has been indicated to prevent digital ulcers in patients at risk, but has no efficacy on existing ulcers (Korn *et al.*, 2004). Iloprost, a prostacyclin (PGI_2) analogue used intravenously, is the only drug approved for the treatment of existing digital ulcers (Wigley *et al.*, 1992). In addition, treprostinil, a PGI_2 analogue used subcutaneously, and epoprostenol (intravenous PGI_2) are used as second- or third-line treatment in scleroderma patients with pulmonary arterial hypertension. However, the therapeutic effect of prostaglandin analogues is counterbalanced by potentially serious systemic side effects related to their potent vasodilator properties, such as severe headaches, facial flushing, tachycardia and systemic hypotension.

In order to get around the toxicity of systemic treatments, topical drugs have been proposed for SSc-related digital ulcers. For example, new topical nitrate formulations (Chung *et al.*, 2009) have been proposed for Raynaud's phenomenon, but they have no efficacy in the treatment of existing ulcers. In addition to classical topical administration methods (i.e. cream or gel), iontophoresis is a simple, non-invasive transdermal drug delivery method using a low-intensity electric current. Some authors have highlighted the potential of iontophoresis of vasodilator drugs as a treatment for digital ulcers (Murray *et al.*, 2005; 2008). Indeed, the iontophoretic route has the advantage of optimizing drug diffusion at the site of injury while limiting systemic drug exposure. We recently showed that iontophoresis of sodium nitroprusside (SNP) leads to a non axon reflex-dependent, increase in cutaneous vascular conductance on the forearm of patients with secondary Raynaud's phenomenon (Roustit *et al.*, 2009). Local administration of PGI_2 analogues could have potential for the treatment of digital ulcers. However, to our knowledge, iontophoresis of PGI_2 analogues has never been tested, either in animals or in humans.

The main objective of the present study was to test whether iontophoretically administered vasodilatator drugs such as PGI_2 (epoprostenol) and PGI_2 analogues (iloprost and treprostinil) induce an increase in cutaneous blood flow in rats. As a secondary objective, we also assessed the cutaneous tolerance of PGI_2 analogues when administered iontophoretically.

Methods

Animals

Fifty male Wistar rats (8 weeks old, 300–400 g), obtained from CERJ (Le Genest-St-Isle, France), were housed in controlled conditions conforming to the current French legislation and fed standard rat chow. The protocol was approved by the Rhone Alps Region Animal Ethics Committee (number 309). The rats were subjected to a day/night cycle of 12 h/12 h and were provided with food and water *ad libitum*. Three days before the iontophoresis, the fur on the back, the hind legs and the back of the neck was removed by shaving with electric clippers followed by the application of hair removal cream (Veet®, Massy, France). The skin was then wiped with a wet compress and dried. After the experiments, rats were kept, as before, for 3 days for close examination of the skin and then killed by lethal dose of pentobarbital.

Drugs

The PGI_2 analogues and intravenous PGI_2 we used were: treprostinil (Bioprojet Pharma, Paris, France) 2.5 mg mL^{-1} solution (MW 390.5 g mol^{-1}; pKa 4.5); iloprost (Bayer Sante, Puteaux, France) 0.1 mg mL^{-1} solution (MW 478.6 g mol^{-1}); and epoprostenol (GlaxoSmithKline, Marly-le-Roi, France) 0.5 mg 50 mL^{-1} solvent (glycine, sodium chloride, sodium hydroxide, water for injection) (PGI_2 MW 352.5 g mol^{-1}; pKa 7.5) (Alexander *et al.*, 2008). Sodium nitroprusside (Serb, Paris, France) (MW 215.9 g mol^{-1}), a nitrate that induces the activation of soluble guanylyl cyclase through NO release, was used as a positive control. Isotonic sodium chloride (NaCl 0.9%) (Aguettant, Lyon, France) was used as a control solution.

Drugs were administered as commercially available solutions for human use: treprostinil solution at 2.5 mg mL^{-1} (6.4 mM), iloprost solution at 0.1 mg mL^{-1} (0.2 mM), epoprostenol solution at 0.01 mg mL^{-1} (0.028 mM) and sodium nitroprusside at 12.5 mg mL^{-1} (57.8 mM). For each solution, the pH was determined before iontophoresis using a microprocessor-based pH meter (pH 210, Hanna Instruments, Woonsocket, RI, USA). Iloprost and treprostinil solutions pH were 6 and 6.5, respectively, which are suitable for epidermal application. However, the epoprostenol solution purchased was spontaneously at pH 12 at 0.01 mg mL^{-1}. We therefore adjusted the pH to 6.5 by adding 1 N HCl, and tested both solutions (pH 12 and pH 6.5). After iontophoresis, the pH of all solutions was retested using the semi-quantitative Universal and Special Indicator papers (Macherey-Nagel, Düren, Germany).

The two most potent drugs (treprostinil and iloprost) were tested at three different concentrations. Solutions of treprostinil (6.4 mM) and iloprost (0.2 mM) were diluted with NaCl 0.9% to 0.64 mM and 0.064 mM, and 0.02 mM and 0.002 mM, respectively; as NaCl has been shown to induce less

axon reflex non-specific vasodilatation in human skin than pure water (Durand *et al.*, 2002). Pilot experiments in rats with our protocol confirmed these findings.

We asked Bayer Sante and Bioprojet to provide us with the exact compositions of the vehicles for iloprost and treprostinil (placebo form), respectively, or of each drug solution in order to reconstitute it. We had a positive answer only for treprostinil, for which the exact composition of the drug, provided by the manufacturer, was: sodium citrate 6.3 mg mL^{-1}, hydrochloric acid 0.2 mg mL^{-1}, metacresol 3 mg mL^{-1}, sodium hydroxide 0.32 mg mL^{-1}, sodium chloride 5.3 mg mL^{-1} and water for injection. Treprostinil vehicle was then reconstituted, adjusted for pH and tested as a control.

All solutions were prepared on the same day as iontophoresis.

Experimental procedures

The rats were anaesthetized with sodium pentobarbital (50 mg kg^{-1} i.p.) and were maintained in the prone position for the duration of the whole experiment, by placing them on the table with the back uppermost (Figure 1); two rats were studied simultaneously. Experiments were performed in a temperature-controlled room, and the rats were placed on a thermal pad, temperature being maintained at 37.5°C, adjusted using a rectal probe in one rat, connected to the thermal pad (Harvard apparatus). Mean arterial blood pressure (three readings) was measured by plethysmography using the tail cuff method, before and immediately after iontophoresis. Before iontophoresis each rat was closely inspected to ensure that the hairless skin in the back and the hind legs was intact. Photographs were taken before the start of iontophoresis, immediately after the iontophoresis and 3 days later. A cutaneous score was used to assess skin tolerance, based on the International Contact Dermatitis Research Group scoring (Fregert, 1981). Negative reactions were coded grade 0; weak reactions (grade 1) are characterized as non-vesicular erythema. Strong positive reactions (grade 2) are characterized as erythema associated with vesicles. Extreme positive reactions (grade 3) are bullous lesions. Irritant reactions (that we coded grade 4) are characterized by necrosis.

Histopathological examination of full-thickness skin biopsies from treated and untreated rats skin areas was realized in experiments 2 and 3 detailed thereafter. Eighty biopsies were fixed in AFA fluid (5% acetic acid, 75% absolute ethylic alcohol, and 18% water; Carlo Erba), embedded in paraffin and stained with haematoxylin, eosin, safran. In order to evaluate the effect of the treatment on the skin, various features were searched for, such as hyperkeratosis and epidermolytic aspects. In the stratum corneum, the degree of hyperkeratosis was evaluated, and the presence of parakeratosis was noted. The granular layer was evaluated for perinuclear vacuolar changes, cytolysis and the appearance of the keratohyaline granules. The spinous layer was investigated for the development of these features. Vasculitis, which is a histological diagnosis defined as inflammation targeting blood vessel walls and compromising their function, leading to haemorrhagic and/or ischaemic events, was also assessed. Furthermore, inflammation accompanied by infiltration of neutrophils, lymphocytes or mast cells at the dermo-epiderma interface was evaluated.

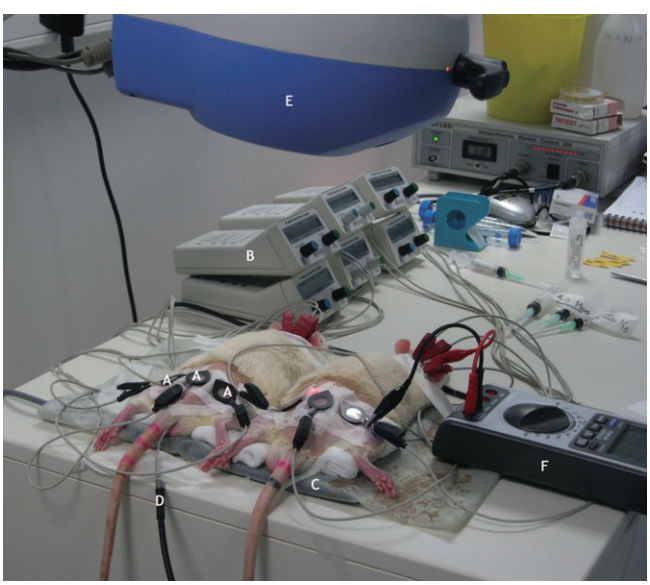

Figure 1

Experimental setup: three iontophoresis chambers (A) connected to power supplies (B) were placed on the back and the hind legs of two shaved rats placed in the prone position on a thermal pad (C), temperature being maintained at 37.5°C and adjusted using a rectal probe (D) in one rat. Laser Doppler imaging (E) was performed over the area including all iontophoresis chambers. Cutaneous resistance was determined during iontophoresis with an amperometric biosensor unit (F).

Iontophoresis protocol and laser Doppler imaging (LDI)

Iontophoresis probes were used together with LDI (PeriIont System and PeriScan PIM 3 System, Perimed, Järfälla, Sweden). Two or three probes, depending on the area of intact skin available, were placed on the hairless skin of the lower back/hind legs, each probe having a 1.2-cm^2 circular contact surface area. A cotton swab was placed under the two hind legs to help maintain the skin surface close to horizontal during the experiment. Passive probes were placed on the back of the neck. Before ionto-

phoresis, baseline LDI data were recorded for 5 min with the laser head placed 20 cm above the skin. The resolution was set at 'medium' (2 mm step length) and LDI scans were taken every minute. Given the fact that only two or three sites were simultaneously available on each individual rat, we randomly assigned the order in which the different drugs and controls were used in each experiment. Following data recording, the regions of interest were chosen beneath the probes and from adjacent untreated skin areas, and monitored to ensure the stability of the blood flux (flow rate per area) in each animal. Under our experimental conditions flux values in these control areas were stable in all animals (data not shown).

Cutaneous resistance was determined during iontophoresis with an amperometric biosensor unit (Multimeter system DVM1200, Velleman, Gavere, Belgium). For these measurements, one electrode was connected to the passive probe on the neck of the rat and the other to a probe positioned on a hind leg. Measurements were commenced 5 min after the start of iontophoresis. This delay was chosen in pilot experiments and was aimed at monitoring voltage after the start of iontophoresis. Voltage (expressed in volts) was recorded and skin resistance calculated and expressed in Ω (Ferrell *et al.*, 2002).

The following experiments were performed:

Experiment 1. Iontophoresis was performed for 20 min with the current intensity set at 100 µA using both anodal and cathodal currents (n = 8 for each series). Iontophoresis chambers contained 180 µL drug solution and NaCl 0.9% was used as a control. LDI acquisition was performed throughout the whole procedure.

Experiment 2. We tested the effects of treprostinil and iloprost in the cathodal direction in comparison with treprostinil vehicle in the same animals. LDI acquisition was again performed throughout the whole procedure.

Experiment 3. We tested the effects of treprostinil and iloprost in the cathodal direction at three different concentrations in the same animal. LDI acquisition was again performed throughout the whole procedure and during an additional 60 min after the end of iontophoresis.

Experiment 4. We determined whether iloprost, treprostinil and NaCl 0.9% induced vasodilatation through passive diffusion. Iontophoresis probes were filled with the same drug solutions, but were not connected to the current generator. The length of acquisition was 20 min from application of the drug, the initial scan being used as a reference for baseline flux.

Data analysis

Data are expressed as area under the curve (AUC_{20min} or AUC_{80min} for the third experiment), and expressed as a percentage of baseline flux (%BL.s). Baseline flux was averaged over 5 min.

Qualitative data are expressed as numerical values and percentage in parenthesis. Quantitative data are expressed as mean and SD and were analysed by ANOVA, and with 2 × 2 *t*-tests for unpaired or paired analyses as required, corrected by Bonferroni's correction. *P*-values less than 0.05 were considered statistically significant.

Results

Effect of cathodal and anodal iontophoresis of iloprost, treprostinil and epoprostenol on cutaneous flux: initial screening

At the cathode, treprostinil 6.4 mM (115 669 ± 49 812%BL.s, $P < 0.001$), iloprost 0.2 mM (60 180 ± 43 069%BL.s, $P = 0.005$) and epoprostenol 0.028 mM pH 12 (42 865 ± 24 922%BL.s, $P = 0.0018$) induced an increase in AUC_{20min} significantly different from 0 (Figure 2A). As expected, SNP induced a significant increase in AUC_{20min} (41 937 ± 28 785%BL.s, P = 0.008). However, epoprostenol 0.028 mM pH 6.5 (14 557 ± 18 012%BL.s) and NaCl (18 071 ± 16 677%BL.s) did not induce a significant increase in skin blood flux, expressed as AUC_{20min}.

At the anode, although we observed a non-significant trend towards a small increase in AUC_{20min} (36 792 ± 49 268%BL.s, P = 0.07) for iloprost 0.2 mM, this was only significantly different from 0 for treprostinil 6.4 mM (AUC_{20min} 24 695 ± 26 339%BL.s, P = 0.048) (Figure 2B). No increase in AUC_{20min} or iontophoresis end flux was observed for SNP, for epoprostenol 0.028 mM at either pH and for NaCl.

Paired analysis of the effect of treprostinil 6.4 mM and iloprost 0.2 mM in comparison with treprostinil buffer at the cathode

Treprostinil 6.4 mM (107 340 ± 42 269%BL.s, $P < 0.0001$) and iloprost 0.2 mM (49 484 ± 21 842%BL.s, $P < 0.001$) induced an increase in AUC_{20min} in comparison with the vehicle for treprostinil (Figure 3).

Concentration-dependent effect of cathodal iontophoresis of iloprost and treprostinil on cutaneous flux

Three different concentrations of iloprost and treprostinil were studied. Treprostinil at 6.4 mM,

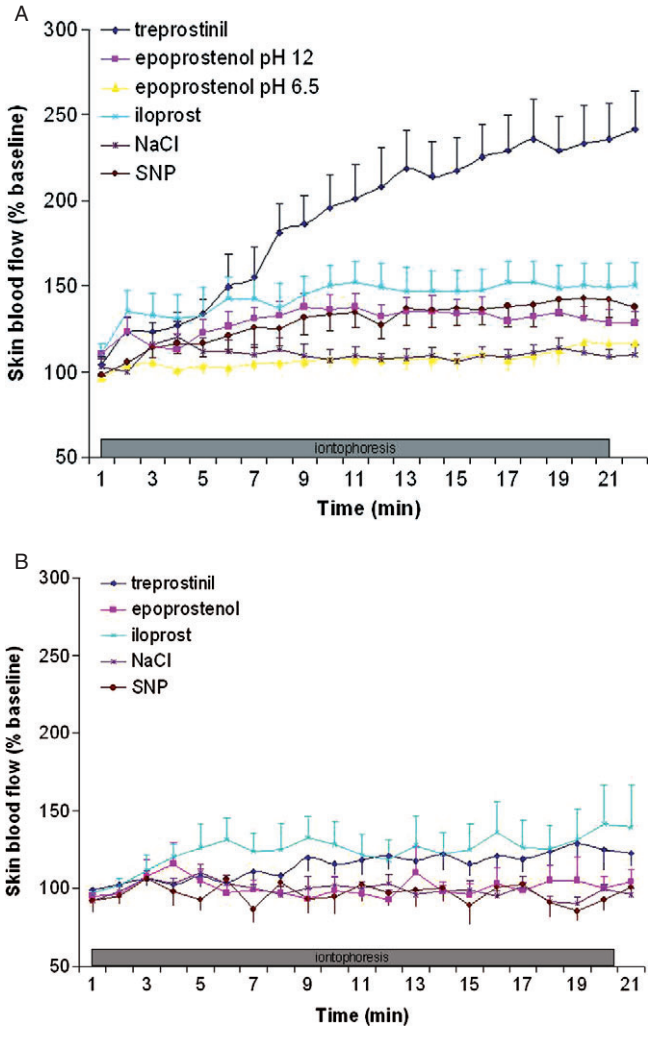

Figure 2

Effects of iontophoresis (A) in the cathodal direction and (B) in the anodal direction, of iloprost 0.2 mM, treprostinil 6.4 mM, epoprostenol 0.028 mM at pH 12 and pH 6.5, NaCl 0.9 % and sodium nitroprussiate (SNP) 57.8 mM, on cutaneous blood flow expressed as % of baseline.

0.64 mM and 0.064 mM induced an increase in AUC_{80min} that did not differ between concentrations (270 963 ± 117 637%BL.s, 342 568 ± 379 205%BL.s, 190 418 ± 108 746%BL.s, respectively, NS) (Figure 4A).

Iloprost at 0.02 mM induced a significant increase in AUC_{80min} in comparison with iloprost 0.002 mM (379 597 ± 196 125%BL.s vs. 96 730%BL.s ± 145 138, respectively, P = 0.02) (Figure 4B). Iloprost at 0.2 mM tended to increase the AUC (270 126%BL.s ± 303 908) compared with 0.002 mM (P = 0.08). Unlike with treprostinil, the maximal effect of iloprost was observed at the end of the iontophoresis (20 min), and decreased thereafter. However, it did not return to baseline at 80 min.

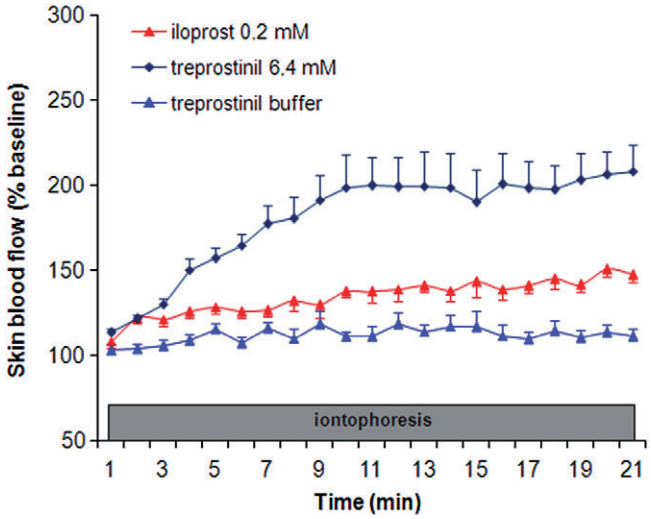

Figure 3

Paired analysis of the effect of treprostinil 6.4 mM and iloprost 0.2 mM in comparison with treprostinil buffer at the cathode, on cutaneous blood flow expressed as % of baseline.

Immediately after iontophoresis, for all solutions the pH was still compatible with its use as a skin application (pH ranging from 6.5 to 7.9).

Cutaneous resistance

The values for cutaneous resistance during iontophoresis of treprostinil 6.4 mM (45 ± 7 KΩ) and iloprost 0.2 mM (44 ± 5 KΩ) were not significantly different from that obtained with NaCl (45 ± 7 KΩ, NS). When these solutions were diluted, skin resistances were still not different from that with NaCl: 46 ± 14 KΩ for treprostinil 0.64 mM, 49 ± 14 KΩ for treprostinil 0.064 mM, 51 ± 14 KΩ for iloprost 0.02 mM and 48 ±8 KΩ for iloprost 0.002 mM.

Passive transdermal diffusion of iloprost and treprostinil

Treprostinil did not increase cutaneous flux when applied without iontophoresis (Figure 5). Iloprost at 0.02 mM but not 0.2 mM induced a small increase in cutaneous flux, as reflected by a non-significant trend towards an increased AUC_{20min} (iloprost 26 048 ± 30 792%BL.s at 0.02 mM vs. 15 178 ± 22 251%BL.s at 0.2 mM, NS).

Skin and systemic tolerance of the iontophoresis

No side effects of the treatments were observed on the skin. All the cutaneous scores were 0 both immediately after the procedure and at day 3. None of the various histopathological features listed in Methods were found in any of the skin biopsies (Figure 6). No

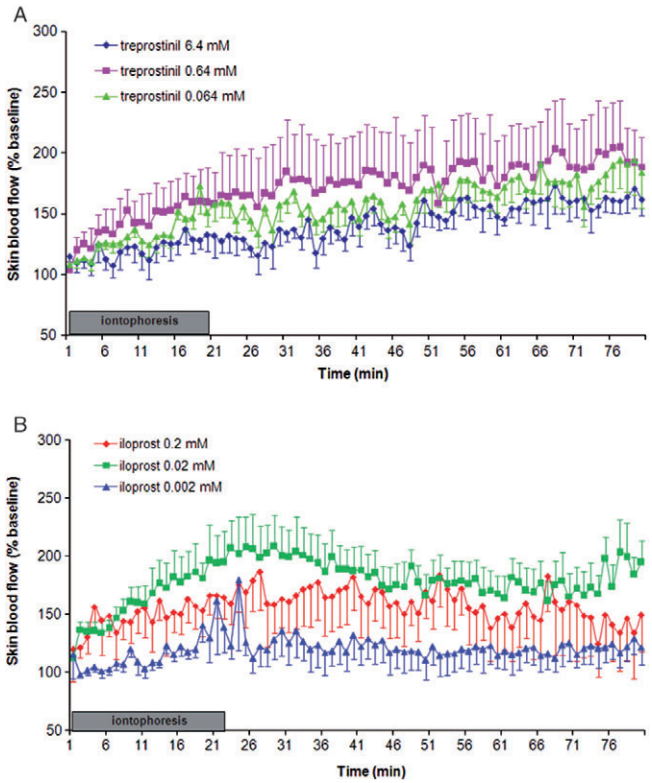

Figure 4

Paired dose-dependent effect of cathodal iontophoresis of (A) treprostinil at 6.4 mM, 0.64 mM and 0.064 mM and (B) iloprost at 0.2 mM, 0.02 mM and 0.002 mM, on cutaneous blood flow expressed as % of baseline.

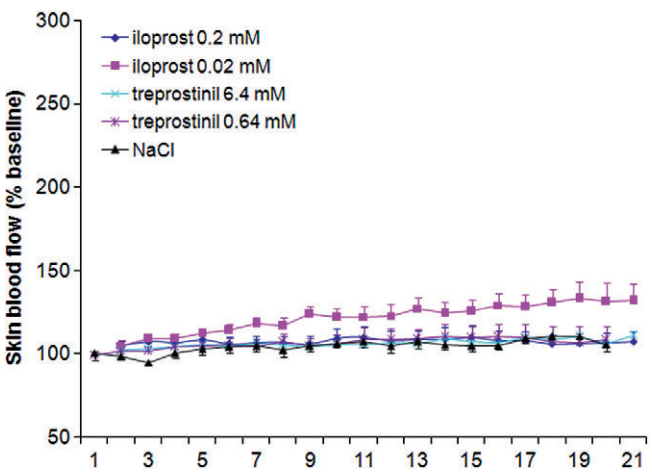

Figure 5

Effect of the passive transdermal diffusion of iloprost 0.2 mM and treprostinil 6.4 mM on cutaneous flux expressed as % baseline blood flow.

significant drop in mean arterial pressure after iontophoresis was observed with any of the drugs used. During the screening phase, systolic blood pressure was 105 ± 32 mmHg before and 113 ± 30 mmHg after iontophoresis of treprostinil 6.4 mM, 105 ± 19 mmHg before and 112 ± 23 mmHg after iloprost 0.2 mM, 120 ± 30 mmHg before and 112 ± 30 mmHg after epoprostenol 0.028 mM pH 12, 100 ± 14 mmHg before and 108 ± 17 mmHg after epoprostenol 0.028 mM pH 6.5, and 109 ± 32 mmHg before and 114 ± 25 mmHg after NaCl. During experiment 2, systolic blood pressure was 136 ± 27 mmHg before iontophoresis and 129 ± 26 mmHg after. During experiment 3, systolic blood pressure was 106 ± 19 mmHg before iontophoresis and 112 ± 26 mmHg after concentration-dependent iontophoresis of treprostinil and 114 ± 28 mmHg before and 111 ± 33 mmHg after concentration-dependent iontophoresis of iloprost.

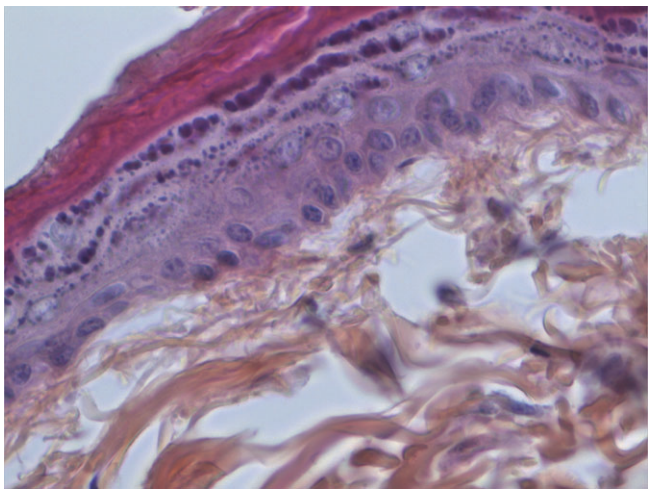

Figure 6

Histopathological examination of a full-thickness skin biopsy taken after iontophoresis of treprostinil 6.4 mM. The sample was embedded in paraffin and stained with haematoxylin, eosin and safran (x40).

Discussion and conclusion

This is the first demonstration that cutaneous cathodal iontophoresis of treprostinil and iloprost, two PGI_2 analogues, induces a large and sustained increase in cutaneous blood flow in rats. This pharmacodynamic effect was produced without any local toxic effects on the skin.

Iontophoresis refers to the facilitated movement of ionized molecules through application of an electrical field (Kalia *et al.*, 2004). It has the advantage over passive diffusion of enabling the delivery of polarized and/or larger drugs across the dermal barrier. Iontophoresis enhances the transport of drugs by two major mechanisms in addition to

passive diffusion, electrorepulsion and electro-osmosis. Electrorepulsion refers to the ion-electric field interaction that provides a force, which drives ionized drugs through the skin. Electro-osmosis refers to the bulk motion of the solvent that carries ionic or neutral solutes with the solvent stream and is mostly observed when iontophoresis is applied using an anodal current (Dixit *et al.*, 2007). Many factors are critical for transdermal drug delivery using iontophoresis, among these the most important are the drug concentration and molecular weight, the drug's charge, the solution pH (which directly influences ionization), the current strength, and skin hydration and resistance (Dixit *et al.*, 2007). In our study, we tried to optimize these factors: all PGI_2 analogues are weak acids that may be theoretically repulsed by a cathodal current when ionized. Our observations are consistent with this hypothesis as we mostly observed increased cutaneous flux with cathodal currents for all drugs. We also observed a weak trend towards increased skin flow for iloprost and treprostinil with anodal currents, and this is probably due to non-specific electro-osmosis. The pH of treprostinil and iloprost solutions, whether diluted or not, was between 5 and 6.5, which is close to skin pH and higher than treprostinil pKa, ensuring a large fraction of ionized molecules. In contrast, skin blood flow increased when the epoprostenol solution was at pH 12, but not when at 6.5, which may be because most epoprostenol is not ionized at neutral pH given its higher pKa value of 7.5. The second possibility is that the stability of PGI_2 is highly dependent on pH being quite stable only at alkaline pH. The molecular weight of the drugs was not a limitation in this study, as they are far below the limit for iontophoresis. We applied a relatively high current (100 μA over 20 min, i.e. 120 mC), and it is unclear whether a lower current strength would provide a similar increase in skin blood flow. However, we have observed similar increases in skin blood flux during iontophoresis of 57.8 mM SNP for 20 min at 100 μA through rat skin to that found when we used a 20-μA current in humans (Blaise *et al.*, 2010). This suggests that hairless rat skin is a suitable model for topical and transdermal drug delivery studies, probably due to the similar lipid content and water uptake properties of rat and human skin (Morimoto *et al.*, 1992). In our study, the choice of drug concentrations was based on the currently available concentrations of these drugs for human use and hence the initial concentrations of the drugs differed, and this was the reason why a comparison of the potency of those drugs was not performed. We did not observe a clear-cut concentration-dependent effect for either treprostinil or iloprost (i.e. all treprostinil concentrations induced a large vasodilator response, while for iloprost there was no effect at 2 μM and a maximal effect at 20 μM). This probably reflects the lack of linearity between drug concentration and drug iontophoretic flux across the skin, which has been observed with most drugs used in solutions containing background electrolytes (Kalia *et al.*, 2004). Indeed, our observation is consistent with the general tendency for the flux concentration profile to plateau as concentration increases. This observation is important, as it will help in the choice of drug concentrations to be used in future experiments with healthy human volunteers. We ruled out the potential confounding effect of the excipients for treprostinil, as we were unable to detect any variation in skin blood flow with them. Unfortunately, we were unable to obtain either an iloprost placebo or the exact composition of the drug's excipient, despite several requests to the manufacturer, and are thus unable to provide such a clear conclusion for iloprost. This is a potential limitation of our study. Iloprost and treprostinil are both PGI_2 analogues acting through stimulation of IP receptors. The difference observed following cessation of iontophoresis is therefore probably due to a different pharmacokinetic profile in the skin.

Skin resistance is an important parameter that influences the degree of permeability and drug diffusion, and can be measured easily during iontophoresis (Ferrell *et al.*, 2002). In order to rule out variations in skin resistance to the different drugs as a confounding factor, we recorded the potential difference at each site 5 min after the start of iontophoresis. We found no difference in skin resistance between treprostinil, iloprost, whether diluted or not, and NaCl.

A good candidate for therapeutic iontophoresis in patients with skin ulcers would be an ionized drug without cutaneous toxicity and with a large and sustained pharmacodynamic effect at the site of transdermal delivery enabling a once a day delivering protocol, without major systemic side effects. Transdermal iontophoresis is considered to be a safe procedure, associated with only moderate erythema and tingling sensations (Kumar and Lin, 2008). In terms of local toxicity, we observed no local side effects in the 3 days following iontophoresis of the different drugs and the pH remained compatible with topical application. We attempted to ascertain whether any skin irritation or burning occurred. No clinical or histopathological effects were observed. In terms of systemic effects, no variation in arterial pressure was observed, as assessed by the tail cuff method.

However, we did not perform continuous blood pressure monitoring for these animals, and this might be considered as a limitation in our study. However, continuous blood pressure monitoring is included in our protocol for future experiments using healthy human volunteers. A fairly recent report showed that SNP and acetylcholine leads to an increased digital flux (assessed using LDI) in eight patients with SSc, five of which exhibited limited cutaneous SSc and three of which diffuse cutaneous SSc (Murray *et al.*, 2008). This suggests that dermal thickening is not a major obstacle to the iontophoresis flux. However, such a question will need to be addressed in our future experiments.

Microvascular function can be routinely studied in human skin using non-invasive LDI (Turner *et al.*, 2008) and thermal hyperaemia is commonly used as a reference for maximal vasodilatation (Cracowski *et al.*, 2006). We did not normalize flux data over maximal vasodilatation in this study as the duration of the experiment would have to have been much longer and more complex, as local heating for 40 min is required to reach a stable thermal plateau. The present study was designed as a drug screening protocol, and given the large effects observed compared with baseline flux, we consider our data as sufficient to initiate human studies in which we will normalize flux over baseline and thermal plateau.

Iontophoresis has been associated with confounding non-specific axon reflex vasodilatation (Cracowski *et al.*, 2006). However, we did not observe this non-specific effect when using NaCl, despite using a current of 100 μA during 20 min. Indeed, in pilot experiments, we tested the effects of both NaCl 0.9% and pure water as vehicles and as a large axon reflex vasodilatation was seen only with pure water, we chose NaCl as the diluent for the drugs (Durand *et al.*, 2002). Also, as a consequence, we did not use lidocaine/prilocaine cream as previously used in human iontophoresis experiments (Blaise *et al.*, 2010).

In conclusion, we showed that cathodal iontophoresis of treprostinil and iloprost induces a large and sustained increase in cutaneous blood flow in rats. This effect was observed using treprostinil concentrations as low as 64 μM and iloprost as low as 20 μM. Weak or no effects of these drugs were observed when they were applied without current. No local side effects were observed. The next step will be to confirm this proof-of-concept in healthy human volunteers. Treprostinil and iloprost cathodal iontophoresis could then be investigated in the future as a new local therapy for digital ulcers in patients with scleroderma.

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Conflicts of interest

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Perspectives and conclusion

Methods for the noninvasive exploration of cutaneous microcirculation have been mainly based on optical microscopy and laser Doppler techniques. Nailfold videocapillaroscopy has found clinical application in the classification and the evaluation of systemic sclerosis. However, when performed on other skin sites, capillaroscopy provides low-contrast images and only allows rough quantification of capillary density. More sophisticated techniques such as orthogonal polarization spectral (OPS) imaging and sidestream dark field (SDF) provide good quality images of superficial microvessels on thin epithelial layers. They are semi-quantitative techniques implemented using small devices that can be used at the bedside. In critically ill patients, OPS and SDF imaging of the sublingual region has been used as a surrogate marker of general microvascular dysfunction.

Laser Doppler techniques (i.e. LDF and LDI) provide a signal linearly correlated to microvascular blood flow. They have been widely used to assess microvascular reactivity, in combination with various tests. Among them, iontophoresis of Ach and SNP have been extensively used to assess endothelium-dependent and independent vasodilation, respectively. However, the complexity of the underlying mechanisms of Ach iontophoresis makes its use as a specific test of endothelial function debatable. Other limitations such as current-induced non specific effects and poor reproducibility have been reported. Therefore, studies using iontophoresis must be carefully designed to limit bias: LDI should be preferred to LDF, current intensity should be <100 µA, and saline should be preferred as the control.

Other reactivity tests such as post-occlusive reactive hyperemia (PORH) or local thermal challenges (heating and cooling) provide interesting information on global

microvascular function (PORH) or on more specific pathways (e.g. local heating). Again, special care should be taken to avoid methodological bias and optimize reproducibility. Indeed, within-subject reproducibility of laser Doppler measurements depends on the technique (spatial variability is highest for single-point LDF), the skin site, as well as the way of expressing data.

Recent advances in methods to assess microvascular function

The recently marketed laser speckle contrast imaging (LSCI) technique theoretically combines the advantages of LDF (high frequency measurements) and LDI (full-field measurements), but data acquisition requires caution, particularly regarding movement artifacts. Although, as yet, limited data is available, LSCI seems to be a powerful tool to assess skin microvascular function. Since the work comparing the reproducibility of LSCI with that of LDI and LDF [104], we have continued with the study of LSCI in healthy subjects, and showed an excellent correlation between skin blood flux assessed with LSCI and LDI, over a wide range of skin perfusion [140].

Recent advances in the noninvasive methods to study the cutaneous microcirculation have been characterized by innovative techniques (e.g. LSCI), as well as a better understanding of the mechanisms underlying the reactivity tests. The assessment of the reproducibility of these tests has also contributed to optimize the way of expressing data. However, further work is needed to establish the relationship between skin microvascular function and systemic vascular disease progression before it can be used as a surrogate outcome [7]. The mechanistic bases of the microvascular reactivity tests also deserve further study (e.g. thermal challenges, PORH, Ach iontophoresis). The heterogeneity in methods is another issue, which may explain the discrepancies observed between different studies when

skin microvascular reactivity has been used as a surrogate marker of general microvascular dysfunction. The standardization of methods may help to improve external consistency.

Abnormal neurovascular responses to thermal challenges

Nevertheless, these techniques have been used to better understand the pathophysiology of diseases which specifically affect skin microcirculation. Particularly, the use of thermal challenges has allowed deeper exploration of the pathophysiology of RP, and we have shown in the second part of this dissertation impaired microvascular function in both primary and secondary RP, by using different tests.

Patients with RP secondary to SSc had impaired axon reflex-dependent vasodilation, suggesting abnormal neurovascular control in these patients [19]. Whether the origin of this abnormality is functional or structural is not known. On the other hand, we found no evidence of such an abnormality in primary RP patients.

However, local cooling allowed us to detect impaired microvascular reactivity in primary RP compared to healthy controls. Our data suggest that sensory nerve inhibition in patients increases transient vasodilation to the level observed in matched healthy volunteers, whereas local anesthesia did not affect the response in controls [21].

This suggests that the mechanism through which microvascular dysfunction to local cooling is mediated in primary RP differs from that involved during local thermal hyperemia. This not surprising considering the opposite effects of axon reflexes to local heating or cooling: when triggered by local heating, the axon reflex leads to vasodilation [83], whereas it inhibits vasodilation in the early phase of local cooling [123, 202]. The mediators involved in those responses have not yet been clearly identified. Moreover, speaking about local ‘heating’ and ‘cooling’ may be an oversimplification, as different potential transient receptor proteins (TRPs) are involved according to the temperature [239], potentially leading to distinct effects

on microvascular tone. This may explain the discrepancy between our results and those by Hodges *et al*, who have shown increased transient vasodilation while locally cooling the anesthetized skin of healthy subjects from 34 to 29°C [202], which we did not observe when cooling down to 15°C [21]. Another reason for this difference could be the studied skin site: Hodges et al cooled the forearm whereas we focused on the dorsum of the finger.

Increased vasoconstriction to local cooling in primary RP

Local cooling also permitted to evidence exaggerated vasoconstriction in primary RP, besides impaired transient vasodilation [21]. Considering the underlying mechanisms of the local cooling test, several pathways could be involved (Figure 18), including increased noradrenaline-mediated vasoconstriction secondary to the translocation of α_{2c}-adrenoreceptors, which involves the RhoA/Rho kinase (ROCK) system [22]. Another mechanism may involve the inhibition of the NO pathway, both through decreased activity of NOS and through other process downstream of NOS [23]. Reactive oxygen species (ROS) generation in the mitochondria of vascular smooth muscle is the earliest cold-induced response in cutaneous arteries [24], through ROCK activation [180]. Moreover, when exposed to oxidative stress, NOS generates superoxide rather than NO [240], suggesting that increased ROS production may be the common step prior to ROCK activation and decreased NO (Figure 18).

Experimental data provide conflicting results regarding cooling-induced ET-1 release in primary RP [25, 176], but available evidence suggest increased release of ET-1 in primary RP; although it does not hold a primary role in the pathophysiology of primary RP, ET-1 may contribute to the maintenance of the vasospasm [13, 25].

To summarize, the local cooling test revealed abnormal microvascular reactivity in primary RP, preexisting even in the absence of vasospasm. Our results suggest that part of the

exaggerated response to local cooling depends on the increased inhibition of a still unknown vasodilator inhibition by sensory nerves. One can imagine that at least similar or even greater abnormalities may be found in patients with SSc, but this remains to be confirmed.

From pharmacology to therapeutics

Enhancement of the NO pathway through PDE5 inhibition may be beneficial in patients with primary RP. Indeed, in the third part of this dissertation we have shown than sildenafil increases skin blood flux at baseline and at all phases of the response to local cooling in primary RP, arguing for a non specific effect of sildenafil on skin perfusion. Although local cooling cannot be considered as a surrogate for paroxystic ischemia such as RP, single dose 100 mg sildenafil has a significant vasodilator effect for the cutaneous microcirculation of primary RP patients exposed to local cooling. These results suggest potential benefit of sildenafil "as required" before exposure to cold to prevent ischemic attacks. This hypothesis should be addressed in a randomized double-blind controlled trial.

Many phase II and III clinical trials have recently evaluated the efficacy of PDE5 inhibitor in primary or secondary RP, such as PF-00489791 (clinicaltrials.gov NCT01090492), tadalafil (clinicaltrials.gov NCT01117298 and NCT00707187), vardenafil (clinicaltrials.gov NCT01291199), SLx-2101 (clinicaltrials.gov NCT00528242), or udenafil compared to calcium-channel blockers (clinicaltrials.gov NCT01280266). Some of them have been recently completed and results should be published soon, while others are still ongoing.

Pathophysiological understanding of RP suggests that other drugs could be beneficial. Among them, inhibitors of the RhoA-Rho kinase (ROCK) pathway such as fasudil could be of great interest. As previously mentioned, the ROCK pathway has been shown to be involved in skin vasoconstriction to local cooling in healthy subjects [131], and growing evidence

suggests its involvement in RP [21, 178, 179]. To our knowledge, a phase III clinical trial is still ongoing to address this issue (clinicaltrials.gov identifier NCT00498615).

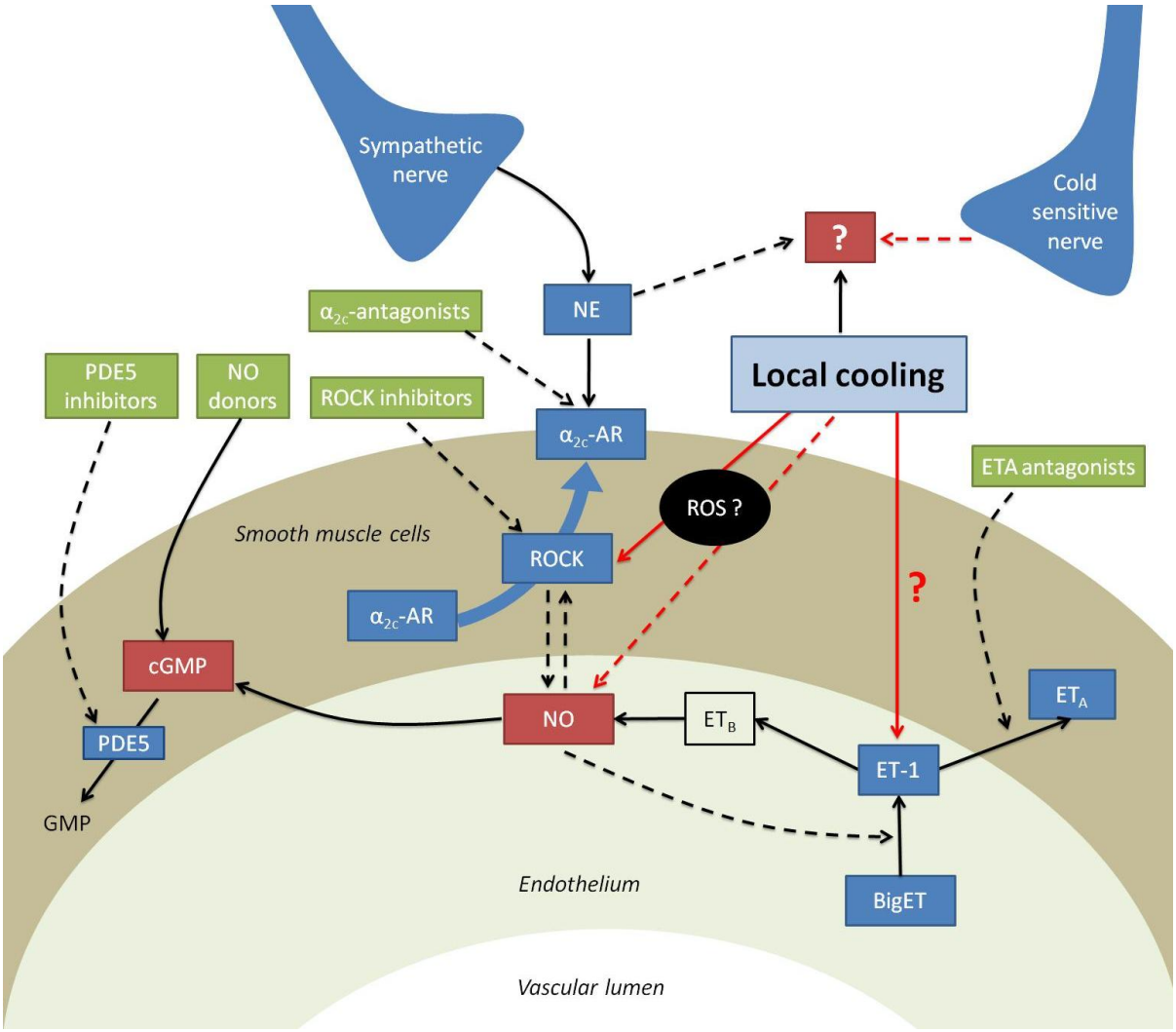

Figure 18. *Representation of the different pathways involved in the response to local cooling. Blue boxes represent mechanisms leading to vasoconstriction whereas those favoring vasodilation appear in red boxes. Plain arrows symbolize activation and dash arrows inhibition. Red arrows represent the mechanisms potentially involved in the impaired microvascular reactivity to local cooling in primary Raynaud's phenomenon, and green boxes represent pharmacological targets under investigation. NE:norepinephrine; α2$_c$-AR: α2$_c$-adrenoreceptors; ROCK: RhoA/Rho kinase; NO: nitric oxide; cGMP: cyclic guanosine-5-monophosphate; ROS: reactive oxygen species; PDE5: phosphodiesterase 5; ET-1: endothelin-1; ET$_A$/ET$_B$: endothelin-1 receptors A and B; BigET: Big-endothelin.*

In the same way OPC-28326, a specific α-adrenergic antagonist with preferential binding to the α_{2c} subtype, decreased the time to rewarming after exposure to cold but did not improve digital blood flow assessed by straingauge occlusion plethysmography in patients with SSc [241]. Another clinical trial evaluating the effect of a α_{2c}-antagonist, ORM-12741, has been recently registered (clinicaltrials.gov NCT01315899).

Despite conflicting results, ET-1 may be involved in the pathophysiology of RP, suggesting a potential interest of ERAs. However, compared with placebo, bosentan did not improve the frequency, duration, pain or severity of RP attacks in SSc patients [242]. Nonetheless, it could be interesting to test the effect of ERA on skin blood flow in primary RP before exposure to cold.

Therapeutic iontophoresis: an innovative therapy for RP?

In basal conditions, iontophoresis of bosentan (in rats) or sitaxentan (in rats and humans) did not increase skin blood flow. However, after ET-1 infusion, cathodal iontophoresis of sitaxentan significantly increased cutaneous blood flux in rats, suggesting that the absence of effect initially observed was due to low ET-1-dependent basal skin microvascular tone rather than the absence of diffusion of the antagonist into the skin.

Therefore, iontophoretically-delivered sitaxentan may be effective to prevent/treat digital ulcerations, although it does not increase basal skin blood flow. Indeed, the relationship between increased skin blood flow in the finger and the prevention of digital ulcer healing has not been established with bosentan [234, 235]. However, there will not be further clinical study with sitaxentan as it has recently been withdrawn. Local therapeutic iontophoresis of other ERAs such as ambrisentan could be evaluated. It is interesting to note that the effect of ambrisentan on skin blood flow is being tested in patients RP and digital ischemia secondary to SSc (NCT01072669).

Therapeutic iontophoresis of PGI_2 analogues gave more encouraging results. Indeed, preliminary data on rats have revealed a sustained increase in skin blood flux after cathodal iontophoresis of treprostinil and iloprost [28]. The considerable increase in skin blood flow induced by treprostinil was confirmed in healthy subjects, suggesting that iontophoresis of treprostinil could be an interesting treatment for SSc-related digital ulcers.

In the continuation of this line of research, a new clinical study is planned to assess the effect of iontophoresis of treprostinil on digital skin blood flow, in healthy subjects and in patients with SSc. Another objective of this study will be to assess the systemic/dermal bioavailability of iontophoretically-delivered treprostinil.

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